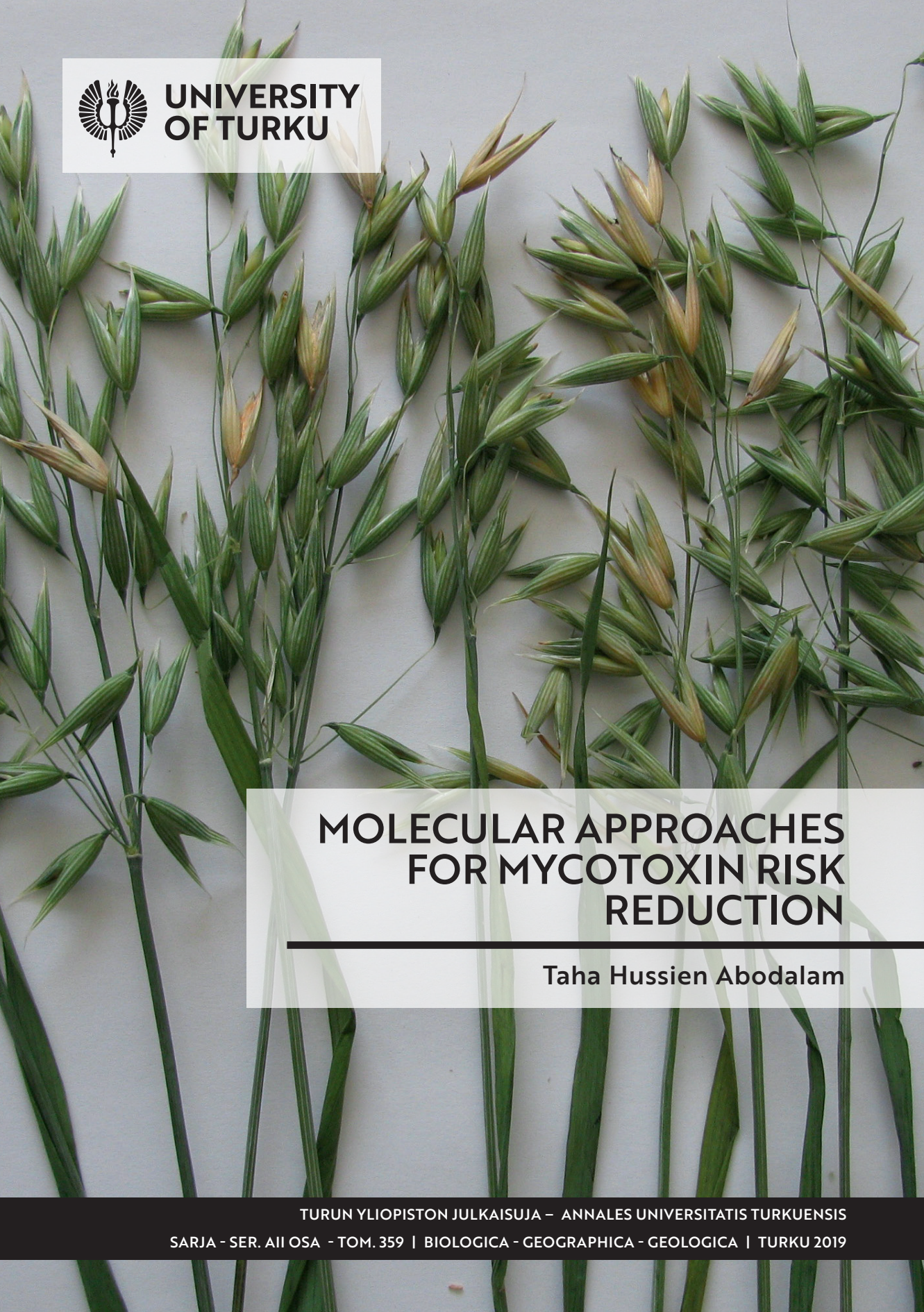


UNIVERSITY OF TURKU

MOLECULAR APPROACHES FOR MYCOTOXIN RISK REDUCTION

Taha Hussien Abodalam

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA - SER. AII OSA - TOM. 359 | BIOLOGICA - GEOGRAPHICA - GEOLOGICA | TURKU 2019

UNIVERSITY OF TURKU

MOLECULAR APPROACHES FOR MYCOTOXIN RISK REDUCTION

Taha Hussien Abodalam

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA - SER. AII OSA - TOM. 359 | BIOLOGICA - GEOGRAPHICA - GEOLOGICA | TURKU 2019

University of Turku

Faculty of Sciences and Engineering
Department of Biochemistry
Laboratory of Molecular Plant Biology

Supervised by

Adjunct Prof. Tapani Yli-Mattila
Laboratory of Molecular Plant Biology
Department of Biochemistry
University of Turku
FI-20014 Turku, Finland

Prof. Dr. Petr Karlovsky
Laboratory of Molecular
Phytopathology
and Mycotoxin Research
Department of Crop Sciences
Georg-August-University Goettingen
37077 Goettingen, Germany

Reviewed by

Prof. Gülruh Albayrak
Department of Molecular Biology and
Genetics/Faculty of Science
Istanbul University
34134, Vezneciler, İstanbul, Turkey

Prof. Jarkko Hantula
Natural Resources Institute Finland,
Finnish Forest Research Institute,
(Luke)
00790 Helsinki, Finland

Opponent

Associate Prof. Matias Pasquali
Department of Food, Enviromental and
Nutritional Sciences DEFENS
University of Milan, 20122 Milano, Italy

The originality of this thesis has been checked in accordance with the University of Turku quality assurance system using the Turnitin Originality Check service.

Cover photo by Tapani Yli-Mattila
ISBN 978-951-29-7694-2 (PRINT)
ISBN 978-951-29-7695-9 (PDF)
ISSN 0082-6979 (Print)
ISSN 2343-3183 (Online)
Grano Oy - Turku, Finland 2019

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following scientific articles, referred to in the text by their Roman numerals.

I. Hussien T., Carlobos-Lopez A.L., Cumagun C.J.R., Yli-Mattila T. (2017) Identification and quantification of Fumonisin-producing *Fusarium* species in grain and soil samples from Egypt and the Philippines. Phytopathologia Mediterranea 56, 1: 146−153.

II. Yli-Mattila T., Rämö S., Hietaniemi V., Hussien T., Carlobos-Lopez A.L., Cumagun C.J.R. (2013) Molecular Quantification and Genetic Diversity of Toxigenic Fusarium Species in Northern Europe as Compared to Those in Southern Europe. Microorganisms. 1: 162-174.

III. Yli-Mattila T., Rämö S., Hussien T., Rauvola M., Hietaniemi V., Kaitaranta J. (2017) Different grain grinding methods affect detection of *Fusarium graminearum* DNA and mycotoxins. Phytopathologia Mediterranea. 56, 1: 167−174.

IV. Yli-Mattila T., Gavrilova O., Hussien T., Gagkaeva T. (2015) Identification of the first *Fusarium sibiricum* isolate from Iran and the first *F. langsethiae* isolate from western Siberia. Journal of Plant Pathology. 97, 1: 183-187.

V. Hussien T., Amra H., Sultana Y.Y., Magan N. Carlobos-Lopez A.L., Cumagun C.J.R., Yli-Mattila T. (2018) New genotypes of Aflatoxin-producing fungi from Egypt and the Philippines. Manuscript.

TABLE OF CONTENTS

ABBREVIATIONS

A.	*Aspergillus*
AF	Aflatoxin
AF B1	Aflatoxin B1
AF B2	Aflatoxin B2
AF G1	Aflatoxin G1
AF G2	Aflatoxin G2
AFPA	*Aspergillus flavus* and *A. parasiticus* agar medium
BLAST	basic local alignment search tool
cfu	Colony forming units
d	Day
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
EC	European Commission
EST	Expressed Sequence Tags
EU	European Union
F.	*Fusarium*
FB	Fumonisin
FB1	Fumonisin B1
FB2	Fumonisin B2
FB3	Fumonisin B3
FHB	*Fusarium* head blight
g	g-force, gram
h	Hour
HPLC	High performance liquid chromatography
HT-2	HT-2 toxin
IARC	International Agency for Research on Cancer
kg	Kilogram

l	Liter
min	Minute
NIV	Nivalenol
OT	ochratoxins
PCR	Polymerase chain reaction
ppt	Parts per trillion
ppb	Parts per billion
qPCR	Real time PCR
s	Second
T-2	T2-toxin
T_t	Time to threshold
Tm	Melting temperatures
v/v	volume / volume
UV	Ultraviolet
w/v	Mass / Volume
YES	Yeast Extract Sucrose
ZEA	zearalenone
µ	micro

ABSTRACT

Mycotoxin contamination in food is a serious concern for human and animal health. Today, we do not know how to detoxify food materials that are contaminated with mycotoxins in ways that retain their edibility. Therefore, avoiding mycotoxins from entering the food chain is an important approach. This needs early and easy identification, detection, and quantification of mycotoxin-producing fungi. The conventional methods for the identification, taxonomy, detection, and quantification of toxigenic fungi are challenging because they require a high level of expertise and a set of sophisticated equipment.

The aim of current study was to use molecular-based approaches (as they are practical, rapid, and more reliable) to identify, classify, detect, and quantify mycotoxin-producing fungi including aflatoxin-producing *Aspergillus* and fumonisin- and trichothecene (TC)-producing *Fusarium* species. The results obtained from this study suggested that there are 2 main populations of *F. graminearum* in Europe. The population of the 3-acetyl-deoxynivalenol (3ADON) chemotype is dominant in northern Europe and it has probably recently been spreading from Finland to north-western Russia, while the population of the 15ADON chemotype is dominant in the central and southern Europe and it has been spreading to the Denmark and Norway.

The results also suggested that the homogenization of the oat flour by milling with a 1 mm sieve is important for the reproducibility of deoxynivalenol (DON) and *F. graminearum* DNA levels, which is evident from a higher correlation between the DON and *F. graminearum* DNA levels in oat grain samples that were sieved after milling.

In the thesis, the *F. langsethiae* isolate obtained from Iranian wheat was re-identified as *F. sibiricum,* and the identification was confirmed by IGS sequencing. This work reports the first record of *F. sibiricum* from Iran, outside northern Asia and Norway, and the first isolation of *F. langsethiae* (a European pathogen) from western Siberia.

Large variations in the DON content and the amounts of *F. graminearum* DNA, and poor coefficient of determination (R^2) between these were detected in oat grain when the RIDA®QUICK SCAN kit was used for DON content estimation. This study confirmed that the coefficient of determination was usually less when DNA or DON levels were estimated from oat flour that was not ground with 0.8 mm or 1 mm sieves. DON levels obtained with the Rida®Quick method were usually higher than those obtained with accredited GC-MS method in the Finnish oat, barley, and wheat samples. The homogenization of the oat flour by sieving is therefore likely to be connected to the variations in the DON detection. Also, it was suggested that the amounts of DON close to the legislative limits should be reconfirmed with accredited quantitative analyses.

In addition, isolation, identification, detection, and quantification of *Fusarium* and *Aspergillus* isolates from Egypt and the Philippines maize, wheat, and soil samples were implemented in this thesis. *A. parasiticus* isolates, which produced higher amounts of aflatoxins, were only found in the Philippines.

TIIVISTELMÄ

Elintarvikkeiden sisältämät mykotoksiinit ovat vakava huolenaihe ihmisten ja eläinten terveydelle. Nykyään emme tiedä, miten mykotoksiineja sisältäviä elintarvikkeita voi puhdistaa niin, että niiden käyttökelpoisuus säilyy. Siksi on tärkeää välttää mykotoksiinien pääsyä elintarvike-ketjuun. Tämä vaatii mykotoksiineja tuottavien sienten varhaista tunnistamista, detektointia ja kvantifiointia helposti käyttöönotettavilla menetelmillä. Perinteiset menetelmät tunnistusta, taksonomiaa, detektointia ja toksigeenisten sienten kvantifiointia varten ovat hyvin haastavia, koska ne vaativat huippuluokan osaamista ja laitteita.

Työn tarkoituksena oli käyttää molekyylibiologisia menetelmiä, koska ne ovat käytännöllisiä, nopeita ja luotettavia tunnistamaan, luokittelemaan, havaitsemaan ja kvantifioimaan mykotoksiinia tuottavia sieniä, mukaan lukien aflatoksiinia tuottavia *Aspergillus*-homeita sekä fumonisiinia ja trikotekeeneitä tuottavia *Fusarium*-punahomeita. Väitöskirjatyön tulosten perusteella esitetään, että Euroopassa on kaksi *F. graminearum*-populaatiota. 3ADON-kemotyyppi on vallalla Pohjois-Euroopassa ja se on ilmeisesti levinnyt hiljattain Suomesta Luoteis-Venäjälle, kun taas 15ADON-kemotyyppi on vallalla Keski- ja Etelä-Euroopassa, ja se on hiljattain levinnyt Tanskaan ja Norjaan.

Lisäksi esitetään, että kauranäytteiden homogenointi jauhamalla 1 mm:n seulalla näyttää olevan tärkeä DON- ja *F. graminearum*-DNA-pitoisuusmittausten toistettavuudelle, mikä on osoitettu kauran korkeammalla korrelaatiolla DON ja *F. graminearum*-DNA-pitoisuuksien välillä.

F. langsethiae -kanta, joka eristettiin Iranissa vehnästä, tunnistettiin myöhemmin *F. sibiricumiksi* ja tunnistus varmistettiin IGS-sekvensoinnilla. Työssä raportoidaan tästä ensimmäisestä iranilaisesta *F. sibiricum*-kannasta, joka oli alun perin määritetty *F. langsethiae*-lajiin, sekä ensimmäisestä Euroopan ulkopuolelta löydetystä *F. langsethiae*-kannasta, joka löytyi Länsi-Siperiasta.

Aikaisemmin on usein havaittu, että kauranjyvän *F. graminearum*-DNA- ja DON-pitoisuuksien välillä on melko huono korrelaatio. Nyt saadut tulokset vahvistivat, että korrelaatio *F. graminearum*-DNA ja DON-pitoisuuden välillä oli pienempi, kun ne uutettiin kauroista, jota ei oltu jauhettu käyttäen 0,8 mm: n tai 1 mm:n seuloja. Rida®Quick-pikamääritysmenetelmällä mitatut DON-pitoisuudet, olivat tavallisesti korkeammat kuin akreditoitujen GC-MS-menetelmällä mitatut DON-pitoisuudet suomalaisessa kaura-, ohra- ja vehnänäytteessä. Kaurajauhojen homogenointi seulan avulla on siten todennäköisesti yhteydessä *Fusarium*-DNA ja DON-määrien vaihteluihin. Pikamääritysmenetelmillä havaitut lähellä suurinta

sallittua määrää olevat DON-pitoisuudet olisikin vahvistettava akreditoiduilla kvantitatiivisilla kromatografisilla analyyseillä.

Ensimmäisessä julkaisussa Egyptistä ja Filippiineiltä peräisin olevista maissi-, vehnä- ja maaperänäytteistä eristettiin *Fusarium*–isolaatteja. Lisäksi *Fusarium*-punahomeita tunnistettiin ja fumonisiinia tuottavien *Fusarium*-punahomeiden DNA:n määrää mitattiin Egyptistä ja Filippiineiltä peräisin olevista maissi- ja vehnänäytteistä. *A. parasiticus*-isolaatteja, jotka tuottivat suurimmat mitatut aflatoksiinimäärät, löydettiin vain Filippiineiltä.

1. INTRODUCTION

1.1 Mycotoxins

Mycotoxins are low-molecular-weight natural toxic chemicals produced by molds (a type of fungi), especially *Aspergillus*, *Penicillium,* and *Fusarium* species. The fungi can contaminate the food-chain with mycotoxins either directly by contaminating the plant-based food and feed materials with mycotoxins or indirectly by growing on food and feed products. Mycotoxins contamination can occur in all agricultural commodities including maize, wheat, barley, soybeans, sorghum, and nuts in the fields, during transportation, or during storage, if conditions are suitable for fungal growth (Alshannaq and Yu, 2017; Streit et al., 2012). Currently, more than 300 mycotoxins are known; however, only a few of them are commonly found in food and feed materials. The most common mycotoxins are Aflatoxins (AFs), Fumonisins (FBs), ochratoxins (OT), patulin, zearalenone (ZEA), trichothecenes (TCs), Deoxynivalenol (DON), and T-2 toxin (Pereira et al., 2014). Mycotoxins cause worries all over the world not only because of the risk related to the health of humans and animals but also because of the economic losses that occur due to mycotoxin-contaminated food and feed materials (Ostry et al., 2017). In African countries, several mycotoxins are encountered, especially AFs and FBs, but in European countries, TCs, OT, and ZEA are mostly encountered. However, with the increase in international trade, all types of mycotoxins might be detected in different areas of the world (Bhat et al., 2010). The exposure of humans and animals to mycotoxins like AFs, TCs, and FBs, usually occur through the ingestion of contaminated materials. Such exposer can cause both acute and chronic toxicities. In fact, mycotoxins have several toxic effects including carcinogenic, hepatotoxic, nephrotoxic, neurotoxic, mutagenic, teratogenic, estrogenic, and immunosuppressive effects (Kensler et al., 2011; Pereira et al., 2014). Due to the toxic effects of mycotoxins, many countries have set-up strict limits for the amounts of mycotoxins in the food and feed materials and have established legislation for controlling their possible contamination (Juan et al., 2012; Moretti et al., 2017). For example, EU mycotoxin limits in Cereals are 4–15, 2–10, 200–1000, 200–500 µg/kg for total AFs, OT, FBs, and DON, respectively (Alshannaq and Yu, 2017).

1.2 *Fusarium* species

Several teleomorph genera have been found for the species of the anamorph genus *Fusarium*. Most of them belong to the phylum–Ascomycota, class–Ascomycetes, order–Hypocreales, and genus–Gibberella (Nelson et al. 1994;

Leslie and Summerell, 2006). At the Nomenclature Session of the Botanical Congress meeting in Melbourne in 2011, it was decided that all names, irrespective of being typified by an anamorph or a teleomorph, will be on equal footing in terms of priority. This means that the second-class nomenclatural status of *Fusarium* as an anamorph genus has ended, allowing the use of the name *Fusarium* without additional teleomorph names. Historically, taxonomic treatments of *Fusarium* have stressed the anamorph nomenclature, because teleomorphs are unknown for most of the species and anamorphs are usually found by plant pathologists and other applied biologists in different cultures (Geiser et al., 2013). *Fusarium graminearum* (anamorph) is the major causative agent of fusarium head blight (FHB) in many regions, worldwide. The teleomorph of this fungus is known as *Gibberella zeae*. In future, only one name should be used for different fungal species (Hawksworth, 2011). According to Geiser et al., 2013), the anamorph name–*Fusarium* should be used as the only genus name for all *Fusarium* species instead of the different teleomorph names, including the *Gibberella*.

The genus *Fusarium* is widespread in all habitats and has both pathogenic and saprophytic species (Liddell, 1991; Nelson et al., 1994; Sandoval-Denis et al., 2018). Many *Fusarium* species cause plant disease in a huge number of crops and cereals. Also, some species of this genus have the ability to become pathogenic for both humans and animals. The isolation of *Fusarium* species can be performed from plant material and soil samples (Summerell et al., 2003; Aoki et al., 2013). The plant diseases, which are caused mainly by the *Fusarium* genus, appear in 80% of all the cultivated plants (Leslie and Summerell, 2006). The symptoms of the infection caused by *Fusarium* species can be recognized at all stages of the plant growth, starting from seed germination to the vegetation, based on the *Fusarium* species involved and the host plant. Several *Fusarium* species can co-infect one host plant causing infection with an ability to produce mycotoxins as secondary metabolites (Desjardins, 2003; Logrieco et al., 2007).

The main mycotoxins produced by *Fusarium* species include TCs and FBs. *F. graminearum*, together with other closely related species of *F. graminearum* species complex, is the most common cause of FHB and the most important TCs producer worldwide. The main species of *F. graminearum* species complex in Europe is *F. graminearum* sensu stricto, which is also the most important DON producer in most countries in Europe (Pasquali and Migheli, 2014; Pasquali et al., 2016; Yli-Mattila and Gagkaeva, 2016).

New T-2/HT-2 toxin-producing *Fusarium* species such as *F. langsethiae* (Torp and Nirenberg) and *F. sibiricum* (Gagkaeva, Burkin, Kononenko, Gavrilova, O'Donnell, T. Aoki, and Yli-Mattila) have been recently found in northern Europe and Asia (Torp and Nirenberg, 2004; Yli-Mattila et al., 2011). Up to now, *F. langsethiae* isolates have only been found in Europe (except one isolate in western Siberia), while *F. sibiricum* is mainly distributed in Siberia and Russian Far East, except for one isolate in Norway (Yli-Mattila et al., 2011; Yli-Mattila, 2012) (and one isolate from Iran, Paper IV), which was considered as an intermediate between *F. sporotrichioides* and *F. langsethiae*.

F. verticillioides and *F. proliferatum* are considered the main producers of FBs (Marin et al., 2004). They belong to *Gibberella fujikuroi* species complex and the identification of this complex based on morphological characteristics is challenging, even for experts (Leslie and Summerell, 2006; Rossi et al., 2009). The early detection and identification of *Fusarium* species infection are substantial in predicting the toxicological risk and in preventing the formation of toxic metabolites (Desjardins, 2003; Hong et al 2018).

1.2.1 Fumonisins

The FBs are an important group of mycotoxins produced mainly by *F. verticillioides* and *F. proliferatum.* They have a long-chain hydrocarbon in their structure, which is considered the source of FBs toxicity (Rheeder et al., 2002). There are around 12 types of FBs including FB1, FB2, and FB3. All of them have similar chemical structures and can contaminate corn (Sydenham et al., 1996), wheat, and barley (Aziz et al., 2004; Levasseur-Garcia, 2018). The FBs can also be detected in rice, sorghum, triticale, cowpea seeds, soybeans, and asparagus (Desjardins, 2006; Schatzmayr and Streit, 2013). The mycotoxins in this group are thermostable even under cooking conditions, but their concentration can be reduced by processing at a high temperature (above 150ºC) (Marasas, 2001).

The main factors promoting FBs production are the infestation of plants by insects, high temperature, and water activities. The FBs are mainly produced under field condition before harvest and during the beginning of drying, but in rare cases, they may be produced during storage under extreme temperature conditions (Mannaa and Kim, 2017).

The FB1 and FB2 (Figure 1) are considered the most important FBs. The FB1 is considered highly toxic for mammals. The International Agency for Research on Cancer (IARC) has classified FB1 as Group 2B mycotoxin (IARC, 2002). The FBs have been reported to cause many health problems in animals, like the

equine leukoencephalomalacia (ELEM) in horse and pulmonary edema in swine (CAST, 2003; Piva et al., 2005). In humans, a relationship has been observed between the occurrence of esophageal cancer and the consumption of corn containing FBs (CAST, 2003). Moreover, FB1 has been reported to possess teratogenic (Marasas et al., 2004) and carcinogenic (Boppana et al., 2017; Gelderblom et al., 2001; Lemmer et al., 1999) properties. Several surveys have raised concerns regarding the extent of FB1 contamination and its impact on mammal's health and productivity (Rodrigues and Naehrer, 2012; Boutigny et al., 2014; Cendoya et al., 2014; Abd-El Fatah el al., 2015).

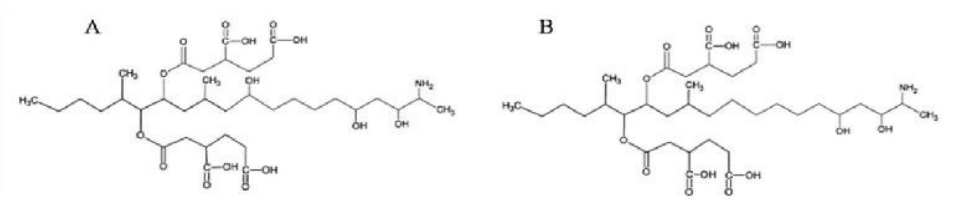


Figure 1. Chemical structures of FB1 (A) and FB2 (B) (modified from Mohamed, 2010).

Table 1 shows the morphological identification and FB production of 51 isolates from maize grains that were collected from three big governorates of Egypt's delta region (B: Al Beheira, K: Al Qalyubia, and D: Al Dakahlia). The most frequent species was *Fusarium verticillioides* that represented 96% of the isolates. Only 4% of the isolates (2 isolates) belonged to *Fusarium proliferatum.*

Table 1. Morphological identification of *Fusarium*, detection and identification of fumonisin-producing fungi, and analysis of FB production by HPLC (Abd-El Fatah et al., 2015).

Isolate code	**Morphological and microscopic identification**	**Molecular detection and identification of FB-producing fungi**			**HPLC analysis of fumonisin B1 mg/kg**
		Taqfum-2F Vpgen-3R PCR	**Verpro-F VERTI-R PCR**	**Fumonisin-producing species**	
B1	*F. verticillioides*	+	+	*F. verticillioides*	N.D.
B2	*F. verticillioides*	+	+	*F. verticillioides*	N.D.
B3	*F. verticillioides*	+	+	*F. verticillioides*	120
B4	*F. verticillioides*	+	+	*F. verticillioides*	100
B5	*F. verticillioides*	+	+	*F. verticillioides*	120
B6	*F. verticillioides*	+	+	*F. verticillioides*	41

B7	*F. verticillioides*	+	+	*F. verticillioides*	N.D.
B8	*F. verticillioides*	+	+	*F. verticillioides*	N.D.
B9	*F. verticillioides*	+	+	*F. verticillioides*	5.3
B10	*F. verticillioides*	+	+	*F. verticillioides*	4.40
B11	*F. verticillioides*	+	+	*F. verticillioides*	N.D.
B12	*F. verticillioides*	+	+	*F. verticillioides*	N.D.
B13	*F. verticillioides*	+	+	*F. verticillioides*	30
B14	*F. proliferatum*	+	+	*F. verticillioides*	25
B15	*F. verticillioides*	+	+	*F. verticillioides*	25
B16	*F. verticillioides*	+	+	*F. verticillioides*	380
B17	*F. verticillioides*	+	+	*F. verticillioides*	90
B18	*F. verticillioides*	+	+	*F. verticillioides*	200
B19	*F. verticillioides*	+	+	*F. verticillioides*	59
B20	*F. verticillioides*	+	+	*F. verticillioides*	175
K1	*F. verticillioides*	+	+	*F. verticillioides*	N.D.
K2	*F. verticillioides*	+	+	*F. verticillioides*	10.5
K3	*F. verticillioides*	+	+	*F. verticillioides*	N.D.
K4	*F. verticillioides*	+	+	*F. verticillioides*	1.4
K5	*F. verticillioides*	+	+	*F. verticillioides*	3.5
K6	*F. verticillioides*	+	+	*F. verticillioides*	20
K7	*F. verticillioides*	+	+	*F. verticillioides*	100
K8	*F. verticillioides*	+	+	*F. verticillioides*	30
K9	*F. verticillioides*	+	+	*F. verticillioides*	50
K10	*F. verticillioides*	+	+	*F. verticillioides*	555
K11	*F. verticillioides*	+	+	*F. verticillioides*	30
K12	*F. verticillioides*	+	+	*F. verticillioides*	54
K13	*F. verticillioides*	+	+	*F. verticillioides*	75
K14	*F. verticillioides*	+	+	*F. verticillioides*	13
K15	*F. verticillioides*	+	+	*F. verticillioides*	38

K16	*F. verticillioides*	+	+	*F. verticillioides*	260
K17	*F. verticillioides*	+	+	*F. verticillioides*	73.5
K18	*F. verticillioides*	+	+	*F. verticillioides*	20
K19	*F. verticillioides*	+	+	*F. verticillioides*	8.3
D1	*F. verticillioides*	+	+	*F. verticillioides*	35
D2	*F. verticillioides*	+	+	*F. verticillioides*	12
D3	*F. verticillioides*	+	+	*F. verticillioides*	65
D4	*F. verticillioides*	+	+	*F. verticillioides*	175
D5	*F. verticillioides*	+	+	*F. verticillioides*	30
D6	*F. verticillioides*	+	+	*F. verticillioides*	53
D7	*F. verticillioides*	+	+	*F. verticillioides*	N.D.
D8	*F. verticillioides*	+	+	*F. verticillioides*	N.D.
D9	*F. verticillioides*	+	+	*F. verticillioides*	30
D10	*F. verticillioides*	+	+	*F. verticillioides*	62.5
D11	*F. verticillioides*	+	+	*F. verticillioides*	285
D12	*F. proliferatum*	+	+	*F. verticillioides*	20

N.D. = not detected

1.2.2 Biosynthesis of Fumonisins

The biosynthesis of FBs in the *Fusarium* species mainly requires a 15-gene cluster (FUM1 and FUM6-FUM19) that can be conserved between several fungal genera (Desjardins, 2006; Proctor et al., 2013). However, unlike the biosynthetic gene clusters of natural products from fungi, there is no pathway-specific regulatory gene for FB biosynthesis pathway in the FUM cluster of the *Fusarium* species (Woloshuk et al., 1994; Proctor et al., 2003; Flaherty and Woloshuk, 2004). The proposed biosynthetic pathway for FBs is shown in Figure 2. The function of some FUM genes are known, for example, FUM13 encodes a C-3 ketoreductase of FBs (Desjardins, 2006; Medina et al., 2013), FUM3 encodes a 2-ketoglutarate-dependent dioxygenase that catalyzes the conversion of FB B3 to B1 (Proctor et al., 2003; 2013), and FUM3P catalyzes the C-5 hydroxylation (Desjardins, 2006; Lazzaro et al., 2012). The known regulatory genes of FB biosynthesis are not linked to the FUM cluster and include FCC1, FCK1, PAC1, ZFR1, and GBP1. It has also been reported that *A. niger* strains are able to produce FB2 (Frisvad et al., 2007; Aerts et al., 2018).

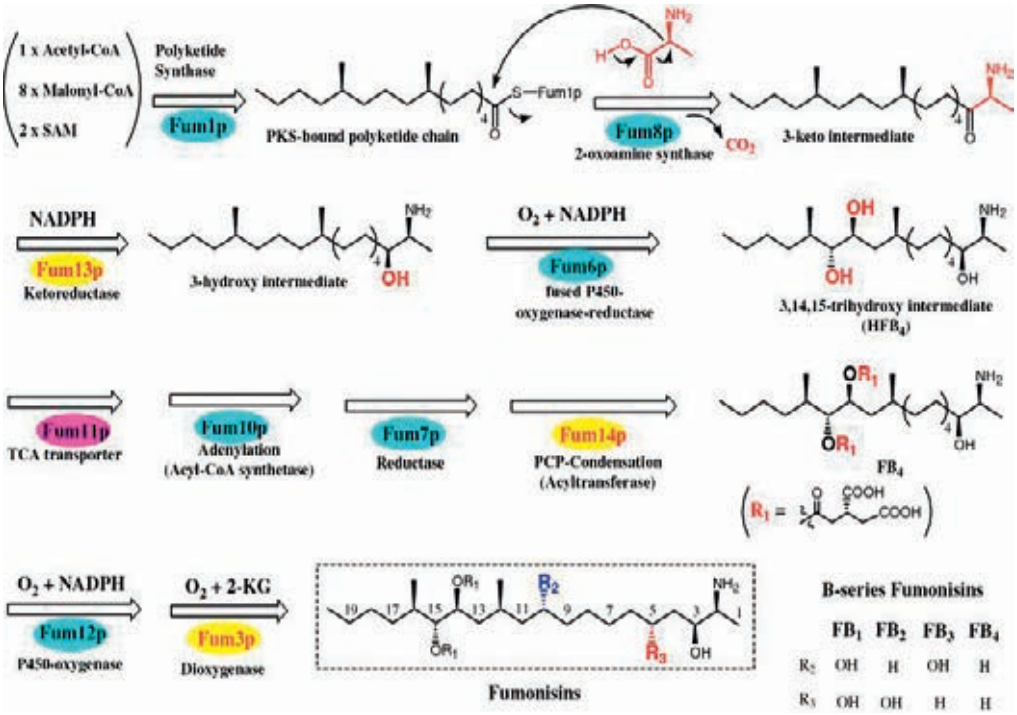


Figure 2. Biosynthesis of FBs (modified from Du et al., 2008).

1.2.3 Trichothecenes

The TCs are a big family of toxic fungal secondary metabolites including many significant mycotoxins related to the crop grains like T-2, NIV, and DON (McCormick et al., 2011; Proctor et al., 2018). They are tricyclic sesquiterpenoid chemically and their toxic effects are caused due to the inhibition of the ribosomal protein biosynthesis. Four main types of TCs have been identified from TC-producing *Fusarium* species. These types are named A, B, C, and D. Types C and D are not linked to FHB. Type A and B are the main toxins associated with FHB and related human and animal health problems (Foroud and Eudes, 2009; Ates et al., 2013). The T-2 and HT-2 TCs belong to type A TCs. *F. sporotrichioides* and *F. langsethiae* are considered the main producers of type A TCs. Type B TCs includes both DON and NIV that are mainly produced by *F. graminearum* and *F. culmorum*. Type A TCs are more toxic for mammals compared to type B TCs, while type B TCs are more phytotoxic than type A TCs. For instance, T-2 has been reported to be approximately 10-times more toxic than DON, which is considered the most important mycotoxin involved in FHB (Foroud and Eudes, 2009).

Generally, TCs are stable under high temperature, storage conditions and food processing (Freire and da Rocha, 2017). The main effects on mammalian health are reduced feed ingestion, vomiting and immune suppression and the effects are mainly dependent on the toxin concentration in the commodity (Milićević et al., 2010; Freire and da Rocha, 2017). Exposures to TCs can cause growth obstruction and reproduction disorder both in humans and animals, but in plants, these *Fusarium* toxins can inhibit seedling growth and plant cells regeneration. TCs may also cause additional toxic effects such as inhibition of DNA synthesis and cell multiplication and destruction of mitochondrial function. Furthermore, these toxins can induce apoptosis of mammals and in plants, they may stimulate programmed cell death (Foroud and Eudes, 2009; Freire and da Rocha, 2017). The chemical structures of different types of TCs are presented in Figure 3.

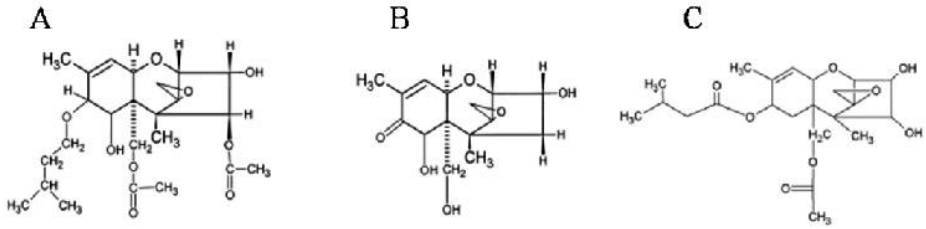


Figure 3. Chemical structures of T-2 toxin (A), DON (B) and HT-2 (C) (modified from De Ruyck et al., 2015).

1.2.4 Biosynthesis of Trichothecenes

TCs are toxic secondary metabolites, which were first found in *Trichothecium roseum*. Therefore, the name of these mycotoxins came from the name of this genus (Freeman and Morrison 1949; Alexander et al., 2009), but they are mainly produced by *Fusarium* species. More than 180 TCs have been isolated from different fungi species including *Fusarium* species*, Myrothecium* species*, Stachybotrys* species*, Trichoderma* species, *and Thrichothecium* species (Eriksen, 2003; Surup et al., 2014). The biosynthesis of different types of TCs is species- and even strain-specific and based on the suitability of environmental conditions. However, it is controlled genetically (Desjardins 2006; Paterson and Lima 2010; Proctor et al 2018).

Primarily, around 15 genes (including catalytic and regulating) take part in the TCs' biosynthesis pathway in *Fusarium* species (Alexander et al., 2009; Merhej et al., 2011). The responsible genes for TCs' biosynthesis are located at 3 loci on various chromosomes in the *Fusarium* species. The main gene cluster in

TCs' biosynthesis pathway (TRI) consists of 12 genes located within a 25-kb genomic region (Kimura et al., 2001; Maeda et al., 2016). TRI1, TRI16, and TRI101 loci are responsible for the remaining 3 genes (Gale et al., 2005; Alexander et al., 2009). During the TCs' biosynthesis, oxygenation, isomerization, acetylation, hydroxylation, and esterification are considered the main steps (Desjardins, 2006; Desjardins and Proctor, 2007; Kimura et al., 2007; Merhej et al., 2011). Trichodiene synthase, that is encoded by TRI5, first transforms farnesyl pyrophosphate into trichodiene. Then, during the following 4 stages, isotrichotriol is synthetized via C-2 hydroxylation and 12, 13 epoxidations, followed by two hydroxylation reactions (TRI4 is the responsible gene as multifunctional cytochrome P450). The following 2 steps are non-enzymatic and produce isotrichodemol. Finally, the TC skeleton is ready across the consistency of a C-O bond between the C-2 oxygen and C-11. After that calonectrin is produced via acetylation at C-3, hydroxylation at C-15 and the catalytic activity of TRI101, TRI1, and TRI3. The above-mentioned biosynthesis process is the same for both type-A and -B TCs (Figure 4).

The remaining steps are specific for different *Fusarium* strains. In the case of DON production by *F. graminearum* strains, calonectrin is transformed to either 3-ADON or 15-ADON. Eventually, DON is produced by the catabolic actions of TRI1 and TRI8. The 3-ADON or 15-ADON production is regulated by variation in the esterase coding parts of TRI8. On the other hand, the NIV is produced via hydroxylation at C-4 of DON, or across transformation of calonectrin to 3,15-diacetoxyscirpenol (3,15-DAS), then adding a ketone at C-8 position with the help of TRI7 and TRI13 genes (Desjardins, 2006; Desjardins and Proctor, 2007; Kimura et al., 2007; Merhej et al., 2011; Nakajima et al., 2015; Maeda et al., 2016). The 3,15-DAS is the core substance for type-A TCs' biosynthesis in *F. sporotrichioides*, (the main source of type-A TCs). In the synthesis T-2 toxin, C-4 acetylation of 3,15-DAS is implemented by the action of TRI7, TRI8, and TRI1 genes. The 3,15-DAS is also considered the primary substrate for HT-2 production. Due to the absence of TRI7 expression, HT-2 is produced via hydroxylation of 3,15-DAS followed by addition of isovalerate to C-8 site (Foroud and Eudes, 2009).

1.3 Toxigenic *Aspergillus* species

The *Aspergillus* genus is considered one of the first studied and documented genera of fungi. These fungi are diverse both ecologically and metabolically (Pildain et al., 2008; Ehrlich and Brian, 2014; Frisvad et al., 2019). The genus acquired its name, in 1729, from the microscopic characteristics of the fungi, spore-bearing constructions that look like an aspergillum (a brush used to spray

holy water in the Roman Catholic Church). Therefore, any fungi that are able to produce aspergillum-like asexual spore heads are classified in the genus *Aspergillus*.

According to, Geiser et al., 2006 and, Geiser et al., 2008 there are around 250 known *Aspergillus* species identified and classified inside the phylum of Ascomycota (Arone et al., 2016). The popular examples of toxigenic *Aspergillus* molds are *A. parasiticus*, *A. flavus*, *A. nidulans*, *A. fumigatus*, *A. clavatus*, and *A. niger*. The *Aspergillus* section *Flavi* including *A. flavus* and *A. parasiticus* are able to produce AFs in a huge number of food and feed materials. Some other mycotoxins producing *Aspergillus* species include *A. nomius*, *A. pseudotamarii*, *A. bombycis*, and *A. ochraceoroseus* (Cotty, 1994; Cotty and Bhatnagar, 1994; Bhatnagar et al., 2003; Frisvad et al., 2005; Frisvad et al., 2019).

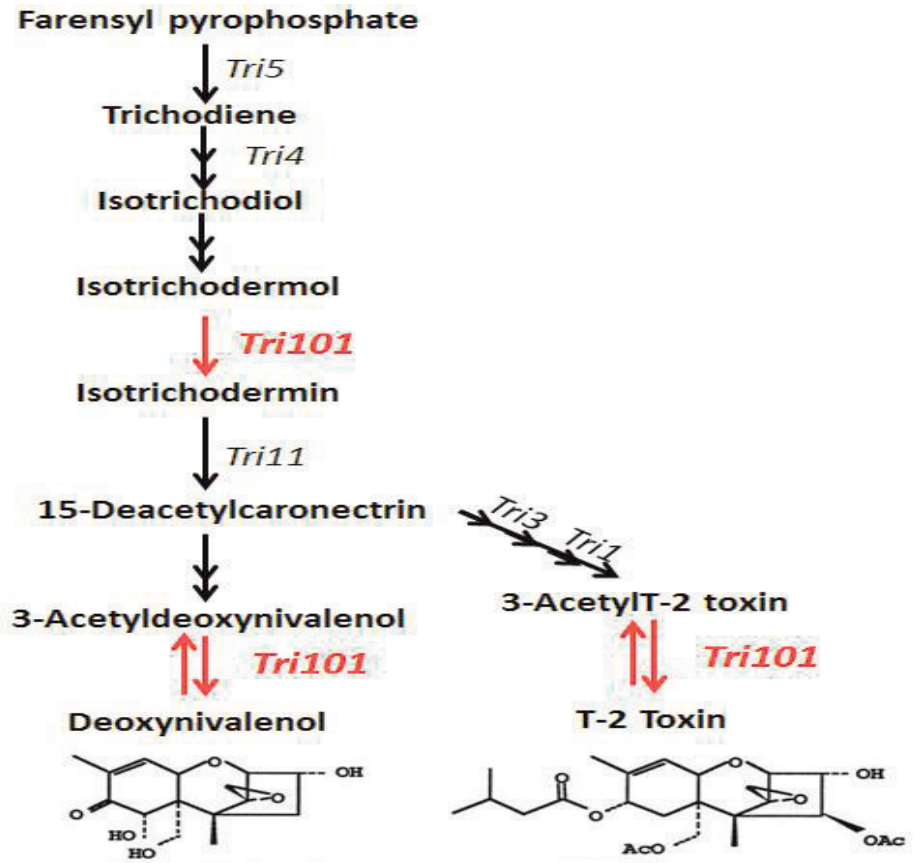


Figure 4. Biosynthesis of trichothecenes (modified from Wang et al., 2015).

Abbreviations: Tri5, the trichodiene synthase gene; Tri4, multifunctional trichodiene hydroxylase; TRI101, trichothecene 3-O-acetyltransferase; Tri11, isotrichodermin 15-hydroxylase; Tri3, trichothecene 15-O-acetyltransferase; Tri1, encoded trichothecene 7,8-dihydroxylase.

Aspergillus species are ubiquitous and can grow in diverse habitats such as soil, water, and different food and feed materials. They do not require any specific nutrients. They play an important role in the natural cycling of nutrients as they decay the plant debris as saprophytes. *Aspergillus* section *Flavi* can grow over a temperature range of 17°C –42°C but the optimum temperature for AFs' production is 25°C–35°C. Czapek dox agar and potato dextrose agar media can be used for mold growth under lab conditions (Hara et al., 1974; Hedayati et al., 2007; Arzanlou et al., 2016).

The *Aspergillus* colonies morphologically represent powder-like surface and hyaline septate hyphae with regular sac at their tips. Microscopically, *Aspergillus* species can be distinguished based on their structure criteria such as sclerotia and the colony color. The traditional identification of *Aspergillus* using culture characteristics such as colony shape and pigment production is not sufficiently efficient and reliable. The detection and identification of other AF-producing fungi are often based on ultraviolet (UV) documentation of secreted toxins in the agar medium (Lin and Dianese., 1976; Basaran and Demirbas, 2010). However, the polymerase chain reaction (PCR)-based methods are more efficient and reliable for identification, detection, and quantification of mycotoxigenic fungi (Godet and Munaut, 2010; Jurjević et al., 2015; Zarrin and Erfaninejad, 2016).

1.3.1 Aflatoxins

AFs are mainly produced by *A. flavus* and *A. parasiticus* species. There are 4 types of AFs, that is, B1, B2, G1, and G2. The chemical structures of these AFs are shown in Figure 5 (Lerda, 2010). AF-B1 is the most abundant and toxic of the 4 AF types (European Commission, 2010). Many crops and food products are susceptible to *Aspergillus* species infections. For example, corn, peanut, cottonseed, Brazil nuts, and pistachio nuts are considered to be at the highest risk of contamination with AFs (Busby and Wogan, 1984; Shephard, 2008; Kara et al., 2015). The name "aflatoxin" was obtained by combining the letter "A" from the "***A**spergillus"* and the letters "fla" from the "**fla**vus". The suffix "B" of AFs B1 and B2 was taken from the **b**lue color fluorescence under UV, on the other hand, the "G" letter came from the yellow-**g**reen color fluorescence of the toxins (AFs G1 and G2) under UV light (Nesbitt et al., 1962; Van der Zijden et al., 1962).

Aflatoxicosis are the diseases caused by AF intake. The disease was reported for the first time in the United Kingdom, which resulted in the death of 100,000 turkeys and was named 'Turkey X disease'. Acute aflatoxicosis may cause death and chronic aflatoxicosis may lead to liver cancer, immune suppression, and other pathological displays (Blount, 1961; Shephard, 2008). AF-producing

fungi are commonly found in soil habitat worldwide and they commonly infest crops like maize, groundnut, and wheat. AFs can enter in food and feed products at any stage of the food production. Due to the increasing transportation of agricultural products, AF is becoming a serious problem all over the world. In this regard, many countries have enacted legislation to reduce the risk of AF in the food and feed materials (Haumann, 1995; Carvajal-Campos et al., 2017). The maximum allowed limit for total AFs (sum of AFs B1, B2, G1, and G2) in many countries ranges from 1 to 35 µg/kg for the food products with an average of 10 µg/kg and from 0 to 50 µg/kg for the feedstuffs with a threshold of 20 µg/kg (Abbas, 2005; Alshannaq and Yu, 2017). The maximum allowed AF limit for peanuts in Brazil and USA is 20 µg/kg, while Canada and the European Union (EU) have established a limit of 15 µg/kg (Fonseca, 2011). In addition, the European Commission (EC) has established a maximum limit of AF at 0.02 mg/kg for the feed products (EC, 2003).

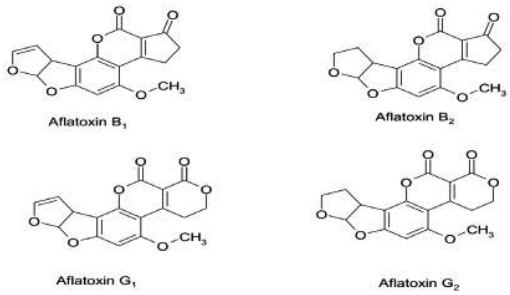


Figure 5. Chemical structures of AFs B1, B2, G1, and G2 (modified from Lerda, 2010).

1.3.2 Aflatoxin biosynthetic pathway

Genetic information for the biosynthesis of fungal natural products like mycotoxins is mainly present in gene clusters that can consist over 10,000 bases (Keller et al., 2005; Brakhage, 2012). A cluster generally consists of 2 or more genes that work together to synthesize a secondary metabolite. In *Aspergillus* species, the complete DNA data is included in 8 chromosomes (Robinow and Caten, 1969; Keller et al., 2005). Within this organization, the genes responsible for AFs biosynthesis are present in the 54th gene cluster that has a span of 80 kb and presents on chromosome 3 (Georgianna and Payne, 2009). The gene cluster consists of 30 genes and the AF biosynthesis pathway is regulated mainly by 2 genes–aflR and aflS (Figure 6) (Chang, 2003; Yu, 2012).

The A. flavus and *A. parasiticus* are the main species involved in the AFB1 contamination; thus, several studies on the AF biosynthesis pathway have

utilized gene cluster in these species (Jiujiang et al., 1995; Yu et al., 2004; Ren et al., 2018). The AF biosynthesis pathway has also been studied in *A. nidulans* (the main source of sterigmatocystin) because sterigmatocystin is the precursor of AFB1 (Ehrlich, 2009). The homology of the AF biosynthesis pathway gene cluster between *A. parasiticus* and *A. flavus* is around 90%–99%, while in the case of *A. parasiticus* and *A. nidulans,* it is about 55%–75% (Yu et al., 2004; Ehrlich et al., 2005). There is a difference between the genes involved in the biosynthesis of B-type and G-type AFs. The 3 genes–aflU, aflF, and nadA participate only in the AFG biosynthesis (Ehrlich et al., 2004; Ehrlich et al., 2005). The first gene (aflU) is responsible for the production of cytochrome P450 monooxygenase, the second gene (aflF) for an aryl alcohol dehydrogenase, and the third gene (nadA) for a reductase. The role of different genes in AFB1 biosynthesis pathway has been analyzed using advanced molecular techniques like Gene cloning and Expressed Sequence Tags (EST) (Zhang et al 2015). The development of these techniques is important in elucidating and predicting the functions of the genes involved in the AFB1 biosynthesis that take place in the presence of various enzymes (Kistler and Broz, 2015; Zhang et al., 2015).

1.4 Detection and identification of mycotoxin-producing fungi

Before the discovery of PCR, the detection and identification of mycotoxigenic fungi were mainly based on conventional mycological methods or chemical analysis of the secondary metabolites (like mycotoxins) produced by the fungus (Berbee and Taylor, 2001; Buchheidt et al., 2017). The conventional mycological methods or chemical analysis-based tests are time-consuming and labor-intensive. The conventional mycological protocols require isolation and cultivation of the fungi on several culture media for about 1 week for their precise identification (Abbas et al., 2004). Furthermore, extensive expertise is needed for the identification of the fungi species, especially the mycotoxin-producing fungi species: *Fusarium*, *Penicillium,* and *Aspergillus*. In the case of chemical analysis of the mycotoxins produced by the fungi, elaborated methods for sample preparation and expensive laboratory tools/materials are required (Berbee and Taylor, 2001).

The PCR-based techniques for the identification and detection of mycotoxin-producing fungi are based on DNA isolation from food/feed materials, which is followed by execution of PCR reaction using fungi species/strain-specific primers (Knoll et al., 2002). In addition, the use of new thermocyclers can reduce the PCR test time to less than 1 hour (Knoll et al., 2002). Nowadays, quantitative PCR is used for the multiplication of copy numbers of the genes

responsible for mycotoxin biosynthesis (Schnerr et al., 2001). The presence of a specific target sequence is the key for the mycotoxigenic fungi detection and identification by the PCR-based method. Thus, the genes that are critical for the mycotoxin biosynthetic are used as the target for precise detection and identification of the fungal species. However, when the genes involved in the mycotoxin production are identified, many sequences can be used as targets (White et al., 2015; Lamoth and Calandra, 2017).

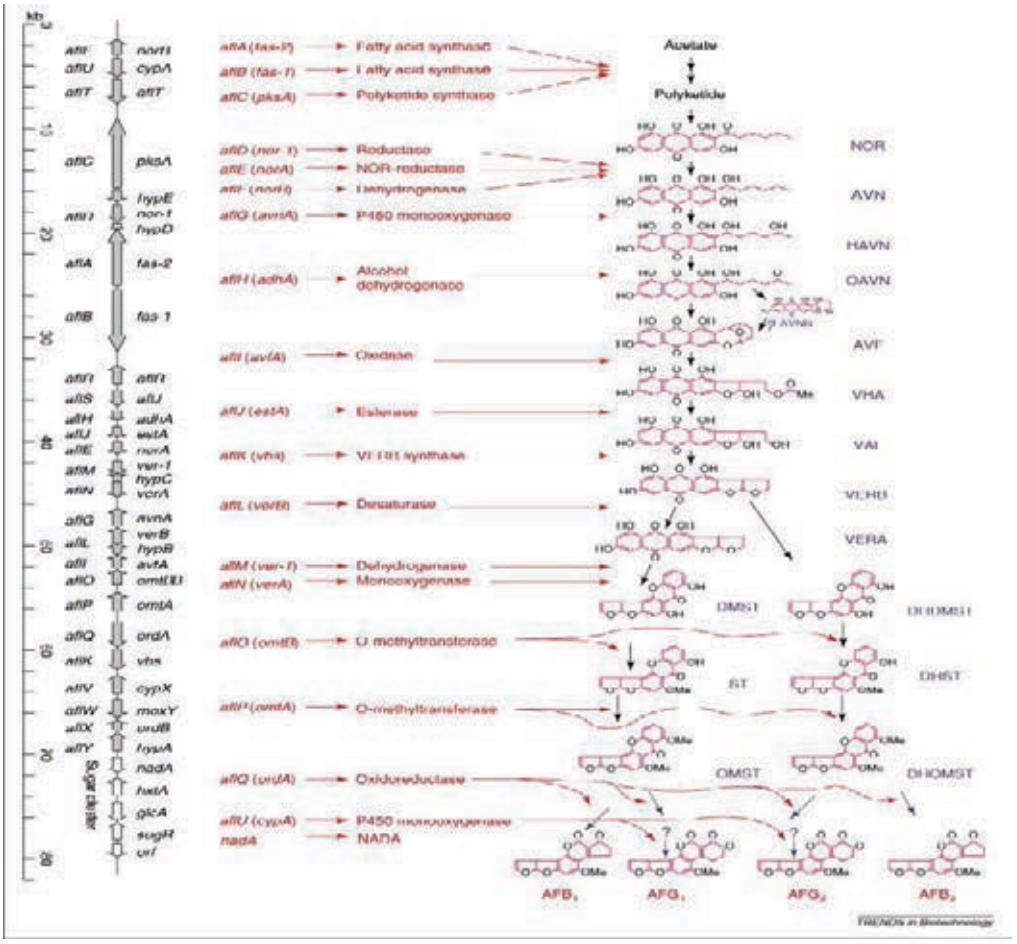


Figure 6. AF Biosynthesis Pathway (modified from Yu et al., 2004).

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.

1.4.1 Detection and identification of *Fusarium* species

The detection and identification of *Fusarium* species are still in some ways dubious. In fact, over 80 species are included in the *Fusarium* genus and the number is changing continuously, because of the taxonomy methods development (Leslie and Summerell, 2006). Therefore, many debates related to fungal taxonomy is still ongoing among the mycologists (Asan, 2011). This genus can be distinguished with the help of few morphological characteristics that are important for the differentiation of the various species. However, only the experts can discriminate the pathogenic *Fusarium* species based on the morphological criteria including macro- and micro-conidia and chlamydospores (Dongyou, 2009; Maryani et al., 2019).

In *Fusarium* species, the macroconidia are sickle form with several septa and look like a banana or a canoebut, while the microconidia are 0–3 septate and evolve from phialides. The chlamydospores have thick walls and can appear in several *Fusarium* species (Leslie and Summerell, 2006). The various forms of macroconidia are considered the most important trait in *Fusarium* species discrimination, which includes the production of asexual spores and the characteristic banana shaped macroconidia (Moretti, 2009). All researchers working on fungal taxonomy agree that to achieve the goal of real identification of the fungal species, single spore should be isolated and grown under optimum growth condition consisting of the appropriate culture media, temperature, and pH (Dongyou, 2009).

In contrast, the PCR-based approaches for fungi identification have helped a lot in revealing the neglected diversity in the *Fusarium* genus (O'Donnell et al., 2015). The fungal phylogeny based on molecular analysis has been applied by many scientists in *Fusarium* species' taxonomy. However, several phylogenetic relationships are still unclear due to the availability of limited data about the phylogenetic analysis implemented for this genus (Watanabe et al., 2011). DNA sequencing of a species-specific gene using the PCR tests must be done to identify a fungus. The internal transcribed spacer (ITS) region was selected as the official "barcode" locus for the fungi identification (Schoch et al., 2012). However, the primers based on ITS region are not entirely species-specific, because some of the *F. sporotrichioides* isolates have an identical ITS sequence with *F. langsethiae* isolates (Yli-Mattila et al., 2004a). This is why for the *Fusarium* genus, the translation elongation factor 1-α (TEF) gene has been chosen as a single-locus marker for the identification of this genus (Geiser et al., 2004; O'Donnell et al., 2015). In addition, many species-specific PCR assays have been developed but they need to be validated widely especially for isolates

from different plant materials and/or in different geographic locations (Van der Lee et al., 2015; Rahjoo et al., 2008). Several mycologists have used species-specific PCR test to identify many *Fusarium* species, such as *F. culmorum* (Nicholson et al., 1998; Sanoubar et al., 2015), *F. graminearum* (Waalwijk et al., 2003; Sanoubar et al., 2015), *F. poae* (Parry and Nicholson, 1996), *F. pseudograminearum* (Aoki and O'Donnell, 1999), *F. langsethiae* (Yli-Mattila et al., 2004a, 2004c; Konstantinova and Yli-Mattila, 2004), and *F. langsethiae* using Random Amplification of Polymorphic DNA (RAPD)-PCR product (Wilson et al., 2004). The gene FUM1 can be used to detect FB1 production by *F. Verticillioides* (López-Errasquín et al., 2007). Some other researchers have used real-time PCR or quantitative PCR (qPCR) to quantify the contamination of crop by *Fusarium* species, for example, determination of the copy number of the TRI5 gene in relation to the DON content of a grain sample (Kim and Yun, 2011; Bilska et al., 2018).

1.4.2 Detection and identification of *Aspergillus* species

AFs may be found in food and feed products even if there is no spoilage in them. The insects (including wasps and bees) and birds are considered the main vectors for fungal spore transmission to food and feed materials where the spores may find the optimal conditions for both growth and AF production (Misra and Thirumalaisamy, 2012). The infestation of egg cells of plants during the flowering stage by AF-producing fungi can be another source for seeds contamination by AFs (Hill et al., 1983).

Although in the *Aspergillus* species, mycelium can have an endotrophic relationship without any harmful effect on the crop's health, AFs may be secreted in the plant in high concentrations, especially during cultivation under drought conditions. Therefore, plant-based products may already contain AFs at harvest stage. However, the toxins level during harvest is usually less than that found in stored commodities and this level can be economically critical (Hansen and Jung, 1973; Moss, 2002). Therefore, the early and precise identification/detection of aflatoxigenic fungi is crucial for food security.

There are several methods for fungal identification and detection including conventional mycological procedures (Pitt and Hocking, 2009), enzyme-linked immunosorbent assays (Notermans et al., 1986), and PCR-based methods (Shapira et al., 1996; Haugland et al., 2002; Luo et al., 2012; Frisvad et al., 2019). The conventional identification and classification of *Aspergillus* species are mainly based on the morphological and the macroscopic criteria of the fungi colony. Initially, the *Aspergillus* genus was identified without using biochemical or physiological criteria of the fungi (Raper and Fennell, 1965). Subsequently,

the colony color and the physiological characteristics were added to the taxonomic methods, as described by Murakami (1976) and Murakami et al. (1979). These included several methods such as the production of pigment in Czapek agar using nitrite as single nitrogen matter, secretion of extracellular enzymes and acids, and other physiological characteristics (Klich and Pitt, 1988). Later, several fungi secondary metabolites were used for fungi taxonomy, based on their bioactive properties (Frisvad, 1989; Frisvad et al., 1998 and 2004; Samson et al., 2004). *Aspergillus* species secrete many mycotoxins and other secondary metabolites, which can be used in fungal taxonomy (Frisvad et al., 2007 and 2008).

However, the conventional mycological taxonomic and identification methods that were used to test the food spoilage were labor-intensive and required many facilities including mycological expertise. Furthermore, these methods required more than 5 days to recognize the fungi infection. After applying the PCR-based methods for identification and detection of microorganisms under lab condition using species-specific primers, several protocols were developed for the detection of fungi in different food commodities (Cocolin et al., 2002; Aymerich et al., 2003; Amagliani et al., 2006). PCR-based methods including both qPCR and conventional PCR have been reported to be more precise and specific for identification, detection, and quantification of mycotoxin-producing fungi (Shapira et al., 1996; Haugland et al., 2002). Few researchers have described these protocols, for example, Geisen et al. (1996) and Shapira et al. (1996). reported a PCR-based method for mycotoxin-producing fungi detection targeting specific DNA sequences in the AF biosynthesis pathway. Also, multiplex RT-PCR containing 4–5 primer pairs of various combinations of aflD, aflO, aflP, aflQ, aflR, and aflS (aflJ) have been used to detect toxigenic fungi (Degola et al., 2007). In addition, Mayer et al. (2003) were able to study the correlation of the copy numbers of the nor-1 gene and colony forming units (cfu) of *A. flavus* in pepper, paprika, and maize. Recently, Luo et al. (2014) used real-time quantitative loop-mediated isothermal amplification for rapid detection of AF producing fungi in food.

Although simple PCR has provided quick and sensitive results, these methods require specific lab tools, which can make such protocols expensive and time-consuming for rapid monitoring. The qPCR assessments are much quicker compared to the simple PCR because the detection is based on the production of fluorescence during PCR cycles and there is no need for DNA gel electrophoresis (Jothikumard and Griffiths, 2002). However, the qPCR requires expensive and sophisticated tools that are not usually available for routine detection in processing facilities, especially in small food processing units.

2. AIMS OF THE STUDY

Mycotoxin contamination is a serious concern for human and animal health. To date, we do not know how to detoxify materials that are contaminated by mycotoxins in ways that retain their edibility too. Therefore, preventing mycotoxins from entering the food-chain is an important approach. This needs early identification, taxonomy, detection, and quantification of mycotoxin-producing fungi. The conventional methods for the identification, taxonomy, detection, and quantification of toxigenic fungi are challenging because they require a high level of expertise and a set of sophisticated equipment. The aim of this study is to use the practical, rapid, and more reliable molecular approaches to identify, classify, detect, and quantify the most important mycotoxin-producing fungi including AF-producing microorganisms, FB-producing fungi, and TC-producing *Fusarium* species.

The specific aims were:

1. To isolate, identify, and quantify FB-producing species in maize, wheat, and soil samples; and to test Potato Dextrose Agar (PDA)-Eppendorf as a long-term preservation method for *F. verticillioides* isolates. (Paper I)
2. To monitor *Fusarium* mycotoxins and the fungal DNA levels, and the correlation between them in Finnish oat samples; and to investigate the main populations of *F. graminearum* isolates in Europe, which may have specialized to different host plants. (Paper II)
3. To investigate DON levels in Finnish cereal grain with accredited GC-MS and Rida®Quick methods; to compare these with *F. graminearum* DNA levels; and to measure the effects of grain grinding methods on DON and *F. graminearum* DNA levels. (Paper III)
4. To confirm the identification of the Iranian *F. sibiricum* isolate and to find new ways for clear-cut discrimination of the closely related species, *F. langsethiae* and *F. sibiricum,* by combining morphological characters and optimized PCR protocols with species-specific primers. (Paper IV)
5. To (a) generate information about AFs production and the diversity of *A. flavus* and *A. parasiticus* species; (b) to test primers designed for AF detection using these isolates; (c) to utilize PCR results and AFs' analysis data in classifying the isolated aflatoxigenic fungi to genotypes and chemotypes. (Paper V).

3. METHODOLOGY

An overview of the key methods used in the thesis is provided below. More comprehensive descriptions of the materials and methods can be found in Papers I, II, III, IV, and V.

3.1 Samples, fungal isolation, and isolates

3.1.1 Grain samples for *Fusarium* DNA and trichothecene analyses

a. In Paper II, oat (30), wheat (20), and barley (20) samples from different parts of Finland were collected for DON and *F. graminearum* DNA level analysis.

b. In Paper III, 10 oat grain samples of the Year 2010, 15 samples of the Year 2011, and 40 samples of the Year 2012 were analyzed for DON, 3ADON, 15ADON, T-2, and HT-2 toxins at MTT Agrifood Research Finland (Jokioinen, Finland;). In addition, 20 oat samples of the Year 2012 were analyzed for DON, 3ADON, 15ADON, T-2, and HT-2 toxins.

3.1.2 Samples for isolation of aflatoxin-producing and fumonisin-producing fungi

Thirty-five samples were collected from each country. The Egyptian samples were represented by 9 soil, 16 maize, 9 wheat samples, and 1 lab bench swab sample. In the Philippines, samples were divided into 23 soil, 6 maize, 2 coconut, and 4 peanut samples (Paper I and V).

3.1.3 *Fusarium* isolates

All isolates analyzed in Paper IV were stored in a collection at All-Russian Plant Protection Institute (VIZR) (St. Petersburg-Pushkin, Russia). The Iranian *Fusarium* isolate, which was previously identified as *F. langsethiae* (Kachuei et al., 2009) was kindly provided by Dr. Reza Kachuei. Dr. Simon Edwards forwarded the isolate to Dr. Tapani Yli-Mattila. Isolate IBT 9959 was kindly provided by Dr. Ulf Thrane. The DNA of the single spore isolate G243 (*F. graminearum*) was used as the standard for quantification of *F. graminearum* DNA, while the DNA of the single spore isolates S55 and C192 were used as the standard for quantification of *F. sporotrichioides/F. langsethiae* DNA and *F. culmorum* DNA as described by Yli-Mattila et al. (2008 and 2011) (Papers II and III).

3.1.4 Isolation of fumonisin-producing and aflatoxin-producing fungi

The isolation was performed on Potato dextrose agar (PDA) medium or on *Aspergillus flavus* and *A. parasiticus* agar (AFPA) medium:

(a) For grain samples; 5 grains of maize and 10 grains of wheat samples were surface-disinfected with 5% sodium hypochlorite (NaOCl) for 1 min followed by rinsing 3 times with sterile water. The disinfected grains (5 maize and 10 wheat grains/dish and 3 petri dishes/sample) were placed on PDA or AFPA for 3-5 days at 25°C.

(b) For soil and coconut samples; 2 grams of each sample was suspended in 6 mL sterile distilled water in sterile polystyrene tubes and mixed on a rolling mixer for 20 min (Donner et al., 2009). 100 µL of each supernatant was inoculated on 90-mm petri dishes containing PDA or AFPA. After the completion of the incubation period, the *Fusarium* colonies were transferred to PDA plates and single spore was derived from each isolate by serial dilutions of spore suspension and spreading them on ager-water plates, this was followed by incubation at 25°C for 12h. Then, 3 single spores were picked under a microscope and incubated on a PDA medium for 24h. The youngest colony was used as the source for inoculation of PDA-Eppendorf. A copy of all single spore isolates was preserved in the small culture collection at the Department of Biochemistry, University of Turku (UTU), Finland and another copy of them was sent to the culture collection of the VIZR.

3.2 Identification and preservation of fungal isolates

3.2.1 Identification of fumonisin- and trichothecene-producing *Fusarium* isolates

a. Traditional identifications

All FB- and TC-producing *Fusarium* isolates were identified based on the macroscopic and microscopic characteristics described by different researchers (Barnett and Hunter, 1972; Nelson et al., 1983) at the Laboratory of Mycology and Phytopathology, VIZR (Paper I).

b. Molecular methods for re-identification

Primer pairs, Verprof, and VERTI-R (which are based on specific PCR products of FB producing *F. verticililoides* strains); and Taqfum- f2, VPgen- R3 (Paper I) (which are specific for *F. verticillioides*, *F. proliferatum, F. globosum,* and *F. nygamai)* were used for the identification of fumonisin-producing fungi as described by Waalwijk et al. (2008).

For the identification of *F. langsethiae* and *F. sporotrichioides*, primer pairs FlangF3/lanspoR1 and FsporF1/lanspoR1 (Paper IV) (which are based on species-specific RAPD-PCR products of European *F. langsethiae* and *F. sporotrichioides* isolates) were used as described by Wilson et al. (2004). Bio-

Rad thermal cycler S1000 was used in VIZR and the PTC-200 DNA Engine thermal cycler was used at the UTU for PCR amplification. Also, another protocol of Yli-Mattila et al. (2004a) with a lower annealing temperature was used. Primer pairs PfusF/FspoR and PfusF/FlanR based on ITS sequences were also used for the identification of *F. sporotrichioides* and *F. langsethiae* as described by Yli-Mattila et al. (2004a and 2011).

Aliquots (5 µL), from each PCR product, were analyzed by electrophoresis in a Tris-borate-EDTA (TBE) buffer in 1.0% agarose gels by using the ChemiDoc MP Imaging System (Bio-Rad) in VIZR and the FluorchemTM Advanced Fluorescence, Chemiluminescence, and Visible Light Imaging (Alpha Innotech Corporation) system at the UTU.

3.2.2 Identification of aflatoxins producing fungi

a. Traditional identifications

The positive AF-producing isolates were identified according to phenotypic characteristics based on the growth patterns on AFPA and Czapek yeast autolysis (CYA) (Rodrigues et al. 2011).

b. Molecular identification of the isolates

The identity of one strain from each genotype and chemotype were performed by molecular analysis, which involved the sequencing of their ITS region. The gene sequence of each isolate was amplified using the primers ITS1 and ITS4 and the PCR cycling conditions were as follows: 3 min pre-denaturation at 94°C, 35 cycles of amplification (30 s at 94°C, 40 s at 55°C, 1 min extension at 72°C), and a final extension step that was performed for 7 min at 72 °C. The amplified PCR products were sequenced by The Institute for Molecular Medicine Finland (FIMM) and the DNA sequences were aligned using advanced BLAST searches (http://www.ncbi.nlm.nih.gov/). The sequences were submitted to GenBank.

3.2.3 Eppendorf-agar long term preservation

The single spore *Fusarium* isolates in addition to single spore *A. flavus* and *A. parasiticus* isolates were cultured in an Eppendorf tube containing half mL of PDA medium for one week at 25°C and then moved to the cold temperature conditions (4°C). The viability of the isolates was tested by growing them on PDA petri dishes every 3 months for 27 months (Paper I).

3.3 DNA extraction and molecular detection and quantification of mycotoxigenic fungi

3.3.1 DNA extraction from fungi

The *Aspergillus* and *Fusarium* isolates were cultured in 0.5 mL of malt extract broth medium (30 g malt extract and 5 g peptone per liter) in Eppendorf tubes at 25 °C for 3 days, after which the mycelium was transferred to a new Eppendorf tube. The DNA of the isolates was extracted using both octanol/isopropanol method as described by Paavanen-Huhtala et al. (1999) and GenElute™ Plant Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO, USA) as described by Yli-Mattila et al. (2008). Quality of the DNA was confirmed by using ITS1 and ITS4 (Paper III) primers and amplifying ITS region of fungal DNA as described by Yli-Mattila et al. (2004). Also, some isolates were cultured on potato dextrose agar (PDA; Scharlau Chemie S.A., Spain) at 25°C and the DNA was extracted using a modified CTAB (Hexadecyltrimethylammonium bromide) method according to Ausubel et al. (2002) (Paper IV). Total DNA was quantified by using a fluorescence-based Qubit fluorometer (Invitrogen, Carlsbad, USA) as described by Yli-Mattila et al. (2009).

3.3.2 DNA extraction from grains

Oat grains were ground using a hammer mill, KT-120 (Koneteollisuus Oy) with a 1 mm sieve in MTT, while the food company oat grains were ground at the UTU without sieving. DNA was extracted from the ground grain samples by using GenElute™ Plant Genomic DNA kit of Sigma, as described by Yli-Mattila et al. (2008). In Paper III, the oat grain samples from MTT and Food Company were ground at the UTU using a mill (Krups KM75 Coffee Grinder) without sieving, as described by Yli-Mattila et al. (Paper II), and DNA was extracted using GenElute™ Plant Genomic DNA kit of Sigma. Total DNA was quantified by using a fluorescence-based Qubit fluorometer (Invitrogen, Carlsbad, USA), as described by Yli-Mattila et al. (2009).

3.3.3 Molecular detection of aflatoxin-producing fungi

Primers pairs ver-1/ver-2 and ordAF/ordAR (Paper V), which are specific for ver-1 and ordA genes in AF biosynthesis pathway, respectively, were used for the detection of AF production by AF-producing isolates, as described by Färber et al. (1997) and Chang et al. (2005) (Table 2). MJ Research thermal cycler (PTC-200) was used for PCR amplification. Aliquots (8 µL) of each PCR product were analyzed by electrophoresis in a TBE buffer in 1.0% agarose gel and visualized by using Alpha Innotech Corporation MultiImage Light cabinet with camera and

filters. The gel photos were analyzed by using the GEL program (Patzekin and Klopov, Petersburg Nuclear Physics Institute, Russia) to determine the molecular weight of the PCR products and the PCR products' profile from different isolates.

3.3.4 Quantitative PCR of fumonisin-producing fungi

The qPCR was performed to quantify the content of FB-producing-fungi DNA in 15 maize samples and 6 wheat samples from Egypt, and in 6 maize samples from the Philippines, according to Waalwijk et al. (2008) using Taqfum-2f and Vpgen-3R primer pairs and FUMp probe. Also, the TMFg12 primers and probe were used for the quantification of *F. graminearum* DNA in the same samples, as described by Yli-Mattila et al. (2008). A Bio-Rad IQTM5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) was used for running qPCR samples. The DNA level of *Fusarium* species in grain samples was counted per total DNA as described by Yli-Mattila et al. (2011).

3.3.5 Quantitative PCR of trichothecene-producing fungi

The TMFg12 primers and probe have been designed for the *F. graminearum* specific RAPD-PCR product (Yli-Mattila et al., 2008). The TMLAN primers and probe for *F. langsethiae/F. sporotrichioides* have been designed by Halstensen et al. (2006) and the culmorum MGB primers and probe for *F. culmorum* by Waalwijk et al. (2004). We used these qPCR primers and probes as described by Yli-Mattila et al. (2008, 2009). A Bio-Rad IQTM5 Real-Time PCR Detection System (Bio-Rad, Hercules, USA) was used for running qPCR samples. The DNA level of *Fusarium* species in grain samples was counted per total DNA quantified by Qubit fluorometer (Invitrogen, Carlsbad, CA, USA), as described by Yli-Mattila et al. (2011). Ten grain samples from 2010, 15 samples from 2011, and 38 samples from 2012 were analyzed by using TMFg12 primers and probe; while only 18 samples from 2012, 18 samples from 2011, and 10 samples from 2010 with high T-2/HT-2 levels were analyzed by using TMLAN primers and probe (Paper II). Also, 30 oats, 20 barley, and 21 spring wheat samples that were ground and sieved (except the Year 2013) were analyzed for *F. graminearum* DNA content (Paper III).

3.4 Mycotoxins analyses

3.4.1 Rida®Quick DON analysis

Oat (30), wheat (20), and barley (20) samples from different parts of Finland were analyzed by Rida®Quick DON analysis method at the Turku University of Applied Sciences (TUAS) and the results were compared with the accredited

GC-MS in MTT. The reproducibility of the method was tested by running 6 parallel samples in all cereals with 3 different DON levels. Twelve ground oat samples were obtained from Laboratory 1 and 12 ground wheat samples were obtained from Laboratory 2. For comparing the 2 methods for measuring DON levels, grain samples were ground by 3 mills. In the Laboratory 1, the samples were ground using mill KT 30 (Koneteollisuus OY) without sieving, while in the Laboratory 2, the samples were ground using mill 3100 Falling number with a sieve of 0.8 mm. In MTT, a laboratory mill with a sieve of 1 mm was used as described above. Ground grain sample (1 g) was taken into a Falcon tube and 15 mL of Rida®Quick DON extraction buffer was added. The mixture was shaken manually for 3 min before centrifugation for 10 min (at 3500 g). The clear supernatant (100 µl) was pipetted onto the application area of the test strip and the result was read after 5 min.

3.4.2 GC-MS analyses of trichothecenes

In MTT Agrifood Research Finland, the TCs were extracted and analyzed with an accredited GC-MS method, as described by Hietaniemi et al. (2004) and Yli-Mattila et al. (2011). In MTT, the grain samples were ground using a mill and passed through a 1 mm sieve prior to the mycotoxin and DNA measurements, to achieve better repeatability.

3.4.3 Fluorescence detection of Aflatoxin-producing isolates

Coconut agar medium (CAM) was used for the detection of isolates producing AF (Lin and Dianese 1976). Briefly, 100 g of shredded coconut was homogenized for 5 min with 200 mL of hot distilled water. The homogenate was filtered through cheesecloth, then agar (2%) was added, and the medium was autoclaved. The plates were inoculated with PDA plugs of *Aspergillus* strains and incubated at 25°C for 5 days. The reverse side of the plates was periodically observed under 365 nm UV light for blue fluorescence.

3.4.4 Aflatoxins HPLC analyses

Each *Aspergillus* isolate was inoculated on 500 µL of yeast extract sucrose (YES) medium in an eppendorf tube and incubated for 7 days. AFs were extracted as follows: 500µL chloroform was added to each eppendorf and vortexed well. The chloroform extract was transferred to a new vial and dried gently under air. The dry film was derivatized according to AOAC method (2000) and then analyzed quantitatively using HPLC. A 200-µL stock solution of AFs mix standard in methanol (MeOH) (Supelco, Bellefonte, Pa., USA) containing 200 ng of B1, 60 ng of B2, 200 ng of G1, and 60 ng of G2, was dried under

nitrogen gas and derivatized. Four concentrations were prepared for HPLC injection. The HPLC system used for AFs' analyses was an Agilent 1200 series system (Agilent, Berks., UK) with a fluorescence detector (FLD G1321A), autosampler (ALS G1329A, FC/ALS thermo G1330B), Degasser (G1379B), Bin Bump (G1312A), and a C18 column (Phenomonex, Luna 5 micron, 150 × 4.6 mm) joined to a pre-column (security guard, 4 × 3 mm cartridge, Phenomenex Luna). The mobile phase used was MeOH/water/acetonitrile (30:60:10, v/v/v) with an isocratic flow rate of 1 mL/min at 360 nm excitation and 440 nm emission wavelengths and 25-min run time for each AF analysis.

3.5 Statistical analysis

The R^2 (coefficient of determination), regression slope, and p-value (significance of regression slope) were calculated using the program SigmaPlot, version 12.0 (SPSS Inc., Chicago, IL, USA). The original DNA and toxin concentrations were transformed to logarithmic values [1 + lg(x)] to obtain a more normal distribution for the toxin and DNA concentrations. Samples showing values <0.5 mg/kg or >5.5. mg/kg with Rida®Quick were excluded from the analysis because these values were not correct. The reproducibility for DON levels estimation with Rida®Quick in oat was tested using 3 samples with the DON levels of 1.4, 1.7, and 2.1 ppm (n=6, Table 1) (Paper II). Commercial standard (Check sample DON in wheat. Romer Labs. 1.431 + 0.2566 ppm) was also used. In barley, the DON levels were 0.81, 1.30, and 1.60, while in wheat the DON levels were 0.79, 1.00, and 1.40. Rida®Quick results were compared with accredited GC-MS results by using regression analysis and paired t-test was applied to reveal the 95% significance level. Relative standard deviation (RSD%) was also calculated (Papers II and III).

Statistical significance was determined using software Statistica Version 9 (StateSoft, Tulsa, OK, USA). The means AFs' concentrations were compared using analysis of variance (ANOVA, two-way analyses) ($P<0.05$). Fisher's LSD method ($P=0.05$) was applied to compare significant differences in AFs production among the strains (Paper V).

4. RESULTS

4.1 Isolation, identification, and preservation of fungi

4.1.1 Isolation and identification of FB-producing fungi

In this study, we isolated 43 fungal single-spore isolates (38 from the Egypt and 5 from the Philippines) from the collected samples (Figure 7). Most *Fusarium* isolates were from maize (53.4% of the isolates), then wheat (35.4%), and finally soil (11.2%). According to the morphological and microscopic identification (Figure 8), 22 isolates from Egypt and 3 isolates from the Philippines belonged to *F. verticillioides*, 2 isolates belonged to *F. proliferatum,* and 2 isolates belonged to *F. nygamai*. The molecular identification showed that all FB-producing *Fusarium* species belonged to *F. verticillioides* (Paper I).

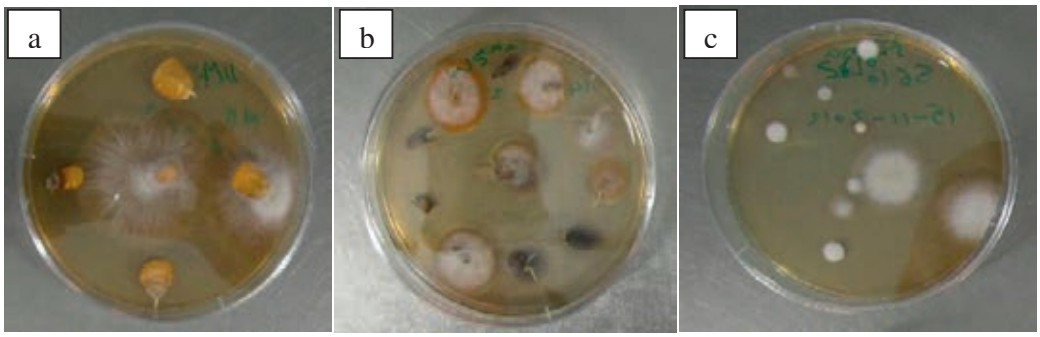


Figure 7. *Fusarium* isolation process; a: maize sample, b: wheat sample, and c: soil sample**.**

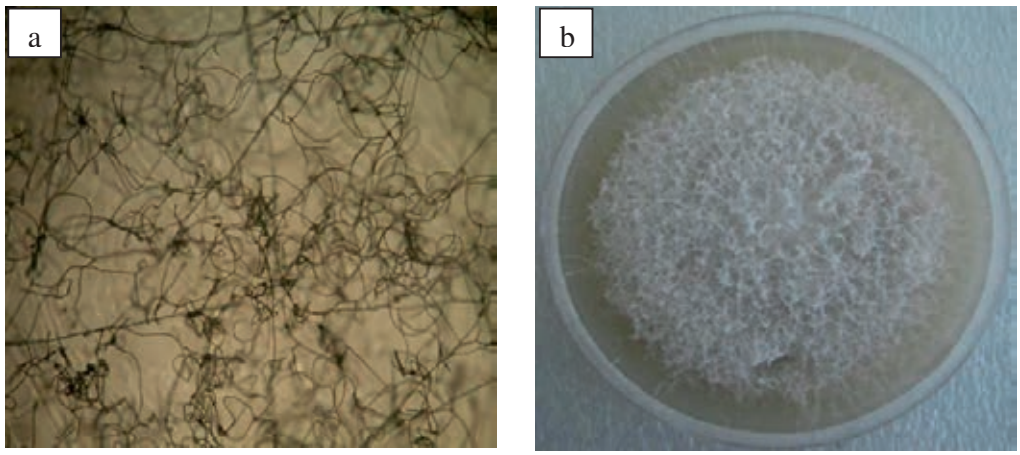


Figure 8. Microscopical (a) and morphological (b) characters of *F. verticillioides* (T. Gagkaeva).

4.1.2 Isolation and identification of aflatoxin-producing fungi

A total of 160 *A. flavus* and *A. parasiticus* isolates that gave a yellow-orange (reverse) color on AFPA medium (Figure 9) were isolated from 70 samples (32 soil, 22 maize, 9 wheat, 4 peanut, 2 coconut, and 1 lab bench swab). Forty-five isolates were from the Egyptian samples and 115 isolates were from the Philippines. Single spore isolates of AF-producing fungi were identified according to their phenotypic properties on AFPA and Czapek Yeast Extract Agar media and their AF production profile (Paper V).

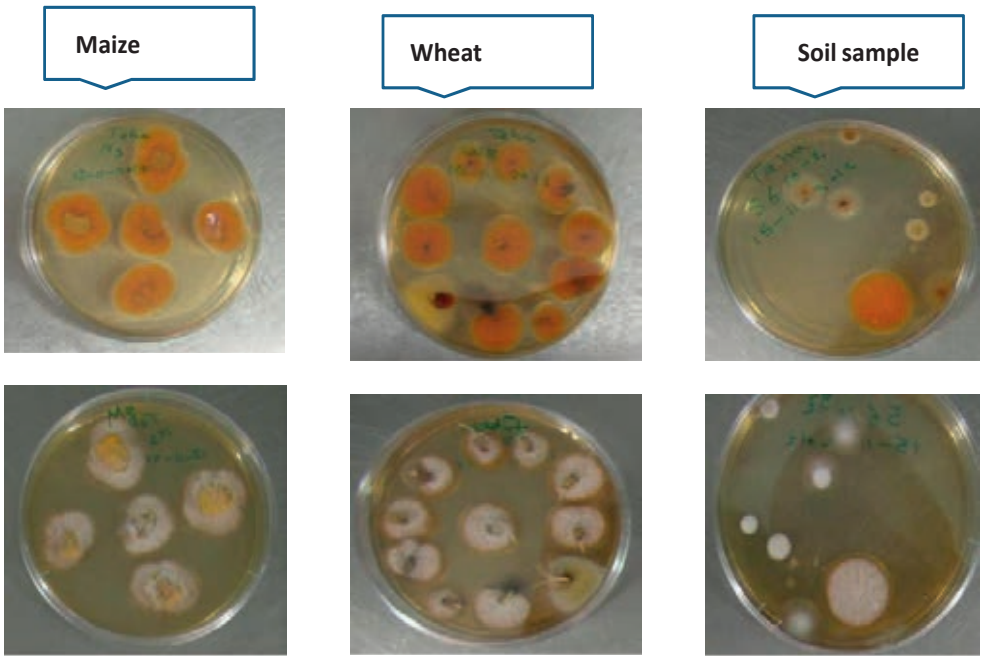


Figure 9. The isolation of AF-producing fungi on AFPA medium

4.1.3 Re-identification of *Fusarium sibiricum*

During morphological identification, colonies of *F. sibiricum* on PSA medium were showing average mycelial growth rates of 6 mm/day at 24°C. Aerial mycelium of *F. sibiricum* was white, typically flocculent, more abundant, and less powder than in *F. langsethiae*. On the reverse side, there were white to cream shades.

Conidiophores of *F. sibiricum* often consisted of a long and nodose stipe terminating with a whorl of phialides, intermingled with the short monophialides

formed directly on the aerial mycelium. Polyphialides were not observed, while in *F. langsethiae* polyphialides with 2 loci were present. Microconidia were apiculate and globose, mostly 0-septate, rarely 1-septate, hyaline, and formed abundantly in false heads, after a few days. Innumerable conidia of the type already described might be present at very outset of *F. langsethiae* colonies growth. Conidia of *F. sibiricum* were formed in mycelium after a few days of growth (Paper IV).

It is interesting to note that in a drop of water under the microscope, false heads of *F. poae* microconidia disintegrate easily, exposing phialides. At the same time, *F. langsethiae* heads are more stable, while *F. sibiricum* heads are difficult to destroy. A noticeable faint sweetish odor was produced by some *F. sibiricum* and *F. poae* strains.

Based on molecular identification, the Iranian *Fusarium* isolates that were identified as *F. langsethiae* had a long TG repeat of 30 bp in its ribosomal IGS region. The results were achieved when the TG repeat was sequenced and it was possible to separate it from *F. sporotrichioides* and *F. langsethiae* isolates based on the longer PCR product after the PCR products were run on a metaphor agarose gel (Paper IV). Otherwise, the partial IGS sequence on both sides of the TG repeat was identical with known *F. sibiricum* isolates.

The TG repeat of the Iranian isolates was shorter than the TG repeat of other *F. sibiricum* isolates (36 bp or longer), but it was still 10 bp longer than the TG repeat of any known *F. sprorotrichioides* or *F. langsethiae* isolate (Yli-Mattila et al., 2011).

The DNA from the Iranian *F. sibiricum* isolates also gave a strong positive signal with TMLAN primers and probe, which was expected, because the ITS sequences of known *F. sibiricum* isolates were identical with *F. langsethiae* isolates.

In PCR assays, previously designed to distinguish *F. langsethiae*, *F. poae*, and *F. sporotrichioides* from other species, none of the 6 primer pairs employed consistently distinguished the *F. sibiricum* isolates and representative strains of *F. langsethiae* or *F. sporotrichioides* (Paper IV), but it was possible to identify *F. sibiricum* isolates by using a combination of primer pairs.

F. sporotrichioides-specific primer pair Fspor F1/lanspoR1 gave a strong positive reaction with both *F. sporotrichioides* and *F. sibiricum* isolates, while F. sporotrichioides-specific primer pair PFusF/FspoR gave a positive reaction with the *F. sporotrichioides* isolate and a negative reaction with both *F. langsethiae* and *F. sibiricum* isolates, when the protocol of Yli-Mattila et al (2004b) was

used. A very weak band was also produced in some cases by *F. sporotrichioides*-specific primer pair Fspor F1/lanspoR1 with *F. langsethiae* DNA (Paper IV). Both *F. langsethiae*-specific primer pairs gave a positive reaction with *F. langsethiae* and *F. sibiricum* isolates. FlanF3/LanspoR1 also gave a positive reaction with the *F. sporotrichioides* isolate when the protocol of Yli-Mattila et al. (2004b) was used, but the reaction was clearly stronger with *F. langsethiae* isolates than with any *F. sibiricum* and *F. sporotrichioides* isolates. PfusF/FlanR gave a positive reaction with *F. langsethiae* and *F. sibiricum* isolates and a negative reaction with the *F. sporotrichioides* isolate (Paper IV).

When the PCR amplifications were performed with primers specific to *F. sporotrichioides* (FsporF1/LanspoR1) and *F. langsethiae* (FlanF3/LanspoR1) using the touchdown PCR protocol suggested by Wilson et al. (2004), the *F. sporotrichioides* and *F. sibiricum* isolates gave a positive reaction with the *F. sporotrichioides*-specific primer pair as obtained with the PCR protocol of Yli-Mattila et al. (2004), although the bands were weaker. With the *F. langsethiae*-specific primer pair (Paper IV) a weak positive reaction was only obtained with *F. langsethiae* strains.

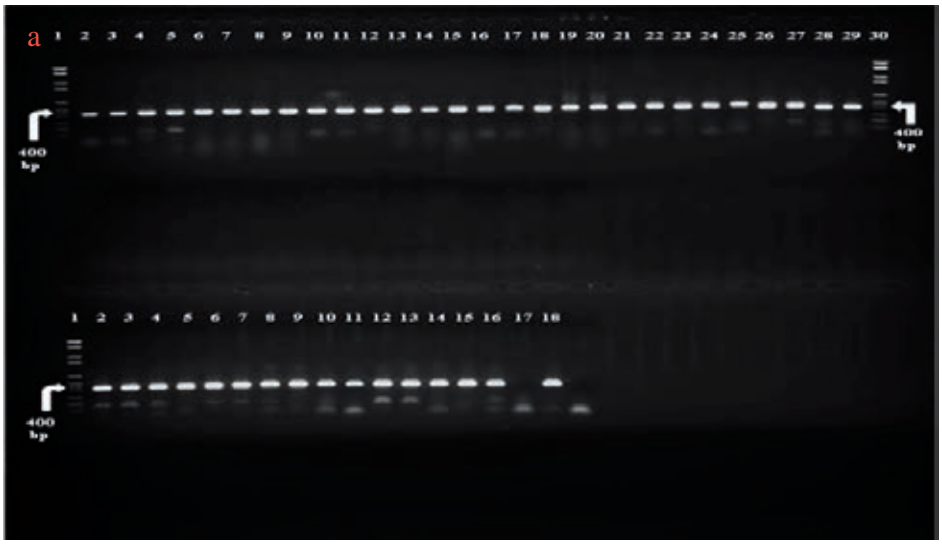
4.1.4 Eppendorf-agar long term preservation

The Eppendorf-agar method showed 100% viability of the preserved fungi during the 27 months storage period (Paper I).

4.2 Molecular detection and quantification of mycotoxin-producing fungi

4.2.1 Molecular detection of aflatoxin-producing fungi

All 43 *A. flavus* and *A. parasiticus* AF-producing isolates had the ver-1 and ordA genes in the AF biosynthetic pathway (Paper V). The PCR with ver-1/ver-2 primer pair (Figure 10b) showed 3 different profiles of PCR products: (a) 1 band at around 537bp (all *A. parasiticus* isolates and most *A. flavus* isolates); (b) 1 band at around 700bp (*A. flavus* isolates 88P, 89P and 90P); and (c) 2 bands– 1 at around 537bp and other at around 700bp (*A. flavus* isolates 23E, 29E, 35E, 41P, 44P, 45P, 59P, 86P, 87P, 96P, 97P, 107P, 108P, 109P, and 112P). However, the PCR product of ordAF/ordAR primer pair gave 1 main band at around 400bp (Figure 10a). Regarding the atoxigenic *A. flavus* isolates, no bands were observed with either primer pair, but they produced PCR products with ITS1/ITS4 primer pair. According to ITS1/ITS4 primer pair, the quality of DNA from all isolates was good enough for PCR.



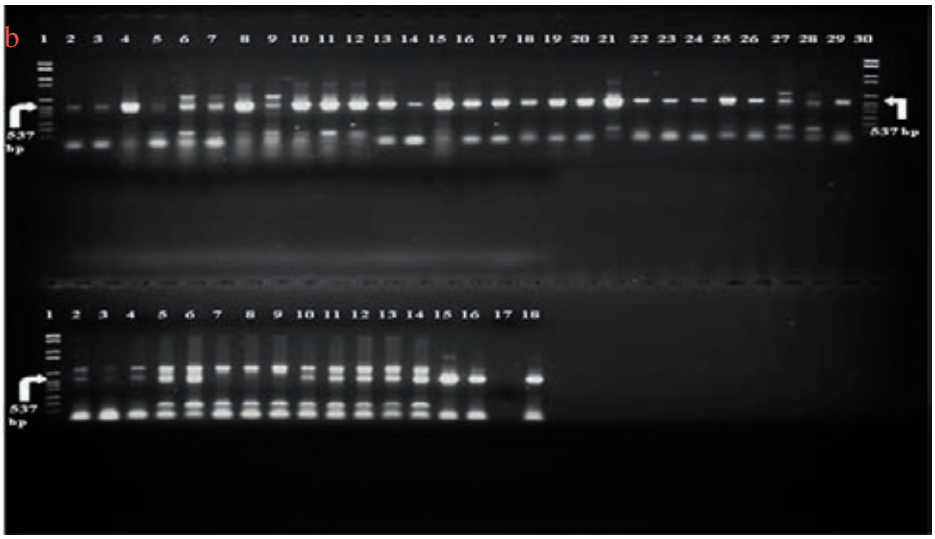


Figure 10. Band pattern of different *Aspergillus* species isolates as resulted from PCR reaction primed by ordAF/ordAR primer pair (a) and ver1/ver2 primer pair (b)

In the upper part of gel, Lanes: Lane 1 = 1 Kb DNA ladder, Lane 2 = IE, Lane 3 = 3E, Lane 4 = 16E, Lane 5 = 21E, Lane 6 = 23E, Lane 7 = 29E, Lane 8 = 34E, Lane 9 = 35E, Lane 10 = 40E, Lane 11 = 41E, Lane 12 = 42E, Lane 13 = 43E, Lane 14 =, Lane 15 = 45E, Lane16=6P, Lane17=7P, Lane18=8P, Lane19=9P, Lane20=10P, Lane21=18P, Lane22=32P, Lane23=33P, Lane24=34P, Lane25=35P, Lane26=36P, Lane27=41P, Lane28=42P, Lane29=43P, Lane30=1 Kb DNA ladder.

In the lower part of gel, Lanes: Lane 1 = 1 Kb DNA ladder, Lane 2 = 44P, Lane 3 = 45P, Lane 4 = 59P, Lane 5 = 86P, Lane 6 = 87P, Lane 7 = 88P, Lane 8 = 89P, Lane 9 = 90P, Lane 10 = 96P, Lane 11 = 97P, Lane 12 = 107P, Lane 13 = 108P, Lane 14 = 109P, Lane 15 = 110P, Lane16=112P, Lane17=atoxigenic *A. flavus*, Lane18= positive

4.2.2 Molecular quantification of fumonisin-producing *Fusarium* species

The qPCR results obtained by using Taqfum-2f, Vpgen-3R primer pairs and FUMp probe for quantification of the FB-producing *Fusarium* species showed that these species were present in 4 maize samples from the Philippines and 8 maize samples from Egypt. The *Fusarium* DNA levels were in the range of 13 x 10^{-3} to 61 x 10^{-1} ng/ng total DNA in positive samples, except in one maize sample from the Philippines with a high concentration of about 1 ng/ng total DNA, which suggests that all DNA was *Fusarium* DNA. No FB-producing *Fusarium* DNA was detected in the wheat samples and in the remaining maize samples (Paper I).

4.3 Mycotoxin analysis results

4.3.1 Aflatoxin production

In general, 23% (43) of the tested isolates (Table 3, Paper III) were able to produce AFs. Fourteen of them were from Egypt and 29 were from the Philippines. The toxin-producing Egyptian isolates were morphologically identified as *A. flavus*. On the other hand, 21% (6 isolates) of the Philippines isolates were morphologically identified as *A. parasiticus* and the remaining as *A. flavus*. Complete agreement was found between the qualitative CAM medium and the quantitative HPLC identification (99.4%). Overall, there was only one false negative isolate (112P) identified when compared with the HPLC results (Table 2) (Paper III). Thirty-one percent of the Egyptian isolates (14) and 25% of the Philippines isolates (29) were able to produce AFs. In general, all AF-producing isolates were able to produce AFB1 in the range of 0.5 ng/mL to 13189 ng/mL in the media. The highest concentration of AFB1 was produced by isolate 6P that was isolated from a soil sample of a coconut field in Situbo, Tampilisan and Mindanao, Philippines. All the 14 Egyptian AF-producing isolates were unable to produce AFG1 and G2, while 12 isolates from the Philippines were AFG1 producers and 7 were AFG2 producers.

According to HPLC analysis, there were 4 chemotypes of AFs producers: (1) isolates producing all 4 AF types (16%, 7 isolates); (2) isolates producing AFB1 only (18.5%, 8 isolates); (3) isolates producing AFB1 and B2 (53.5%, 23 isolates); and (4) isolates producing AFB1 and G1 (12%, 5 isolates). All the 6 *A. parasiticus* isolates belonged to chemotype 1 and they produced all types of AFs (total amount 2400–40400 ng/mL). Whereas, the other isolates (37 isolates) which belonged to *A. flavus* produced less than 1200 ng/mL total AFs. Only one *A. flavus* isolate from the Philippines could produce all four AF types (Paper III).

4.3.2 The correlation between trichothecenes and *Fusarium* DNA Levels

For the Year 2010–2012 samples, the highest levels of DON were found in Finnish oats in Year 2012 sample, when about 10–25% of oats samples contained >1.75 mg/kg of DON. In the samples from MTT, the DON levels varied between <25–10000 ppb (Paper V) and the T-2/HT-2 toxin levels were between <25 and 3000 µg/kg (Figure 3, Paper V). In the oats samples from a food company, the DON levels ranged between 530 and >5500 µg/kg (Paper V). The coefficient of determination (R^2) for the relation between *F. graminearum* DNA and DON levels was 0.95 ($p<0.001$) for 15 oats samples of the year 2011 and 0.87 ($p<0.001$) for 40 oats samples of the Year 2012, which were obtained from MTT (Figure 2a, Paper V). The R^2 value for 20 oats samples from a food company was only 0.22 ($p<0.05$) (Paper V).

For year 2013 Finnish oats samples, the correlation between *F. graminearum* DNA analyzed by qPCR (Figure 11) and DON levels was higher ($R^2 = 0.76$) when the DNA was extracted and measured from grain samples ground in MTT with a mill having a sieve compared with the same grain samples ground at the UTU without a sieve ($R^2 = 0.31$, Figure 2, Paper II). The R^2 value for 20 oats samples from a food company was 0.71, which was also lower than in the grain samples ground in MTT with a mill having a sieve (Paper II). The R^2 values for correlation between DON levels obtained with GC-MS in MTT and Rida®Quick in TUAS for oats (0.88), barley (0.80), and wheat (0.89) were high, although the DON levels obtained from Rida®Quick were usually higher. This caused 7 false positive oats, 5 false positive barley, and 5 false positive wheat samples with Rida®Quick. There was a good correlation between the DON levels obtained for 30 oats, 20 barleys, and 21 spring wheat samples analyzed using accredited GC-MS and Rida®Quick methods (Paper II). Pearson correlation values were 0.935, 0.931, and 0.935; and R^2 values were 0.88, 0.80, and 0.89 for oats, barley, and spring wheat, respectively. However, paired t-test (t-values = 5.694, 7.854, and 3.544) showed differences on the 95% significance level between the Rida®Quick and GC-MS results in all the 3 kinds of cereals. This was mainly due to the higher DON values obtained by Rida®Quick method.

When the DON levels obtained with accredited GC-MS for oat or wheat grain samples ground in MTT with a mill having a sieve were compared with those obtained from Rida®Quick method, the correlation was good (R^2 usually above 0.89 both in wheat and oat, Paper II), although DON levels obtained with GC-MS were usually lower in oat, wheat, and barley (Paper II). The correlation was also good when DON levels from wheat grain samples ground in Laboratory 2 with a mill having a sieve were compared with Rida®Quick method in 3

laboratories (R^2 >0.9, Paper II). But when DON levels from grain oat samples ground in Laboratory 1 with a mill without a sieve were compared with those obtained with Rida®Quick method in 3 laboratories, the correlation was lower (usually <0.8, Paper II).

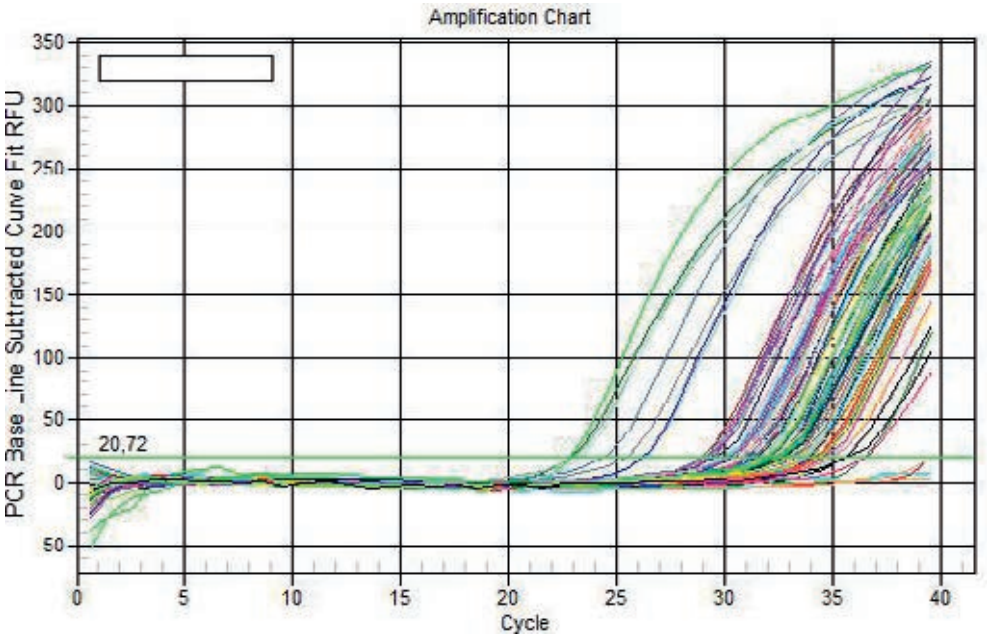


Figure 11. qPCR chart for *F. graminearum* DNA quantification using TMFg12 primers and probe

5. DISCUSSION

5.1 Fungi isolation, identification, and preservation

5.1.1 Isolation and identification of fumonisin-producing fungi

In this doctoral study, isolates of different FB-producing *Fusarium* species from maize, wheat, and soil samples from Egypt and the Philippines were collected. In addition, several reports have indicated the presence of them in Egyptian corn and feed samples (El-Habbaa et al., 2003; Heidy Abo El Yazeed, et al., 2011; Abd-El Fatah el al., 2015) and in the Philippines (Cumagun et al., 2009). Abd-El Fatah et al. (2015) were also able to find the correlation between fumonisin production and *F. verticillioides* isolates in maize samples.

The molecular detection of FB based on Verprof, VERTI-R primer pair for *F. verticilloides* and Taqfum-f2, VPgen-R3 for all fumonisin producers using the method described by Waalwijk et al. (2008) showed that all *Fusarium* isolates were able to produce the toxin. Similar results have been reported by Abd-El Fatah et al. (2015) (Table 1). However, the identification based on morphological and microscopic characteristics is mainly in agreement with the PCR results obtained using species-specific primers, except in 4 isolates (2 of them were identified as *F. proliferatum* and the others as *F. nygamai*). This may be due to difficulties in identifying *Fusarium* species using the conventional methods (Leslie and Summerell, 2006, Rossi et al., 2009). The results showed that we could use PCR based methods as a fast and economical way for the detection of contamination of grain samples with FB-producing fungi (Paper I).

5.1.2 Isolation and identification of aflatoxin-producing fungi

In the present study, a total of 160 *A. flavus* and *A. parasiticus* isolates obtained from soil, maize, wheat, coconut, and peanut showed yellow-orange (reverse) color on AFPA medium. These results confirmed the efficiency of this medium for the detection of *A. flavus* and *A. parasiticus,* as reported by Pitt et al. (1983). *A. parasiticus* isolates were only found in soil samples from coconut fields in the Philippines and all of them were able to produce all the 4 types of AFs, while all Egyptian isolates were identified as *A. flavus*. The toxin profile of 5 *A. flavus* isolates from Philippines producing AFB1 and AFG1 differed from the typical profile of both species. Similar isolates have been reported in West Africa (Atehnkeng et al., 2008; Donner et al., 2009); Argentina (Pildain et al., 2008); and Thailand (Saito and Tsuruta, 1993) (Paper V).

5.1.3 Re-identification of *Fusarium sibiricum*

Based on morphological characteristics, *F. sporotrichioides* can usually be clearly distinguished from *F. langsethiae* and *F. sibiricum*, but *F. sibiricum* and *F. langsethiae* may be confused with each other and with other members of the section Sporotrichiella. Thus, only molecular methods are reliable enough for the identification of all isolates of these species. Until now, the only known reliable species-specific primer pair CNL12/PulvIGSr for *F. sibiricum* is based on the length of the IGS sequences. In the present work (Paper IV), it was confirmed that a combination of 4 *F. langsethiae*- and *F. sporotrichioides*-specific primer pairs can also be used for the identification of *F. sibiricum* isolates. The results are in agreement with those of Wilson et al. (2004), Konstantinova and Yli-Mattila. (2004), Yli-Mattila et al. (2004a, 2011), and Edwards et al. (2012).

The identification of the *F. sibiricum* isolates from Iran was confirmed by IGS sequencing. It could be detected and quantified by the same TMLAN primers and probe used for the detection and quantification of closely related *F. sporotrichioides* and *F. langsethiaea* isolates. The partial IGS sequence of the Iranian isolate was identical with other *F. sibiricum* isolates (Yli-Mattila et al., 2011), except for the length of the TG repeat, which was variable. However, the TG repeat of the Iranian isolate was still longer than the TG repeat of any *F. sporotrichioides* strain, which made it possible to separate it from *F. sporotrichioides* (Paper IV).

The results of the present work (Paper IV) showed that the primer pairs PfusF/FspoR and Pfusf/FlanR based on ribosomal ITS sequences can be used for separating *F. langsethiae* and *F. sibiricum* isolates from the *F. sporotrichioides* isolates. But, according to Yli-Mattila et al. (2004a, 2011), ribosomal ITS sequences and primers based on ITS sequences cannot be used for reliable identification of *F. sporotrichioides*, *F. sibiricum,* and *F. langsethiae*, because ITS sequences of some (4/33 isolates sequenced) *F. sporotrichioides* isolates and all known *F. sibiricum* isolates (1 isolate sequenced) were identical to those of *F. langsethiae* isolates (21 isolates sequenced). Thus, the primer pairs PfusF/FspoR and Pfusf/FlanR cannot be completely species-specific between all *F. sporotrichioides* and *F. langsethiae*/*F. sibiricum* isolates.

The results obtained with *F. langsethiae*-specific primer pair Flang F3/lanspoR1 in the present paper (Paper IV) and in the paper of Yli-Mattila et al. (2011) with the lower annealing temperature (Yli-Mattila et al., 2004c) differ from those obtained by Edwards et al. (2012), who used the touchdown PCR protocol of Wilson et al. (2004). When the protocol of Yli-Mattila et al. (2004c) was used, it

also gave a weak band with *F. sibiricum* and *F. sporotrichioides* isolates and a stronger band with *F. langsethiae* isolates, while the *F. sporotrichioides*-specific primer pair FsoprF1/lanspoR1 gave a strong band only with *F. sporotrichioides* isolates. When the touchdown PCR protocol of Wilson et al. (2004) was used with both primer pairs in the present work, fewer PCR products were obtained, but the reaction was completely specific to *F. langsethiae* isolates. Thus, the touchdown protocol is more specific with *F. langsethiae*-specific primers, while the protocol of Yli-Mattila et al. (2004c) is more sensitive because more PCR product was obtained. With both the protocols it was possible to separate *F. sibiricum* from *F. langsethiae*, but *F. sibiricum* isolates could not be separated from *F. sporotrichioides* isolates. It might be possible to design an *F. sibiricum*-specific primer pair by sequencing the PCR product of Wilson et al. (2004) from more isolates including *F. sibiricum* isolates.

Based on the present data, *F. langsethiae* is a European species, which has been found in the north (Norway, Finland), in the south (Italy), in the west (France and UK), and in the east (Poland and European part of Russia). The main distribution of *F. sibiricum* is in northern Asia. In the present paper (Paper IV) a new *F. sibiricum* isolate was found in Iran. The single isolate of *F. sibiricum* IBT 9959 was found in Norway (Yli-Mattila et al., 2011) and the single *F. langsethiae* isolate was found in Tyumen (in Western Siberia), which is may be due to sporadic transfers of individual isolates as a result of human activities. The situation resembles that of *F. vorosii*, which was first detected in Hungary, but which according to phylogenetic multilocus sequence data belongs to the Asian clade and the main area of distribution is in Asia (Starkey et al., 2007).

This means that the actual distribution of *F. sibiricum* may be much larger in Eurasia than what is presently known. No *F. langsethiae* and *F. sibiricum* isolates have been found outside Europe and Asia (Paper IV).

5.1.4 Eppendorf-agar long term preservation

The Eppendorf-agar method was found suitable for preserving microorganisms for more than 2 years and it was assayed for different types of microbes including fungi, bacteria, and actinomycetes. This method is easy to perform and reduces the time, cost, and the risk of contamination. Several other methods have been used for microorganisms like continuous subculturing on agar slants (Onions, 1971), preservation under mineral or paraffin oil (Perrin, 1979), storage in sterile water (Richter and Bruhn, 1989), drying of fungal cultures (Nakasone et al., 2004), Freeze-drying (lyophilization) (Tan et al., 2007), and cryopreservation (Homolka and Lisa, 2008). However, all of them are more time-consuming and expensive than eppendorf-agar method.

Nevertheless, further work should be done to study the effect of storage on the genetic stability of preserved microbes and the suitability of this method for different microbial species (Paper I).

5.2 Molecular detection and quantification of mycotoxin-producing fungi

5.2.1 Molecular detection of aflatoxin-producing fungi

Fungal material preparation for DNA isolation was performed over a short time period (3 days incubation) in 500µL of broth medium in eppendorf tubes. This approach reduces the time and the amount of the medium used for preparing mycelium, whereas in most studies 20 mL of medium for 7 days is required (Rodrigues et al., 2009; Sultan and Magan, 2010).

For molecular detection of AF production, 2 genes were chosen: (a) the ver-1 gene that codes for versicolorin A dehydrogenase, an enzyme that converts the versicolin A to sterigmatocystin in the middle of the AF biosynthesis pathway (Yu et al., 2004); and (b) the ord1 gene that is considered to be the only gene involved in the final step of transforming O-methylsterigmatocystin (OMST) into AFB1, a crucial step in the AF biosynthesis pathway that seems to be unique to aflatoxigenic species (Prieto and Woloshuk, 1997).

Molecular detection of AF production by using the ver-1/ver-2 (Färber et al. ,1997) and ordAF/ordAR (Chang et al., 2005) primer pairs agree with the results obtained from HPLC analysis of AFs and CAM methods. However, the presence of the AF biosynthesis genes does not always mean that phenotypic production of AF occurs (Rodrigues et al., 2009). The results implied that we can use PCR as a fast and economical method for screening the contamination of grain samples with *Aspergillus* section *Flavi* instead of the time-consuming conventional methods (Degola et al., 2007) or expensive agar-plate methods for fungal contamination analysis (Zheng et al., 2004).

PCR products profile with ver-1/ver-2 primer pair showed differences in the isolated *A. flavus* and *A. parasiticus*. Two new PCR profiles with ver gene primers were detected in our isolates. The first one included 1 band at a molecular weight higher than the reported one, while the second one included 2 bands, 1 at the ordinary place and second at higher than the first one. A similar PCR product was reported by Geisen (1996) but in *Penicillium roqueforti* using primers for the same gene. Based on these results, we introduced the isolates as new genotypes, which gave two new PCR profiles with ver gene. This revealed that this primer pair could be used to identify the diversity of AF-

producing fungi, but it could not be used as a molecular marker for other aflatoxigenic fungi that contained ver gene in the biosynthesis pathway of the natural products (Paper V).

5.2.2 Molecular quantification of fumonisin-producing fungi

The qPCR results showed that DNA of FB-producing fungi was detected only in the maize samples. This is in agreement with the number of isolated *Fusarium* isolates from maize, which indicates that corn is a highly susceptible crop for *F. verticillioides* and *F. proliferatum* infection. In addition, no *F. graminearum* isolates and DNA was isolated or detected from the samples. This may be due to the unsuitable weather condition in Egypt and the Philippines for this species (Paper I).

5.3 Mycotoxins

5.3.1 Correlation between trichothecene and *Fusarium* DNA Levels

DNA levels of *F. graminearum* in oats were in agreement with DON levels measured by accredited GC-MS in all cases. When the Rida®Quick kit results (for DON level) were compared with DNA levels of *F. graminearum*, the variation was much higher. The homogenization of the oats grain with sieving or without sieving seems to be connected to the variation of DON and *F. graminearum* DNA levels. These results (Paper III) are in agreement with previously reported results (Paper II).

More false-positive results were found in all the 3 kinds of cereals when Rida®Quick method was used compared with the accredited GC-MS method. These false-positive results were due to higher DON levels obtained by using the Rida®Quick method.

The DON levels obtained with Rida®Quick method were usually higher than those obtained with accredited GC-MS method in all the 3 kinds of cereals. However, in this study, the semiquantitative Rida®Quick DON method met the requirement of the Commission regulation (EC) 401/2006 for the quantitative DON determination (RSD% </=20%) in most cases. Due to the low DON levels in barley and wheat samples, the RSD% was above 20%. Thus, the results suggest that Rida®Quick results with the DON content close to the legislative limits should be reconfirmed with an accredited quantitative analysis.

5.3.2 Chemotypes of *Fusarium* species in Finland during the years 2010–2012

Mycotoxin–3ADON was found in all grain samples with high DON level, but 15ADON was not found (Tables S1-S4, Paper II). This is supporting the results obtained by other researchers (Yli-Mattila et al., 2008; Nielsen et al., 2011; Yli-Mattila and Gagkaeva, 2013), according to which the 3ADON molecular chemotype is dominant in northern Europe. The NIV was probably produced by *F. poae* (Parikka et al., 2008; Parikka et al., 2012; Hietaniemi et al., 2013), while *F. langsethiae* was found to be the most common T-2/HT-2-producer in Finnish oats.

The difference in correlation might be caused by different milling methods, which is supported by our previous results. The correlation between DON and *F. graminearum* levels was lower, when DNA was extracted from oat flour ground using a coffee mill without a sieve (Hietaniemi, 2013) compared with the present paper, in which both DNA and DON were extracted from the oat flour obtained using a mill having a 1 mm sieve for homogenization. According to Rauvola et al. (2013), the correlation between RIDA® QUICK SCAN and GC-MS results was good, when, DON levels were measured from oat flour ground with a 1 mm sieve. Finnish food companies, however, have been using RIDA® QUICK SCAN for oat samples ground without the 1 mm sieve, which might have caused variation in the results.

According to Rauvola (2013), RIDA® QUICK SCAN often gives oats DON levels above the EU limit (1.75 mg/kg), while the GC-MS results for the DON levels are mostly close (1.4–1.7 mg/kg) to the EU limit. This might have partially explained why according to the food companies 25% of oats samples from the Year 2012 in Finland contained too much DON for human consumption (Hakulin et al., 2013), while according to the MTT (Nielsen et al., 2011) only 10% of the oats samples exceeded the limit. Lower DON values with RIDA® QUICK SCAN were also obtained in wheat and barley (Rauvola 2013), especially close to the EU limit (1.25 mg/kg) (EC, 2005). It should also be taken into consideration that there can be some variations in the GC-MS results among different measurements in different laboratories. These variations may be due to differences in sample preparation and sample selection.

For consumers, the lower limit for DON values guarantee that the DON values are below the EU limit, but for farmers, they mean that the yield may be rejected due to false-positive results. Thus, both RIDA® QUICK SCAN and qPCR can be used simultaneously for screening DON levels in cereal grain samples, but the samples with DON levels close to the EU limit, should be checked by GC-MS

method as well to avoid false positive or false negative results; and even then, some cases may be difficult to analyze precisely.

Only low levels of *F. culmorum* DNA were found in a few oat samples (Tables S1–S5) (Paper II) and no correlation was found between *F. culmorum* DNA and DON levels. *F. graminearum* DNA levels were in agreement with DON levels in all cases, when DON was measured using GC-MS method. When compared with RIDA®QUICK SCAN kit results the variation in the DNA levels was much higher. Thus, the homogenization of the oat flour by sieving seems to be important for achieving reproducible DON and *F. graminearum* DNA levels. According to Gagkaeva and Gavrilova (2013), filtration of oat flour through a 0.4 mm sieve after milling decreases *Fusarium* biomass connected to the hulls. This may be because bigger particles are formed during milling from hulls, which contain more *Fusarium* DNA than the central part of oats grains. Based on our results, *F. graminearum* is clearly the main DON producer in Finnish oats.

There was also a significant correlation (Figure 3) (Paper II) between the combined T-2 and HT-2 levels and combined *F. langsethiae* and *F. sporotrichioides* DNA levels in samples from the Year 2010–2012. For the combined data of the years 2010–2012, the R^2 value was 0.40. The high DNA levels in oat samples found by TMLAN primers and probe were mainly due to high *F. langsethiae*/*F. sporotrichioides* contamination in the outer grain layer, which gets removed during the de-hulling process before they are used for food (Rämö et al., 2008). These results (Figure 3) (Paper II) are in agreement with the previously reported results (Yli-Mattila et al., 2008; Supronienė et al., 2010; Hietaniemi, 2013).

5.3.3 Aflatoxins production

Results obtained from the fluorescence detection on CAM were in agreement with the results obtained from the HPLC analysis of AFs, except for 1 isolate (112P) that gave no fluorescence on CMA. Similar results were obtained by Sultan and Magan (2010) who examined the potential AF production by isolates of *A. flavus* and *A. parasiticus* from Egyptian peanuts. They found that there was about 90% correlation between the results obtained from the CAM and the HPLC method. Also, Rodrigues et al. (2009) reported that the analysis of AF production by fluorescence in CAM showed a good correlation with the HPLC results (Paper V).

Although the ratio of AF-producing isolates from Egyptian samples was higher than that of isolates from the Philippine samples, all Egyptian isolates were unable to produce AFG1 and G2, and the total AF production of the Egyptian

isolates was less than the Philippine isolates ranging from 0.5 to 852 ng/mL of the media. This may be because of the difference in the source of isolates; the Egyptian isolates were mainly from maize, wheat, and soil samples, while most of the Philippines isolates were from soil samples (associated with coconut or peanut). The isolates produced high levels of AFs and belonged to *A. parasiticus*. The occurrence of *A. parasiticus* as predominant species in the coconut field's soil in the Philippines has been reported for the first time. This indicates that there is an urgent need to reduce *A. parasiticus* levels from the soil through proper soil management in order to address the issue of high AF content in copra (dried coconut meat), which resulted in the suspension of the Philippines from exporting the copra to Europe in 2004 (Bawalan, 2004). In addition, all isolated species from maize, wheat, and soil associated with these crops have been identified as *A. flavus*. Thus, this indicates that there is a relationship between the crop and the type of AF-producing fungi. Data obtained by Pildain et al. (2004) showed that the frequencies of *Aspergillus* section *Flavi* varied among fields and crops. Even though *A. flavus* does not apparently have host specificity (St-Leger et al., 2000), distributions of different *A. flavus* species suggest that they may have adapted to specific niches and exhibit competitive advantages in special soils, hosts, regions, and seasons (Jaime-Garcia and Cotty 2006). Variation in the quantity and types of produced AFs of each isolate indicates the diversity of the isolated fungi (Paper V). Similar data was shown by Cotty (1989).

6. CONCLUSIONS

The data presented here demonstrate the role of PCR-based detection and quantification of mycotoxin-producing fungi in reducing the risk of mycotoxin contamination. The following findings were revealed as part of the thesis work:

- Eppendorf-agar is a suitable method for long-term fungi preservation. The PCR-based techniques can be used to identify FB-producing *Fusarium* species and quantify the risk of grain-contamination using qPCR, especially in the developing countries.
- The Rida®Quick results indicating the DON content close to the legislative limits should be reconfirmed with an accredited quantitative analysis.
- Securing the homogenization of the oat flour using a 1mm sieve and the grinding without a sieve was associated with the variations in the DON and *F. graminearum* DNA levels.
- The actual distribution of *F. sibiricum* may be much larger in Eurasia than what is currently known. No *F. langsethiae* and *F. sibiricum* isolates have been found outside the Eurasian continent.
- The isolated AF-producing fungi showed variability in the types and the quantity of AFs production and it was mainly related to the source of isolation. The PCR results with ver-1/ver-2 primer pair indicate that this primer pair can be used to study the diversity of AF-producing fungi, but it cannot be used as a molecular marker for aflatoxigenic fungi. However, the PCR results with ordAF/ ordAR primer pair imply that PCR reaction can be used as a fast and economical technique for screening the contaminated grain samples with *Aspergillus* section *Flavi*.

7. ACKNOWLEDGEMENTS

At first, I would like to express my sincere gratitude to my supervisor Adjunct Professor Tapani Yli-Mattila, who gave me the opportunity to work in the Laboratory of Molecular Plant Biology at the University of Turku. His passion for science and creative excellence in research has always inspired me to put my best in my PhD life.

I am deeply grateful to my co-supervisors, Prof Petr Karlovsky for his support right from the early stages of my PhD study.

I gratefully acknowledge my Advisory committee members Prof. (emerita) Marjatta Raudaskoski, Adjunct Prof. Kirsi Lehto, Dr. Asko Hannukkala and Adjunct Prof. Jukka-Pekka Suomela for their valuable suggestions.

I am grateful to Prof. Gülruh Albayrak and Prof. Jarkko Hantula for reviewing this thesis and providing constructive comments and suggestions. Manuscript edit Support Team Reseapro Scientific Services (P) Ltd is warmly thanked for revising the language of the thesis.

I am thankful to the technical staff (Mika, Kurt, Eve, Anniina, Maija and Tapio), as well as the administrative staff for their help in many different circumstances. I would remember the marvelous company of my lab mates for their cooperation and help.

This work was financially supported by Egyptian mission department.

Most importantly, I would like to thank my wife Hend Shahin, for her love and constant support throughout my research period.

Last but not the least it was the support, love, and care of my parents, brother, sister, friends, all relatives, and well-wishers, throughout these years got me here without which it would have remained as an unfulfilled dream.

8. REFERENCES

Aamot HU, Hofgaard IS, Brodal G, Elen O, Jestoi M, Klemsdal SS (2012) Evaluation of rapid test kits for quantification of DON in naturally contaminated oats and wheat. World Mycotoxin Journal 5:329-350

Aamot HU, Ward TJ, Brodal G, Vrålstad T, Larsen GB, Klemsdal SS, Elameen A, Uhlig S, Hofgaard IS (2015) Genetic and phenotypic diversity within the *Fusarium* graminearum species complex in Norway. European Journal of Plant Pathology 142:501-519

Abbas HK (2005) Aflatoxin and Food Safety. CRC Press, Boca Raton

Abbas HK, Zablotowicz RM, Weaver MA, Horn BW, Xie W, Shier WT (2004) Comparison of cultural and analytical methods of determination of Aflatoxin production by Mississippi Delta *Aspergillus* isolates. Canadian Journal of Microbiology 50:193-199

Abd-El Fatah SI, Naguib MM, El-Hossiny EN, Sultan, YY, Abodalam TH, Yli-Mattila T (2015) Molecular versus Morphological Identifcation of *Fusarium* spp. isolated from Egyptian corn. Research Journal of Pharmaceutical, Biological and Chemical Sciences 6: 1813–1822

Aboul-Nasr MB, Obied-Allah MRA (2013) Biological and chemical detection of Fumonisins produced on agar medium by Fusarium verticillioides isolates collected from corn in Sohag, Egypt. Microbiology 159:1720-1724

Aerts, D., Hauer, E.E., Ohm, R.A. et al. Antonie van Leeuwenhoek (2018) The FlbA-regulated predicted transcription factor Fum21 of *Aspergillus niger* is involved in fumonisin production. Antonie van Leeuwenhoek. 111, 311–322

Alberts JF, Engelbrecht Y, Steyn PS, Holzapfel WH, van Zyl WH (2006) Biological degradation of Aflatoxin B1 by Rhodococcus erythropolis cultures. Int J Food Microbiol 109:121-126

Alexander NJ, Proctor RH, McCormick SP (2009) Genes, gene clusters, and biosynthesis of trichothecenes and Fumonisins in *Fusarium*. Toxin Reviews 28:198-215

Alshannaq A, Yu J- (2017) Occurrence, Toxicity, and Analysis of Major Mycotoxins in Food. International Journal of Environmental Research and Public Health 14:632

Amagliani G, Omiccioli E, Campo A, Bruce IJ, Brandi G, Magnani M (2006) Development of a magnetic capture hybridization PCR assay for Listeria monocytogenes direct detection in milk samples. Journal of applied microbiology 100:375-383

AOAC (2000) Natural toxins. In: Official methods of analysis, 17th edn. Association of Official Analytical Chemists, Washington D.C.

Aoki T, O'Donnell K (1999) Morphological and molecular characterization of *Fusarium pseudograminearum* sp. nov., formerly recognized as the Group 1 population of *F. graminearum*. Mycologia 91:597-609

Aoki T, Smith JA, Mount LL, Geiser DM, O'Donnell K (2013) *Fusarium torreyae* sp. nov., a pathogen causing canker disease of Florida torreya (Torreya taxifolia), a critically endangered conifer restricted to northern Florida and southwestern Georgia. Mycologia 105:312-319

Arim, Rosario H (2004) Mycotoxin Contamination of Foods and Feeds in the Philippines:13

Arone L, Augusto J, Bandyopahyay R (2016) Diversity of *Aspergillus* section Flavi S morphotype in Mozambique. Phytopathology 106-24

Arzanlou M, Samadi R, Frisvad JC (2016) Two novel *Aspergillus* species from hypersaline soils of The National Park of Lake Urmia, Iran. Mycological Progress15: 1081-1092

Asan A (2011) Checklist of *Fusarium* Species Reported from Turkey. Mycotaxon 116:479

Atehnkeng J, Ojiambo PS, Donner M, Ikotun T, Sikora RA, Cotty PJ, Bandyopadhyay R (2008) Distribution and toxigenicity of Aspergillus species isolated from maize kernels from three agro-ecological zones in Nigeria. Int J Food Microbiol 122:74-84

Ates E, Mittendorf K, Stroka J, Senyuva H (2013) Determination of *Fusarium* mycotoxins in wheat, maize and animal feed using on-line clean-up with high resolution mass spectrometry. Food Additives and Contaminants Part A 30:156-165

Audenaert K, De Boevre M, Vanheule A, Callewaert J, Bekaert B, Höfte M, De Saeger S, Haesaert G (2013) Mycotoxin glucosylation in commercial wheat varieties: Impact on resistance to *Fusarium graminearum* under laboratory and field conditions. Food Control

Audenaert K, Van Broeck R, Bekaert B, De Witte F, Heremans B, Messens K, Höfte M, Haesaert G (2009) *Fusarium* head blight (FHB) in Flanders: population diversity, inter-species associations and DON contamination in commercial winter wheat varieties. European Journal of Plant Pathology 125:445-458

Ausubel FM, Brent R, Kingston RE, Moore DD, et al (2002) Short Protocols in Molecular Biology. 5th edn. John Wiley & Sons Publications, New York.

Aymerich MT, Martín B, Garriga M, Hugas M (2003) Microbial quality and direct PCR identification of lactic acid bacteria and nonpathogenic staphylococci from artisanal low-acid sausages. Applied and Environmental Microbiology 69:4583-4594

Aziz NH, Moussa LAA, Far FME (2004) Reduction of fungi and mycotoxins formation in seeds by gamma-radiation. J Food Saf 24:109-127

Barnett HL (L [, Hunter BB (1998) Illustrated genera of imperfect fungi / H.L. Barnett, Barry B. Hunter. APS Press

Basaran P, Demirbas RM (2010) Spectroscopic detection of pharmaceutical compounds from an aflatoxigenic strain of *Aspergillus parasiticus*. Microbiological Research 165:516-522

Bawalan DD (2004) Copra: The Philippines. In: Rick H, Graham F (eds) Crop Post-Harvest: Science and Technology, 2nd edn. Blackwell Science Ltd, Blackwell Science Ltd, 9600 Garsington Road, Oxford OX4, pp 197-206

Berbee ML, Taylor JW (2001) Fungal molecular evolution: gene trees and geologic time. In: McLaughlin DJ, McLaughlin E, Lemke PA (eds) The Mycota, Springer, Berlin, 229–246

Berbee ML, Taylor JW (2001) Fungal molecular evolution: gene trees and geologic time. In: McLaughlin DJ, McLaughlin E, Lemke PA (eds) The Mycota, Springer, Berlin, 229–246

Bhat R, Rai RV, Karim AA (2010) Mycotoxins in food and feed: present status and future concerns. Food Science and Food Safety 9:57-81

Bhatnagar D, Cary JW, Ehrlich K, Yu J, Cleveland TE (2006) Understanding the genetics of regulation of Aflatoxin production and *Aspergillus flavus* development. Mycopathologia 162:155-166

Bhatnagar D, Ehrlich KC, Cleveland TE (2003) Molecular genetic analysis and regulation of aatoxin biosynthesis. Applied Microbiology and Biotechnology 61:83-93

Bilska K, Kulik T, Ostrowska-Kołodziejczak A, Buśko M, Pasquali M, Beyer M, et al. (2018) Development of a Highly Sensitive FcMito qPCR Assay for the Quantification of the Toxigenic Fungal Plant Pathogen Fusarium culmorum. Toxins 10:211

Blount WP (1961) Turkey “X” disease. Turkeys 9:52-77

Boppana NB, Kraveka JM, Rahmaniyan M, Li L, Bielawska A, Bielawski J, et al. (2017) Fumonisin B1 Inhibits Endoplasmic Reticulum Stress Associated-apoptosis After FoscanPDT Combined with C6-Pyridinium Ceramide or Fenretinide. Anticancer research 37:455-463

Bottalico A, Perrone G (2002) Toxigenic *Fusarium* species and mycotoxins associated with head blight in small grains cereals in Europe. European Journal of Plant Pathology 108:611-624

Boutigny A, Ward TJ, Ballois N, Iancu G, Ioos R (2014) Diversity of the *Fusarium graminearum* species complex on French cereals. Eur J Plant Pathol 138:133-148

Boutigny AL, Ward TJ, Ballois N, Iancu G, Ioos R (2014) Diversity of the *Fusarium graminearum* species complex on French

cereals. European Journal of Plant Pathology 138:133-148

Brakhage AA (2012) Regulation of Fungal Secondary Metabolism. Nature Reviews Microbiology 11:21-32

Buchheidt D, Reinwald M, Hofmann W, Boch T, Spiess B (2017) Evaluating the use of PCR for diagnosing invasive aspergillosis. Expert Review of Molecular Diagnostics 17:603-610

Busby WF, Wogan GN (1984) Aflatoxins. In: Chemical Carcinogens, 2nd ed., pp. 945–1136, (C.E. Searle, ed.), American Chemical Society Monograph 182, Washington, D.C.

Carvajal-Campos A, Manizan AL, Tadrist S, Akaki DK, Koffi-Nevry R, Moore GG, Puel O (2017) *Aspergillus korhogoensis*, a Novel Aflatoxin Producing Species from the Côte d'Ivoire. Toxins 9:353

Cary JW, Ehrlich KC, Kale SP, Calvo AM, Bhatnagar D, Cleveland TE (2006) Regulatory elements in Aflatoxin biosynthesis. Mycotoxin Res 22:105-109

CAST (2003) Mycotoxins: Risks in Plant, Animal and Human Systems. Report No. 139 Council for Agricultural Science and Technology, Ames, Iowa, USA

Cendoya E, Monge M, Palacios S, Chiacchiera S, Torres A, Farnochi M, Ramirez M (2014) Fumonisin occurrence in naturally contaminated wheat grain harvested in Argentina. Food Control 37:56-61

Chang PK (2003) The *Aspergillus parasiticus* protein AFLJ Interacts with the Aflatoxin Pathway-Specific Regulator AFLR . Molecular Genetics and Genomics 268:711-719

Chang PK, Horn BW, Dorner JW (2005) Sequence breakpoints in the Aflatoxin biosynthesis gene cluster and flanking regions in nonaflatoxigenic *Aspergillus flavus* isolates. Fungal Genet Biol 42:914-923

Chang PK, Skory CD, Linz JE (1992) Cloning of a gene associated with Aflatoxin B1 biosynthesis in *Aspergillus parasiticus*. Curr Genet 21:231-233

Chen RS, Tsay JG, Huang YF, Chiou RY (2002) Polymerase chain reaction-mediated characterization of molds belonging to the *Aspergillus flavus* group and detection of *Aspergillus parasiticus* in peanut kernels by a multiplex polymerase chain reaction. J Food Prot 65:840-844

Cocolin L, Aggio D, Manzano M, Cantoni C, Comi G (2002) An application of PCR-DGGE analysis to profile the yeast populations in raw milk. International Dairy Journal 12:407-411

Cotty PJ (1989) Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathology 79:808-814

Cotty PJ (1994) Comparison of four media for the isolation of *Aspergillus flavus* group fungi. Mycopathologia 125:157-162

Cotty PJ, Bhatnagar D (1994) Variability among atoxigenic *Aspergillus flavus* strains in ability to prevent Aflatoxin contamination and production of Aflatoxin biosynthetic pathway enzymes. Applied and Environmental Microbiology 60:2248-2251

Cotty PJ, Jaime-Garcia R (2007) Influences of climate on Aflatoxin producing fungi and Aflatoxin contamination. Int J Food Microbiol 119:109-115

Cullen JM, Ruebner BH, Hsieh LS, Hyde DM, Hsieh DP (1987) Carcinogenicity of dietary Aflatoxin M1 in male Fischer rats compared to Aflatoxin B1. Cancer Res 47:1913-1917

Cumagun CJ, R., Ramos JS, Dimaano AO, Munaut F, Van Hove F (2009) Genetic characteristics of *Fusarium verticillioides* from corn in the Philippines. Journal of General Plant Pathology: JGPP 75:405-412

De Ruyck K, De Boevre M, Huybrechts I, De Saeger S (2015) Dietary mycotoxins, co-exposure, and carcinogenesis in humans: Short review. Mutation Research/Reviews in Mutation Research. doi: https://doi.org/10.1016/j.mrrev.2015.07.003

Degola F, Berni E, Dall'Asta C, Spotti E, Marchelli R, Ferrero I, Restivo FM (2007) A multiplex RT-PCR approach to detect aflatoxigenic strains of *Aspergillus flavus*. J Appl Microbiol 103:409-417

Desjardins A (2006) Fusarium mycotoxins: chemistry, genetics, and biology.. Phytopathological Society Press, St. Paul (MN): American

Desjardins AE (2003) Gibberella from A (venaceae) to Z (eae) Annual Review. Phytopathology 41:177-198

Desjardins AE (2006) *Fusarium* mycotoxins. Chemistry, genetics, and biology. APS Press, St. Paul, MN, USA

Desjardins AE, Proctor RH (2007) Molecular biology of *Fusarium* mycotoxins. International Journal of Food Microbiology.

Dongyou L (2009) Molecular Detection of Foodborne Pathogens. *Fusarium*. (Ed.). Dongyou Liu, RCPA Biosecurity QAP, New South Wales, Australia.Publications of CRC Press. Chapter 41. pp.577-592

Donner M, Atehnkeng J, Sikora RA, Bandyopadhyay R, Cotty PJ (2009) Distribution of *Aspergillus* section Flavi in soils of maize fields in three agroecological zones of Nigeria. Soil Biol Biochem 41:37-44

Doohan, Parry, Jenkinson, Nicholson (1998) The use of species-specific PCR-based assays to analyse Fusarium ear blight of wheat. Plant Pathol 47:197-205

Du L, Zhu X, Gerber R. et al. (2008) Biosynthesis of sphinganine-analog mycotoxinsJ Ind Microbiol Biotechnol 35: 455

Edwards SG, Imathiu SM, Ray RV, Back M, Hare MC (2012) Molecular studies to identify the *Fusarium* species responsible for HT-2 and T-2 mycotoxins in UK oats. Int. J. Food Microbiol 156:168–175

Ehrlich KC (2009) Predicted Roles of the Uncharacterized Clustered Genes in Aflatoxin Biosynthesis. Toxins (Basel) 1:37-58

Ehrlich KC, Chang PK, Yu J, Cotty PJ (2004) Aflatoxin Biosynthesis Cluster Gene cypA Is Required for G Aflatoxin Formation . Applied and Environmental Microbiology 70:6518-6524

Ehrlich KC, Mack BM (2014) Comparison of Expression of Secondary Metabolite Biosynthesis Cluster Genes in *Aspergillus flavus, A. parasiticus*, and *A. oryzae*. Toxins 6:1916-1928

Ehrlich KC, Yu J, Cotty PJ (2005) Aflatoxin biosynthesis gene clusters and flanking regions. Journal of Applied Microbiology 99: 518–527

El-Habbaa GM, Mohamed FG, Abou-El-Ella MF, Sabek IA (2003) Evaluation and control of yellow corn grain deterioration caused by *Fusarium* spp. 10th Congress of the Egyptian Phytopathology Society. 7-10 December 2003. Giza, Egypt, 77–96

El-Maghraby OMO, El-Kady IA, Soliman S (1995) Mycoflora and *Fusarium* toxins of three types of corn grains in Egypt with special reference to production of trichothecene-toxins. Microbiol Res 150:225-232

El-Sayed AMAA, Soher EA, Sahab AF (2003) Occurrence of certain mycotoxins in corn and corn-based products and thermostability of Fumonisin B1 during processing. Food / Nahrung 47:222-225

El-Tahan FH, el-Tahan MH, Shebl MA (2000) Occurrence of Aflatoxins in cereal grains from four Egyptian governorates. Nahrung 44:279-280

Eriksen GS (2003) Metabolism and toxicity of trichothecenes. Doctoral thesis, Swedish University of Agricultural Science

Eskola M, Parikka P, Rizzo A (2001) Trichothecenes, ochratoxin A and zearalenone contamination and Fusarium infection in Finnish cereal samples in 1998. Food Additives & Contaminants 18:707-718

European C (2003) Commission Direcctive 2003/100/EC of 31 October 2003 ammending Annex 1 to Directive 2002/32/EC of the European Parliament and of the Council on undesirable substances in animal feed . Official Journal of the European Union L 285:33-37

European C (2005) Commission Recommendation on the preven-tion and reduction of *Fusarium*-toxins in cereals and cereal products. DraftSANCO/1719/2005 – rev 2. European Commission, Brussels, Belgium

European C (2010) Commission regulation (EU) . Official Journal of the European Union 105:7-8

Fadl Allah EM (1998) Occurrence and toxigenicity of shape *Fusarium moniliforme*from freshly harvested maize ears with special references to Fumonisin production in Egypt. Mycopathologia 140:99-103

Farber P, Geisen R, Holzapfel WH (1997) Detection of AFogenic fungi in figs by a PCR reaction. Int J Food Microbiol 36:215-220

Flaherty JE, Woloshuk CP (2004) Regulation of Fumonisin biosynthesis in *Fusarium verticillioides* by a zinc binuclear cluster-type gene ZFR1. Applied and Environmental Microbiology 70:2653-2659

Fonseca H (2011) International legislation on mycotoxins:http://www.micotoxinas.com.br/Legis-i.pdf

Foroud NA, Eudes F (2009) Trichothecenes in Cereal Grains. International Journal of Molecular Sciences 10:147-173

Fredlund E, Gidlund A, Sulyok M, Börjesson T, Krska R, Olsen M, Lindblad M (2013) DON and other selected *Fusarium* toxins in Swedish oats - Occurrence and correlation to specific *Fusarium* species. International journal of food microbiology 167:276-283

Freeman GG, Morrison RI (1949) The isolation and chemical properties of trichothecin, an antifungal substance from Trichothecium roseum Link. Biochemical Journal 44:1-5

Freire FDCO, da Rocha, MEB (2017) Impact of mycotoxins on human health, in Fungal Metabolites, eds Mérillon J. M., Ramawat K., editors. (Cham: Springer;), 239–261

Frisvad JC (1989) The use of high-performance liquid chromatography and diode array detection in fungal chemotaxonomy based on profiles of secondary metabolites. Botanical Journal of the Linnean Society 99:81-95

Frisvad JC, Andersen B, Thrane U (2008) The use of secondary metabolite profiling in chemotaxonomy of filamentous fungi. Mycological research 112:231-240

Frisvad JC, Bridge PD, Arora DK (1998) Chemical fungal taxonomy

Frisvad JC, Frank JM, Houbraken JAMP, Kuijpers AFA, Samson RA (2004) New ochratoxin A producing species of *Aspergillus* section *Circumdati*. Studies in Mycology 50:23-43

Frisvad JC, Hubka V, Ezekiel CN, Hong S-B, Nováková A, Chen AL, Arzanlou M, Larsen TO, Sklenár F, Mahakarnchanakul W, Samson RA, Houbraken J (2019) Taxonomy of *Aspergillus* section Flavi and their production of aflatoxins, ochratoxins and other mycotoxins. Studies in Mycology 93:1–63

Frisvad JC, Skouboe P, Samson RA (2005) Taxonomic comparison of three different groups of Aflatoxin producers and a new efficient producer of Aflatoxin B1, sterigmatocystin and 2-O-methylsterigmatocystin, *Aspergillus rambelli*i sp. nov. Systematic and Applied Microbiology 28:442-453

Frisvad JC, Smedsgaard J, Samson RA, Larsen TO, Thrane U (2007) Fumonisin B2 production by Aspergillus niger. J Agric Food Chem. 55:9727–9732

Gagkaeva TY, Gavrilova OP (2013) Production of T-2 Toxin and Diacetoxyscirpenol by *Fusarium* Fungi on Different Nutrient Media. Agronomia 8:84-89

Gagkaeva TY, Gavrilova OP, Levitin MM (2008) Current views on the *Fusarium* taxonomy in section *Sporotrichiella*. Mycology and Phytopathology 42:201-214

Gagkaeva TY, Levitin MM, Nazarova LN, Sanin SS (2009) Infected grain and complex of *Fusarium* fungi on the territory of Russia Federation for 2004-2006 years. Agro XXI 4:4-6

Gale LR, Bryant JD, Calvo S, Giese H, Katan T, O'Donnell K, Suga H, Taga M, Usgaard TR, Ward TJ, Kistler HC (2005) Chromosome complement of the fungal plant pathogen *Fusarium graminearum* based on genetic and physical mapping and cytological observations . Genetics 1:171-17

Garcia R, Ilag L (1986) Aflatoxin in the Philippines, Workshop of CIMMYT:365-372

Geisen R (1996) Multiplex polymerase chain reaction for the detection of potential Aflatoxin and sterigmatocystin producing fungi. Syst Appl Microbiol 19:388-392

Geiser DM, Aoki T, Bacon CW, Baker SE, Bhattacharyya MK, Brandt ME, et al (2013). One fungus, one name: defining the genus Fusarium in a scientifically robust way that preserves longstanding use. Phytopathology 103, 400–408

Geiser DM, del Mar Jiménez-Gasco M, Kang S, Makalowska I, Veeraraghavan N, et al. (2004) *Fusarium*-ID v. 1.0: a DNA sequence database for identifying *Fusarium*. European Journal of Plant Pathology 110:473-479

Geiser DM, Gueidan C, Miadlikowska J, Lutzoni F, Kauff F, Hofstetter V, Fraker E, Schoch CL, Tibell L, Untereiner WA, et al.(2006) Eurotiomycetes: Eurotiomycetidaeand Chaetothyriomycetidae. Mycologia 98:1053-1065

Geiser DM, Samson RA, Varga J (2008) A review of molecular phylogenetics in *Aspergillus* and prospects for a robust genus-wide phylogeny. In: Varga K., Samson R.A., editors. *Aspergillus* in the genomic era. Academic Publishers; Wageningen 17–32

Gelderblom WC, Abel S, Smuts CM, Marnewick J, Marasas WF, Lemmer ER, Ramljak D (2001) Fumonisin-induced hepatocarcinogenesis: mechanisms related to cancer initiation and promotion. Environ Health Perspect 109 Suppl 2:291-300

Georgianna DR, Payne GA (2009) Genetic Regulation of Aflatoxin Biosynthesis: From gene to Genome . Fungal Genetics and Biology 46:113-125

Godet M, Munaut F (2010) Molecular strategy for identification in *Aspergillus* section Flavi. FEMS Microbiology Letters 304:157-168

Hakulin S (2013) Miten homemyrkkyjä jaetaan viljaketjussa? Proceedings of the Producer Seminar in Hämeenlinna, 31 January 2013; Available online: http://www.vyr.fi/www/fi/tapahtumat/menneet_tapahtumat/viljelijaseminaari_31012013.php (accessed on 26 November 2013). (in Finnish)

Halstensen AS, Nordby KC, Eduard W, Klemsdal SS (2006) Real-time PCR detection of toxigenic *Fusarium* in airborne and settled grain dust and associations with trichothecene mycotoxins. Journal of Environmental Monitoring 8:1235-1241

Hansen E, Jung M (1973) Control of Aflatoxins in the food industry. Pure and Applied Chemistry 35:239

Hara S, Fennell DI, Hesseltine CW (1974) Aflatoxin-producing strains of *Aspergillus flavus* detected by fluorescence of agar medium under ultraviolet light. Appl Microbiol 27:1118-1123

Haugland RA, Brinkman N, Vesper SJ (2002) Evaluation of rapid DNA extraction methods for the quantitative detection of fungi using real-time PCR analysis. Journal of Microbiological Methods 50:319-323

Haumann F (1995) Eradicating mycotoxins in food and feeds. Inform 6:248-256

Hawksworth DL, Crous PW, Redhead SA, Reynolds DR, Samson RA, Seifert KA, et al (2011). The Amsterdam Declaration on fungal nomenclature. IMA Fungus, 2,105–112

Hedayati M, Pasqualotto A, Warn P, Bowyer P, Denning DW (2007) *Aspergillus flavus*: human pathogen, allergen and mycotoxin producer. Microbiology (Reading, England) 153:1677-1692

Hietaniemi V, Cerveg Database (in Finnish). MTT Agrifood Research Finland: Jokioinen, Finland, (2013) Available online: https://portal.mtt.fi/portal/page/portal/kasper/pelto/peltopalvelut/cerveg (accessed on 26 November 2013).

Hietaniemi V, Kontturi M, Rämö S, Eurola M, Kangas A, Niskanen M, Saastamoinen M (2004) Contents of trichothecenes in oats during official variety, organic cultivation and nitrogen fertilization trials in Finland. Agricultural and Food Science 13:54-67

Hill RA, Blankenship PD, Cole RJ, Sanders TH (1983) Effects of soil moisture and temperature on preharvest invasion of peanuts by the *Aspergillus flavus* group and subsequent Aflatoxin development. Applied and Environmental Microbiology 45:628-633

Homolka L, Lisá L (2008) Long-term maintenance of fungal cultures on perlite in cryovials -- An alternative for agar slants. Folia Microbiol (Praha) 53:534-6

Hong SW, Kim DR, Kim JS, Cho G, Jeon CW, Kwak YS (2018) Development qRT-PCR Protocol to Predict Strawberry *Fusarium* Wilt Occurrence. The Plant Pathology Journal 34:163-170

Hussien T, Yli-Mattila T (2013) PCR for aflatoxigenic fungi as an alternative way for aflatoxin detection and quantification in developing countries:323-325

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (2002) Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. IARC Monogr Eval Carcinog Risks Hum 82:1-556

Ilang L (1984) Workshop on Mycotoxin Research in the Philippines:8-19

Iyer P, Zekri AR, Hung CW, Schiefelbein E, Ismail K, Hablas A, Seifeldin IA, Soliman AS (2010) Concordance of DNA methylation pattern in plasma and tumor DNA of Egyptian hepatocellular carcinoma patients. Exp Mol Pathol 88:107-111

Jaime-Garcia R, Cotty PJ (2006) Spatial Relationships of Soil Texture and Crop Rotation to *Aspergillus flavus* Community Structure in South Texas. Phytopathology 96:599-607

Jennings P, Coates ME, Walsh K, Turner JA, Nicholson P (2004) Determination of DON- and nivalenol-producing chemotypes of *Fusarium graminearum* isolates from wheat crops in England and Wales. Plant Pathology 53:643-652

Jiujiang Y, Chang P, Cary JW, Wright M, Bhatnagar D, Cleveland TE, Payne GA, Linz JE (1995) Comparative Mapping of Aflatoxin Pathway Gene Clusters in *Aspergillus parasiticus* and *Aspergillus flavus*. Applied and Environmental Microbiology 61:2365-2371

Jothikumard N, Griffiths MW (2002) Rapid detection of *Escherichia coli* O157:H7 with multiplex real-time PCR assays. Applied and Environmental Microbiology 68: 3169-3171

Juan C, Ritieni A, Mañes J (2012) Determination of trichothecenes and zearalenones in grain cereal, flour and bread by liquid chromatography tandem mass spectrometry. Food Chemistry.

Jurjevic Ž, Kubátová A, Kolařík M (2015) Taxonomy of *Aspergillus* section Petersonii sect. nov. encompassing indoor and soil-borne species with predominant tropical distribution. Plant Systematics and Evolution 301:2441-2462

Kachuei R, Yadegari MH, Rezaie S, Allameh A, Safaie N, Zaini F, Khanezad Yadzi F (2009) Investigation of stored wheat mycoflora, reporting the *Fusarium cf. langsethiae* in three provinces of Iran during 2007. Annals of Microbiology 59:383-390

Kara GNK, Ozbey F, Kabak B (2015) Co-occurrence of Aflatoxins and ochratoxin A in cereal flours commercialised in Turkey. Food Control 54:275-281

Keller NP, Turner G, Bennett JW (2005) Fungal secondary metabolism - from biochemistry to genomics. Nature Reviews Microbiology 3:937-947

Keller NP, Turner G, Bennett JW (2005) Fungal secondary metabolism - from biochemistry to genomics. Nature Reviews Microbiology 3:937-947

Kensler TW, Roebuck BD, Wogan GN, Groopman JD (2011) Aflatoxin: a 50-year odyssey of mechanistic and translational toxicology. Toxicological Sciences 120:28-48

Kim HK, Yun SH (2011) Evaluation of potential reference genes for quantitative RT-PCR analysis in *Fusarium graminearum* under different culture conditions. The Plant Pathology Journal 27:301-309

Kimura M, Anzai H, Yamaguchi I (2001) Microbial toxins in plant-pathogen interactions: biosynthesis, resistance mechanisms, and significance. The Journal of General and Applied Microbiology 47:149-160

Kimura M, Tokai T, Takahashi-Ando N, Ohsato S, Fujimura M (2007) Molecular and genetic studies of *Fusarium* trichothecene biosynthesis pathways, genes, and evolution.

. Bioscience, Biotechnology, and Biochemistry 71:2105-2123

Kistler HC, Broz K (2015) Cellular compartmentalization of secondary metabolism. Frontiers in Microbiology 6:68

Klich M, Pitt JI (1988) A laboratory guide to *Aspergillus* species and their teleomorphs. CSIRO Division of Food Processing, North Ryde, NWS, Australia

Knoll S, Mulfinger S, Niessen L, Vogel RF (2002) Rapid preparation of Fusarium DNA from cereals for diagnostic PCR using sonification and an extraction kit. Plant Pathology 51:728-734

Konstantinova P, Yli-Mattila T (2004) IGS-RFLP analysis and development of molecular markers for identification of *Fusarium poae*, *Fusarium langssethiae*, *Fusarium sporotrichioides* and *Fusarium kyushuense*. International journal of food microbiology 95:321-331

Kristensen R, Torp M, Kosiak B, Holst-Jensen A (2005) Phylogeny and toxigenic potential is correlated in *Fusarium* species as related by partial translation elongation factor 1 alpha gene sequences. Mycological Research 109:173-186

Lamoth F, Calandra T (2017) Early diagnosis of invasive mould infections and disease . Journal of Antimicrobial Chemotherapy 72:19-28

Lazzaro I, Falavigna C, Dall'Asta C, Proctor RH, Galaverna G, Battilani P (2012) Fumonisins B, A and C production and masking in *Fusarium verticillioides* and *F. proliferatum* grown with different water activity regimes. International Journal of Food Microbiology 159:93-100

Lee HJ, Ryu D (2015) Advances in Mycotoxin Research: Public Health Perspectives. J Food Sci 80:2970-2983

Lemmer ER, Pauline de la Motte Hall, Omori N, Omori M, et al (1999) Histopathology and gene expression changes in rat liver during feeding of Fumonisin B1, a carcinogenic mycotoxin produced by *Fusarium moniliforme*. Carcinogenesis 20:817

Lerda D (2010) Mycotoxin factsheet. 3rd edition. JRC technical notes, IRRM, Geel, Belgium

Leslie JF, Summerell BA (2006) The *Fusarium* laboratory manual. Blackwell Publishing Ltd, Oxford, UK

Levasseur-Garcia C (2018) Updated Overview of Infrared Spectroscopy Methods for Detecting Mycotoxins on Cereals (Corn, Wheat, and Barley). Toxins 10:38

Liddell CM (1991) Introduction: recent advances in *Fusarium* systematics. Phytopathology 81:1044-1045

Lin M, Dianese J (1976) A coconut agar medium for rapid detection of aflatoxin production by *Aspergillus* species. Phytopathology 66:1466-1469

Logrieco A, Moretti A, Perrone G, Mulè G (2007) Biodiversity of complexes of mycotoxigenic fungal species associated with *Fusarium* ear rot of maize and *Aspergillus* rot of grape. International Journal of Food Microbiology.

López-Errasquín E, Vázquez C, Jiménez M, González-Jaén MT (2007) Real-Time RT-PCR assay to quantify the expression of fum1 and fum19 genes from the Fumonisin-producing *Fusarium verticillioides*. J Microbiol Methods 68:312-317

Lubulwa G, Davis J (1994) Estimating the Social Costs of the Impacts of Fungi and Aflatoxins, 6th International Working Conference on stored-product Protection (6th IWCSPP):10

Luo C-F, Cai B, Hou N et al (2012) UDP-glucuronosyltransferase 1A1 is the principal enzyme responsible for puerarin metabolism in human liver microsomes. Archives of Toxicology 86(11):1681–1690

Luo J, Vogel RF, Niessen L (2014) Rapid detection of Aflatoxin producing fungi in food by real-time quantitative loop-mediated isothermal amplification. Food Microbiology 44:142-148

Maeda K, Tanaka A, Sugiura R, Koshino H, Tokai T, Sato M, et al (2016), Hydroxylations of trichothecene rings in the biosynthesis of *Fusarium* trichothecenes: evolution of alternative pathways in the

nivalenol chemotype. Environ Microbiol 18: 3798-3811

Mannaa M, Kim KD (2017) Influence of Temperature and Water Activity on Deleterious Fungi and Mycotoxin Production during Grain Storage. Mycobiology, 45(4), 240–254

Marasas WF (2001) Discovery and occurrence of the Fumonisins: a historical perspective. Environ Health Perspect 109 Suppl 2:239-243

Marasas WF, Riley RT, Hendricks KA, Stevens VL, Sadler TW, Gelineau-van Waes J, et al. (2004) Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and in vivo: a potential risk factor for human neural tube defects among populations consuming Fumonisin-contaminated maize. The Journal of Nutrition 134:711-716

Marasas WFO (1995) Fumonisins: their implications for human and animal health. Natural toxins. 3:193-198

Marin S, Magan N, Ramos AJ, Sanchis V (2004) Fumonisin-Producing Strains of *Fusarium*: A Review of Their Ecophysiology. J Food Prot 67:1792-1805

Maryani N, Lombard L, Poerba YS, Subandiyah S, Crous PW, Kema GHJ (2019) Phylogeny and genetic diversity of the banana *Fusarium* wilt pathogen *Fusarium oxysporum* f. sp. cubense in the Indonesian centre of origin. Studies in Mycology 92:155-194

Mayer Z, Bagnara A, Färber P, Geisen R (2003) Quantification of the copy number of nor-1, a gene of the Aflatoxin biosynthetic pathway by real-time PCR, and its correlation to the cfu of *Aspergillus flavus* in foods. International Journal of Food Microbiology 82:143-151

McCormick SP, Stanley AM, Stover NA, Alexander NJ (2011) Trichothecenes: from simple to complex mycotoxins. Toxins (Basel) 3:802-814

Medina A, Schmidt-Heydt M, Cárdenas-Chávez DL, Parra R, Geisen R, Magan N (2013) Integrating toxin gene expression, growth and Fumonisin B1 and B2 production by a strain of *Fusarium verticillioides* under different environmental factors. Journal of the Royal Society Interface 10:20130320

Merhej J, Richard-Forget F, Barreau C (2011) Regulation of trichothecene biosynthesis in *Fusarium*: recent advances and new insights. Applied Microbiology and Biotechnology 91:519

Milićević DR, Škrinjar M, Baltić T (2010) Real and perceived risks for mycotoxin contamination in foods and feeds: challenges for food safety control. Toxins 2:572-592

Misra JB, Thirumalaisamy PP (2012) Bulletin on Management of Aflatoxin. Junagadh. Directorate of Groundnut Research:52

Mohamed E Zain (2010) Impact of mycotoxins on humans and animals J. Saudi Chem. Soc 15:129–144

Moretti A (2009) Taxonomy of *Fusarium* genus, a continuous fight between lumpers and splitters. Zbornik Matice srpske za prirodne nauke 117:7-13

Moretti A., Logrieco A.F., Susca A. (2017) Mycotoxins: An Underhand Food Problem. In: Moretti A., Susca A. (eds) Mycotoxigenic Fungi. Methods in Molecular Biology, vol 1542. Humana Press, New York, NY

Moss MO (2002) Risk assessment for Aflatoxins in foodstuffs. International Biodeterioration and Biodegradation 50:137-142

Moubasher AH, el-Kady IA, Shoriet A (1977) Toxigenic *Aspergilli* isolated from different sources in Egypt. Ann Nutr Aliment 31:607-615

Murakami H (1976) A brief history of classification of the black aspergilli including the Kuro-koji molds. Taxonomic studies on Japanese Industrial strains of the *Aspergillus*. Journal of the Society of Brewing, Japan 71:952-955

Murakami H, Yoshida K, Yoshida K, Noro F (1979) Tables of mycological characters of the representative strains of the black aspergilli. Taxonomic studies on Japanese industrial strains of the *Aspergillus*. Journal of the society of Brewing, Japan 74:466-470

Nakajima Y, Tokai T, Maeda K, Tanaka A, Takahashi-Ando N, Kanamaru K, et al

(2014) A set of heterologous promoters useful for investigating gene functions in *Fusarium graminearum*. JSM Mycotoxins 64:147-152

Nakasone Karen K, Peterson Stephen W, Jong S (2004) Preservation and distribution of fungal cultures. Biodiversity of fungi : inventory and monitoring methods. Amsterdam: Elsevier Academic Press, 37-47

Nelson PE(E) [, Toussoun TA, [1925-], Marasas WFO, [1941-] (1983) *Fusarium* species: an illustrated manual for identification / Paul E. Nelson, T.A. Toussoun, and W.F.O. Marasas. Pennsylvania State University Press

Nelson PE, Dignani MC, Anaissie EJ (1994) Taxonomy, biology and clinical aspect of *Fusarium* species. Clinical Microbiology Review 7:479-504

Nesbitt BF, O'Kelly J, Sargeant K, Sheridan A (1962) Toxic Metabolites of *Aspergillus flavus*. Nature 195:1062-1063

Nicholson P, Chandler E, Draeger RC, Gosman NE, Simpson DR, Thomsett M, Wilson AH (2003) Molecular tools to study epidemiology and toxicology of *Fusarium* head blight of cereals. European Journal of Plant Pathology 109:691-703

Nicholson P, Simpson DR, Weston G, Rezanoor HN, Lees AK, Parry DW, Joyce D (1998) Detection and quantification of *Fusarium culmorum* and *Fusarium graminearumin* cereals using PCR assays. Physiological and Molecular Plant Pathology

Nielsen LK, Jensen JD, Nielsen GC, Jensen JE, Spliid NH, Thomsen IK, Justesen AF, Collinge DB, Jorgensen LN (2011) *Fusarium* head blight of cereals in Denmark: Species complex and related mycotoxins. Phytopathology 101:960-969

NOTERMANS S, HEUVELMAN CJ, VAN EGMOND HP, PAULSCH WE, BESLING JR (1986) Detection of Mold in Food by Enzyme-Linked Immunosorbent Assay. Journal of Food Protection 10:786-791

O'Donnell K, Ward TJ, Robert VARG (2015) DNA sequence-based identification of *Fusarium*: Current status and future directions. Phytoparasitica 15:583-595

Onions AHS (1971) Preservation of fungi. In: Methods in microbiology, Vol. 4. (C. Booth, ed.), V Academic Press, London, UK, 113–151

Ostry V, Malir F, Toman J, et al (2017). Mycotoxins as human carcinogens—the IARC Monographs classification. Mycotoxin Res 33: 65

Paavanen-Huhtala S, Hyvonen J, Bulat S, Yli-Mattila T (1999) RAPD-PCR, isozyme, rDNA RFLP and rDNA sequence analyses in identification of Finnish *Fusarium oxysporum* isolates. Mycol Res 103:625-634

Parikka P, Hakala K, Tiilikkala K (2012) Expected shifts in *Fusarium* species' composition on cereal grain in Northern Europe due to climatic change. Food Additives and Contaminants: Part A, 29 (10): 1543–1555

Parikka P, Hietaniemi V, Ramo S, Jalli H (2008) *Fusarium* infection and mycotoxin contents of oats under different tillage treatments. Session V, 8th International Oat Conference, Minneapolis, MN

Parry DW, Jenkins P, McLeod L (1995) *Fusarium* ear blight (scab) in small grain cereals – a review. Plant Pathology 44:207-238

Parry DW, Nicholson P (1996) Development of a PCR assay to detect *Fusarium poae* in wheat. Plant Pathology 45:383-91

Pasquali M, Beyer M, Logrieco A, Audenaert K, Balmas V, Basler R, Boutigny A-L, et al (2016). A European Database of *Fusarium graminearum* and *F. culmorum* Trichothecene Genotypes Front. Microbiol. 7:406

Pasquali M, Giraud F, Brochot C, Cocco E, Hoffmann L, Bohn T (2010) Genetic *Fusarium* chemotyping as a useful tool for predicting nivalenol contamination in winter wheat. International journal of food microbiology 137:246-253

Pasquali M, Migheli Q (2014) Genetic approaches to chemotype determination in type B-trichothecene producing *Fusaria*. International journal of food microbiology 189:164-182

Paterson RRM, Lima N (2010) How will climate change affect mycotoxins in food?. Food Research International 43:1902-1914

Pereira VL, Fernandes JO, Cunha SC (2014) Mycotoxins in cereals and related foodstuffs: a review on occurrence and recent methods of analysis. Trends in Food Science and Technology 36:96-136

Perrin PW (1979) Long-term storage of cultures of wood-inhabiting fungi under mineral oil. Mycologia 71: 867–869

Pettersson H, Hedman R, Engstrom B, Elwinger K, Fossum O (1995) Nivalenol in Swedish cereals - occurrence, production and toxicity towards chickens. Food Additives and Contaminants 12:373-376

Pildain MB, Frisvad JC, Vaamonde G, Cabral D, Varga J, Samson RA (2008) Two novel Aflatoxin-producing *Aspergillus* species from Argentinean peanuts. Int J Syst Evol Microbiol 58:725-735

Pildain MB, Vaamonde G, Cabral D (2004) Analysis of population structure of *Aspergillus flavus* from peanut based on vegetative compatibility, geographic origin, mycotoxin and sclerotia production. Int J Food Microbiol 93:31-40

Pitt J, Hocking A (2009) Fungi and Food Spoilage. Blackie Academic and Professional, London

Pitt JI, Hocking AD, Glenn DR (1983) An improved medium for the detection of *Aspergillus flavus* and *A. parasiticus*. J Appl Bacteriol 54:109-114

Piva A, Casadei G, Pagliuca G, Cabassi E, Galvano F, Solfrizzo M, Riley RT, Diaz DE (2005) Inabilityof activated carbon to prevent the toxicity of culture material containing fumonisin B1 when fed to weanedpiglets. J. Anim. Sci. 83, 1939–1947

Prieto R, Woloshuk CP (1997) ord1, an oxidoreductase gene responsible for conversion of O-methylsterigmatocystin to Aflatoxin in *Aspergillus flavus*. Appl Environ Microbiol 63:1661-1666

Proctor RH, Brown DW, Plattner RD, Desjardins AE (2003) Co-expression of 15 contiguous genes delineates a FUMONISIN biosynthetic gene cluster in Gibberella moniliformis. Fungal genetics and biology FG & B. 38:237-249

Proctor RH, Busman M, Seo J, Lee YW, Plattner RD (2008) A Fumonisin biosynthetic gene cluster in *Fusarium oxysporum* strain O-1890 and the genetic basis for B versus C Fumonisin production. Fungal genetics and biology: FG & B 45:1016-1026

Proctor RH, McCormick SP, Kim H-S, Cardoza RE, Stanley AM, Lindo L, et al (2018) Evolution of structural diversity of trichothecenes, a family of toxins produced by plant pathogenic and entomopathogenic fungi. PLoS Pathog 14(4)

Proctor RH, Van Hove F, Susca A, Stea G, Busman M, Van der Lee T, et al. (2013) Birth, death and horizontal gene transfer of the Fumonisin byosinthetic gene cluster during the evolutionary diversification of *Fusarium*. Molecular Microbiology 90:290-306

Prodi A, Salomoni D, Bertacchini E, Alkadri D, Pisi A, Tonti S, Alberti I, Dal Pra M, Pancaldini D, Covarelli L, Nipoti P (2011) Determination of DON and nivalenol producing chemotypes of *Fusarium graminearum* isolated from durum wheat in dfferent Italian regions. Plant Breeding and Seed Science 64:75-79

Rahjoo V, Zad J, Javan-Nikkhah M, Gohari AM, Okhowat SM, Bihamta MR, Razzaghian J, Klemsdal SS (2008) Morphological and molecular identification of *Fusarium* isolated from maize ears in Iran. J Plant Pathol 90:463-468

Raper KB, Fennell DI (1965) The genus *Aspergillus*. The Williams and Wilkins Co. Baltimore, Md

Rauvola M (2013) Determination of deoxynivalenol in grain by semi-quantitative quick test. (in Finnish). Bachelor's Thesis, Turku University of Applied Sciences, Turku, Finland, 2013

Rauvola M, Hovinen T, Hietaniemi V, Kaitaranta J, Rämö S (2013) Screening Deoxynivalenol in Oat using a Quickmethod with Comparison to a Quantitative GC-MS Analysis. In Proceedings of the 12th European *Fusarium* Seminar, Bordeaux, France, 12–16 May 2013; p. 127.

Ren S, Yang M, Yue Y, Ge F, Li Y, Guo X, et al. (2018) Lysine Succinylation Contributes to Aflatoxin Production and Pathogenicity in *Aspergillus flavus*. Molecular & Cellular Proteomics: MCP 17:457-471

Rheeder JP, Marasas WF, Vismer HF (2002) Production of fumonisin analogs by *Fusarium* species. Applied and environmental microbiology, 68(5), 2101–2105

Richter DL, Bruhn JN (1989) Revival of saprotrophic and mycorrhizal basidiomycete cultures from cold storage in sterile water. Can. J. Microbiol. 35: 1055-1060

Robinow CF, Caten CE (1969) Mitosis in *Aspergillus nidulans*. Journal of Cell Science 5:403-431

Rodrigues I, Naehrer K (2012) A Three-Year Survey on the Worldwide Occurrence of Mycotoxins in Feed stuffs and Feed. Toxins 4:663-675

Rodrigues P, Santos C, Venancio A, Lima N (2011) Species identification of *Aspergillus* section *Flavi* isolates from Portuguese almonds using phenotypic, including MALDI-TOF ICMS, and molecular approaches. J Appl Microbiol 111:877-892

Rodrigues P, Venancio A, Kozakiewicz Z, Lima N (2009) A polyphasic approach to the identification of aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* Section *Flavi* isolated from Portuguese almonds. Int J Food Microbiol 129:187-193

Rossi V, Scandolara A, Battilani P (2009) Effect of environmental conditions on spore production by *Fusarium verticillioides*, the causal agent of maize ear rot. Eur J Plant Pathol 123:159-169

Saito M, Tsuruta O (1993) A new variety of *A. flavus* from tropical soil in Thailand and its Aflatoxin productivity. Japan Association of Mycotoxicology 37:31-36

Samson RA, Houbraken JAMP, Kuijpers AFA, Frank JM, Frisvad JC (2004) New ochratoxin A or sclerotium producing species of *Aspergillus* section *Nigri*. Studies in Mycology 50:45-61

Sandoval-Denis M, Swart WJ, Crous PW (2018) New *Fusarium* species from the Kruger National Park, South Africa. MycoKeys 34:63-92

Sanoubar R, Bauer A, Seigner L (2015) Detection, Identification and Quantification of *Fusarium graminearum* and *Fusarium culmorum* in Wheat Kernels by PCR Techniques. Journal of Plant Pathology and Microbiology 6:287

Sarver BAJ, Ward TJ, Gale LR, Broz K, Kistler HC, Aoki T, Nicholson P, Carter J, O'Donnell K (2011) Novel *Fusarium* head blight pathogens from Nepal and Louisiana revealed by multilocus genealogical concordance. Fungal Genetics and Biology 48:1096-1107

Schatzmayr G, Streit E (2013) Global occurrence of mycotoxins in the food and feed chain: facts and figures. World Mycotoxin Journal 6:213-222

Schnerr H, Niessen L, Vogel RF (2001) Real time detection of the tri5 gene in Fusarium species by LightCycler™-PCR using SYBR® Green I for continuous fluorescence monitoring. Int. J. Microbiol 71:53-61

Schoch CL, Seifert K A, Huhndorf S, Robert V, Spouge JL, Levesque C A, et al (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences USA, 109, 6241–6246

Shapira R, Paster N, Eyal O, Menasherov M, Mett A, Salomon R (1996) Detection of aflatoxigenic molds in grains by PCR. Appl Environ Microbiol 62:3270-3273

Shephard GS (2008) Risk assessment of Aflatoxins in food in Africa. Food Additives & Contaminants: Part A 25:1246-1256

Shephard GS (2009) Aflatoxin analysis at the beginning of the twenty-first century. Anal Bioanal Chem 395:1215-1224

St Leger RJ, Screen SE, Shams-Pirzadeh B (2000) Lack of host specialization in *Aspergillus flavus*. Appl Environ Microbiol 66:320-324

Starkey DE, Ward TJ, Aoki T, Gale LR, Kistler HC, Geiser DM, Suga H, Tóth B, Varga J, O'Donnell K (2007) Global molecular surveillance reveals novel *Fusarium* head blight species and

trichothecene toxin diversity. Fungal Genet Biol 44(11):1191-204

Stenglein SA (2009) *Fusarium poae*: a pathogen that needs more attention. Journal of Plant Pathology 91:25-36

Stepien L, Popiel D, Koczyk G, Chelkowsky J (2008) Wheat-infecting *Fusarium* species in Poland – their chemotypes and frequencies revealed by PCR assay. Journal of Applied Genetics 49:433-441

Streit E, Schatzmayr G, Tassis P, Tzika E, Marin D, Taranu I, Tabuc C, Nicolau A, Aprodu I, Puel O, Oswald IP (2012) Current Situation of Mycotoxin Contamination and Co-occurrence in Animal Feed—Focus on Europe. Toxins 4:788-809

Suga H, Gale RL, Kistler HC (2004) Development of VNTR markers for two *Fusarium graminearum* clade species for two *Fusarium graminearum*cladespecies . Molecular Ecology Notes 4:468-470

Sugiura Y, Fukasaku K, Tanaka T, Matsui Y, Ueno Y (1993) *Fusarium poae* and *Fusarium crookwellense*, fungi responsible for the natural occurrence of nivalenol in Hokkaido. Applied and. Environmental Microbiology 59:3334-3338

Sultan Y, Magan N (2010) Mycotoxigenic fungi in peanuts from different geographic regions of Egypt. Mycotoxin Res 26:133-140

Summerell BA, Salleh B, Leslie JF (2003) A utilitarian approach to *Fusarium* identification. Plant Disease 87:117-127

Sundheim L, Brodal G, Hofgaard IS, Rafoss T (2013) Temporal variation of mycotoxin producing fungi in Norwegian cereals. Microorganisms 1:188-198

Suproniene S, Justesen AF, Nicolaisen M, Mankeviciene A, Dabkevicius Z, Semaskiene R, Leistrumait A (2010) Distribution of trichothecene and zearalenone producing *Fusarium* species in grain of different cereal species and cultivars grown under organic farming conditions in Lithuania. Annals of Agricultural and Environmental Medicine 17:73-80

Suproniene S, Sakalauskas S, Stumbriene K, Zvirdauskiene R, Svegzda P (2016) Variances in trichothecene chemotype distribution in Lithuanina wheat grain and within pure culture *Fusarium graminearum*isolated from the same grain samples. European Journal of Plant Pathology 144:371-381

Surup F, Kuhnert E, Lehmann E, Heitkämper S, Hyde KD, Fournier J, Stadler M (2014) Sporothriolide derivates as chemotaxonomic markers for Hypoxylon monticulosum. Mycology 5:110–119

Sydenham EW, Shephard GS, Thiel PG, Stockenstrom S, Snijman PW, Van Schalkwyk DJ (1996) Liquid chromatographic determination of Fumonisins B1, B2, and B3 in corn: AOAC-IUPAC collaborative study. Journal of AOAC International. 79:688-696

Talas F, Parzies HK, Miedaner T (2011) Diversity in genetic structure and chemotype composition of *Fusarium graminearum* sensu stricto population causing wheat head blight in individual fields in Germany. European Journal of Plant Pathology 131:39-48

Tan CS, van Ingen CW, Stalpers JA (2007) Freeze-drying fungi using a shelf freeze-drier. Methods Mol Biol 368:119-125

Torp M, Langseth W (1999) Production of T-2 toxin by a *Fusarium* resembling *Fusarium poae*. Mycopathologia 147:89-96

Torp M, Nirenberg HI (2004) Description of *Fusarium langsethiae* sp. nov. International Journal of Food Microbiology 95:89-96

Torp M, Nirenberg HI (2004) *Fusarium langsethiae* sp. nov. on cereals in Europe. International journal of food microbiology 95:247-256

Uoti J, Ylimäki A (1974) The occurrence of *Fusarium* species in cereal grain in Finland. Annales Agriculturae Fenniae 13:5-17

Van der Fels-Klerx HJ, Klemsdal S, Hietaniemi V, Lindblad M, Iannou-Kakouri E, Van Asselt ED (2012) Mycotoxin contamination of cereal grain commodities in relation to climate in North West Europe. Food Additives & Contaminants 29:1581-1592

Van der Lee T, Zhang H, van Diepeningen A, Waalwijk C (2015) Biogeography of *Fusarium graminearum* species complex and chemotypes: A review. Food Additives & Contaminants: Part A 32:453-460

van Egmond HP, Schothorst RC, Jonker MA (2007) Regulations relating to mycotoxins in food: perspectives in a global and European context. Analytical and bioanalytical chemistry 389:147-157

Waalwijk C, Kastelein P, de Vries I, Kerenyi Z, van der Lee T, Hesselink T, Kohl J, Kema G (2003) Major changes in *Fusarium* spp. in the Netherlands. European Journal of Plant Pathology 109:743-754

Waalwijk C, Koch S, Ncube E, Allwood J, Flett B, de Vries I, Kema G (2008) Quantitative detection of *Fusarium* spp. and its correlation with Fumonisin content in maize from South African subsistence farmers. World Mycotoxin Journal 1:39-47

Waalwijk C, van der Heide R, de Vries I, van der Lee T, Schoen C, Costrel-de Corainville G, Häuser-hahn I, Kastelein P, Köhl J, Lonnet P, Demarquet T, Kema GH (2004) Quantitative Detection of *Fusarium* Species in Wheat Using TaqMan. Eur J Plant Pathol 110:481-494

Wang W, Soliman S, Li X, Zhu H, Yang L, Yin Y, Zhou T (2015) Transformation of trichothecene acetyldeoxynivalenol to deoxynivalenol by bacterial acetyltransferase. 10.1201/b18418-145.

Ward TJ, Bielawski JP, Kistler HC, Sullivan E, O'Donnell K (2002) Ancestral polymorphism and adaptive evolution in the trichothecene mycotoxin gene cluster of phytopathogenic *Fusarium*. Proceedings of the National Academy of Sciences of the United States of America 99:9278-9283

Ward TJ, Clear RM, Rooney AP, O'Donnell K, Gaba D, Patrick S, Starkey DE, Gilbert J, Geiser DM, Nowicki TW (2008) An adaptive evolutionary shift in fusarium *Fusarium* head blight pathogen populations is driving the rapid spread of more toxigenic Fusarium graminearum in North America. Fungal Genetics and Biology 45:473-484

Watanabe M, Yonezawa T, Lee KI, Kumagai S, Sugita-Konishi Y (2011) Molecular phylogeny of the higher and lower taxonomy of the Fusarium genus and differences in the evolutionary histories of multiple genes. BMC Evolutionary Biology 11:322

White PL, Wingard JR, Bretagne S, Löffler J, Patterson TF, Slavin MA, et al. (2015) *Aspergillus* polymerase chain reaction: systematic review of evidence for clinical use in comparison with antigen testing. Clinical Infectious Diseases 61:1293-1303

Wilson A, Simpson D, Chandler E, Jennings P, Nicholson P (2004) Development of PCR assays for the detection and differentiation of *Fusarium sporotrichioides* and *Fusarium langsethiae*. FEMS Microbiology Letters 233:69-76

Wogan GN, Kensler TW, Groopman JD (2012) Present and future directions of translational research on Aflatoxin and hepatocellular carcinoma. A review. Food Addit Contam Part A. Chem Anal Control Expo Risk Assess 29:249-257

Woloshuk CP, Foutz KR, Brewer JF, Bhatnagar D, Cleveland TE, Payne GA (1994) Molecular characterization of aflR, a regulatory locus for Aflatoxin biosynthesis. Applied and Environmental Microbiology 60:2408-2414

Ylimäki A, Koponen H, Hintikka E, Nummi M, Niku-Paavola M, Ilus T, Enari TM (1979) Mycoflora and occurrence of *Fusarium* toxins in Finnish grain. Technical Research Centre of Finland, Materials and Processing Technology 21:1-28

Yli-Mattila T (2010) Ecology and evolution of toxigenic Fusarium species in cereals in northern Europe and Asia. Plant Pathology 92:7-18

Yli-Mattila T, Gagkaeva T (2016) *Fusarium* Toxins in Cereals in Northern Europe and Asia. In: Fungi: Applica-tions and Management Strategies (S.H. Deshmukh, J.K Mis-ra, J.P. Tewari, P. Tamas, ed.), CRC Press, Taylor & Francis Group, 293–317

Yli-Mattila T, Gagkaeva T (2010) Molecular chemotyping of *Fusarium graminearum*, *F. culmorum* and *F. cerealis* isolates from Finland and Russia. In: Gherbawy Y, Voigt K (eds) Molecular

identification of fungi. Springer Verlag, Berlin, Heidelberg, New York, pp 159-177

Yli-Mattila T, Mach R, Alekhina IA, Bulat SA, Koskinen S, Kullnig-Gradinger CM, Kubicek C, Klemsdal SS (2004a) Phylogenetic relationship of *Fusarium langsethiae* to *Fusarium poae* and *F. sporotrichioides* as inferred by IGS, ITS, b-tubulin sequence and UP-PCR hybridization analysis. International Journal of Food Microbiology 95:267-285

Yli-Mattila T, Mach RL, Alekhina IA, Bulat SA, Koskinen S, Kullnig-Gradinger CM, Kubicek CP, Klemsdal SS (2004) Phylogenetic relationship of *Fusarium langsethiae* to *Fusarium poae* and *Fusarium sporotrichioides* as inferred by IGS, ITS, beta-tubulin sequences and UP-PCR hybridization analysis. Int J Food Microbiol 95:267-285

Yli-Mattila T, Paavanen-Huhtala S, Jestoi M, Parikka P, Hietaniemi V, Gagkaeva T, Sarlin T, Haikara A, Laaksonen S, Rizzo A (2008) Real-time PCR detection and quantification of *Fusarium poae*, *F. graminearum*, *F. sporotrichioides* and *F. langsethiae* in cereal grains in Finland and Russia. Archives of Phytopathology and Plant Protection, 41 (4) pp.243-260, 2008

Yli-Mattila T, Paavanen-Huhtala S, Parikka P, Hietaniemi V, Jestoi M, Gagkaeva T, Sarlin T, Haikara A, Laaksonen S, Rizzo A (2008) Real-time PCR detection and quantification of *Fusarium poae*, *F. graminearum*, *F. sporotrichioides* and *F. langsethiae* as compared to mycotoxin production in grains in Finland and Russia. Archives of Phytopathology and Plant Protection 41:243-260

Yli-Mattila T, Paavanen-Huhtala S, Parikka P, Hietaniemi V, Jestoi M, Rizzo A (2004b) Real-time PCR detection and quantification of *Fusarium poae* as compared to mycotoxin production in grains in Finland:422-425

Yli-Mattila T, Paavanen-Huhtala S, Parikka P, Konstantinova P, Gagkaeva T (2004c) Molecular and morphological diversity of *Fusarium* fungi in Finnish and north-western Russia. European Journal of Plant Pathology 110:573-585

Yli-Mattila T, Parikka P, Lahtinen T, Ramo S, Kokkonen M, Rizzo A, Jestoi M, Hietaniemi V (2009) *Fusarium* DNA levels in Finnish cereal grains. In: Gherbawy Y, Mach RL, Rai M (eds) Current Advances in Molecular Mycology. Nova Science Publishers, New York, USA, pp 107-138

Yli-Mattila T, Rämö S, Tanner R, Loiveke H, Hietaniemi V (2011) *Fusarium* DNA levels as compared to mycotoxin levels in Finnish and Estonian grain samples. Plant Breeding and Seed Science 64:131-140

Yli-Mattila T, Ward TJ, O'Donnell K, Proctor RH, Burkin AA, Kononenko GP, Gavrilova OP, Aoki T, McCormick SP, Gagkaeva TY (2011) *Fusarium sibiricum* sp. nov, a novel type A trichothecene-producing Fusarium from northern Asia closely related to *F. sporotrichioides* and *F. langsethiae* . International journal of food microbiology 147:58-68

Yöruk EG, Albayrak EG (2012) Chemotyping of *Fusarium graminearum* and *F. culmorum* isolates from Turkey by PCR assay. Mycopathologia 173:53-61

Yu J (2012) Current Understanding on Aflatoxin Biosynthesis and Future Perspective in Reducing Aflatoxin Contamination. Toxins (Basel) 4:1024-1057

Yu J, Bhatnagar D, Cleveland TE (2004) Completed sequence of Aflatoxin pathway gene cluster in *Aspergillus parasiticus*. FEBS Lett 564:126-130

Yu J, Chang PK, Ehrlich KC, Cary JW, Bhatnagar D, Cleveland TE, Payne GA, Linz JE, Woloshuk CP, Bennett JW (2004) Clustered pathways genes in aflatoxin biosynthesis. Appl Microbiol 70:1253–1262

Zarrin M, Erfaninejad M (2016) Molecular variation analysis of *Aspergillus flavus* using polymerase chain reaction-restriction fragment length polymorphism of the internal transcribed spacer rDNA region. Experimental and Therapeutic Medicine 12:1628-1632

Zhang F, Zhong H, Han X, Guo Z, Yang W, Liu Y, et al (2015) Proteomic profile of *Aspergillus flavus* in response to water activity. Fungal Biol 119: 114–124

Zhang ZG, Zhang JY, Zheng XB, Yang YW, Ko WH (2004) Molecular Distinctions Between Phytophthora capsici and Ph. tropicalis Based on ITS Sequences of Ribosomal DNA. J Phytopathol 152:358-364

Annales Universitatis Turkuensis

UNIVERSITY OF TURKU

Grano Oy, Turku, Finland 2019

ISBN 978-951-29-7694-2 (PRINT)
ISBN 978-951-29-7695-9 (PDF)
ISSN 0082-6979 (Print)
ISSN 2343-3183 (Online)