TECHNISCHE UNIVERSITÄT MÜNCHEN
TUM School of Life Sciences der Technischen Universität München
Lehrstuhl für Technische Mikrobiologie

# Taxonomy, diagnosis and mycotoxicology of species belonging to the *Fusarium fujikuroi* species complex (FFSC)

**Évelin Francine Tatsch**

Vollständiger Abdruck der von der TUM School of Life Sciences der Technischen Universität München zur Erlangung des akademischen Grades eines

**Doktor der Naturwissenschaften (Dr. rer. nat.)**
genehmigten Dissertation.

Vorsitzender: Prof. Dr. Michael Rychlik

Prüfende der Dissertation: 1. Prof. Dr. Rudi F. Vogel
2. Prof. Dr. Johan Philipp Benz

Die Dissertation wurde am 12.04.2021 bei der Technischen Universität München eingereicht und durch die TUM School of Life Sciences am 06.07.2021 angenommen.

I dedicate this Ph.D. thesis to my husband
for your constant support, patience and love.

## ACKNOWLEDGMENTS

My personal gratitude goes to:

Prof. Dr. Rudi F. Vogel for the great opportunity to conduct my doctoral thesis in Germany, and for his great support during the years.

Prof. Dr. Ludwig Niessen for sharing his knowledge in food mycology with me. Thank you for encouraging me to speak german and to challenge myself.

The technical asssistants Margarete Schreiber, Andrea Pape and Rene Mamet for their skillful technical assistance and our secretary Angela Seppeur for organizational support.

Dr. Karsten Meyer for the great cooperation, which contributes to this thesis.

The best colleagues in the world: Roman, Doro, Maik, Di, Lisa, Magdalena, Lena, Katharina and Monika for all the good times we spent together at the office, at the lab and in private life.

Dr. Alexander Lauterbach for sharing his MALDI-TOF MS knowledge with me.

Dr. Elisabeth Vogt and Meike Kliche for the initial guidance when I came to Germany.

Apart from the laboratory, I would like to thank Prof. Dr. Marina Copetti, who encouraged me to start a Ph.D. abroad. Thanks to my mother and Oma for their love and encouragement throughout my education. Thanks to my best friends for all the great times.

My husband Gabriel for his never-ending encouragement that kept me going throughout my Ph.D. I would have never made it without you. Muito obrigada, meu amor!

# ABBREVIATIONS

| | |
|---|---|
| °C | degree Celcius |
| µg | micrograms |
| Kg | kilograms |
| AT | Ambient temperature |
| aw | water activity |
| BBA | Bundesforschungsinstitut für Kulturpflanzen |
| BIP | Backward inner primer |
| BFE | Max-Rubner-Institut, Bundeserforschungsanstalt für Ernährung |
| BLAST | Basic local alignment search tool |
| bp | Base pairs |
| Bst | *Bacillus stearothermophilus* |
| cDNA | Complementary DNA |
| CBS | Westerdijik Fungal Biodiversity Institute Utrecht |
| CML | Coleção micológica de Lavras |
| CMW | Collection of the Forestry and Agricultural Biotechnology Institute |
| CTAB | Cetyltrimethylammonium bromide |
| DAPC | Discriminant analysis of principal component |
| DNA | Desoxyribonucleic acid |
| dNTP | Desoxy nucleoside triphosphate |
| DSM | Deutsche Sammlung von Mikroorganismen und Zellkulturen |
| EDTA | Ethylenediaminetetraacetic acid |
| EU | European Union |
| $FB_1$ | Fumonisin $B_1$ |
| FFSC | *Fusarium fujikuroi* species complex |
| FIP | Forward inner primer |
| FUMB | Fumonisin inducing liquid medium |
| *F.* | *Fusarium* |
| gDNA | Genomic DNA |
| IBT | Institut for Bioteknologi strain collection at DTU Bioengineering in Denmark |
| IMI | Institute of Microbiology and Infection |
| ITEM | Istituto Tossine e Micotossine da Parassiti Vegetali |
| HPLC | High performance liquid chromatography |
| LAMP | Loop-mediated isothermal amplification |
| LC-MS/MS | Liquid chromatography-mass spectrometry |
| LOD | Limit of detection |
| MALDI-TOF MS | Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry |
| MDS | Multidimensional scaling |
| MEA | Malt extract |
| min | minute |
| MOPS | 3-Morpholinopropanesulfonic acid |
| mRNA | Messenger RNA |
| MRC | South African Medical Research Council |
| MSP | Main spectrum profile |
| MUM | Micoteca da Universidade de Minho |

| | |
|---|---|
| NCBI | National Center for Biotechnology Information |
| NRRL | Northern Regional Research Laboratory |
| PCR | Polymerase chain reaction |
| PDR | Peak detection rate |
| PMF | Peptide mass fingerprint |
| PKS | Polyketide synthase |
| ppb | Parts per billion |
| RBG | Royal Botanic Gardens Victoria |
| RNA | Ribonucleic acid |
| RT-LAMP | Reverse transcription Loop-mediated isothermal amplification |
| RT-PCR | Reverse transcription polymerase chain reaction |
| SDA | Sabouraud Dextrose Broth |
| SDB | Sabouraud Dextrose Agar |
| SNA | Synthetic Nutrient Agar |
| SNR | Signal to noise ratio |
| TEF 1α | Translation elongation factor 1 α |
| TMW | Technische Mikrobiologie Weihenstephan |
| w/v | Weight per volume |

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# 1 Introduction

## 1.1 Economic and health impacts of *Fusarium fujikuroi* species complex (FFSC)

Species of the fungal genus *Fusarium* (*F.*) are known globally as causal agents of extensive and recurring damages to various fields of economy. Agriculture is the most affected area since several species cause severe and devastating plant diseases. Many species in the genus *Fusarium* have been associated with diseases affecting the quality of crops that are of high economic value for countries worldwide (Beccari et al., 2019; Windels, 2000). It is estimated that *Fusarium* species cause at least one disease in 80 % of all cultivated plants (Leslie and Summerell, 2006). Depending on the species concept that is followed, the genus *Fusarium* includes between 65-70 up to more than 1000 species (Moretti, 2009). Following the lower-number concept, most species are well defined, mostly on morphological grounds. However, modern molecular taxonomic concepts show that many of the well-defined species consist of a high number of taxa that cannot be separated morphologically but show high divergence when DNA-based taxonomy is applied. Today, such species are summarized to form species complexes. Therefore, many of the former species in *Fusarium* are now regarded as species complexes. The *Fusarium fujikuroi* species complex (FFSC) comprises one of the most important phytopathogen-containing species complexes, which cause drastic yield reduction in many countries worldwide. Typical diseases such as “bakanae” or “foolish seedling” of rice, pitch canker of pine as well as ear, seed and root rot of maize and other commodities (Aoki et al., 2014).

*F. fujikuroi, F. verticillioides, F. andiyazi and F. proliferatum* are potential pathogens of the gibberellin-induced bakanae disease of rice causing considerable reduction rice yields in Asia and Africa (Wulff et al., 2010). Maize ear and root rot caused mainly by *F. verticillioides* and *F. nygamai*, respectively, impact the productivity of this cereal consumed

worldwide (Leyva-Madrigal et al., 2015). Maize infections by mixed FFSC members such as *F. verticillioides*, *F. nygamai*, *F. thapsinum* and *F. andiyazi* developed the same symptomatology including root rot, as well as wilting, stalk thinning, and reduced aerial and root growth to different degrees (Leyva-Madrigal et al., 2015). Pitch canker in pine was originally associated only with *F. circinatum* in the United States but the pathogen has spread to European and other countries and is becoming a global problem (Vainio et al., 2019). Additionally, recently described new species such as *F. marasasianum*, *F. parvisorum* and *F. sororula* displayed similar levels of pathogenicity to *Pinus* sp. and symptoms like stem canker, branch die-back on trees in plantations and root or collar rot of seedlings make the symptoms indistinguishable from *F. circinatum*-like infections (Herron et al., 2015). Post-harvest diseases by FFSC species affect the export-import balance of bananas, the crown rot has been received importance due the negative impact (Lassois et al., 2010). Recently, Molnár et al. (2015) and Kamel et al. (2016) reported the occurrence of *F. verticillioides* and *F. musae* on banana fruits from Dominican Republic, Ecuador, Ivory Coast, Columbia and Costa Rica. Although both species are involved on the pathogenicity, *F. musae* strains demonstrate greater ability to cause infection on banana fruits than *F. verticillioides* (Moretti et al., 2004).

Relevant information about the FFSC species is included in table 1 based on the available literature. FFSC species are able to infect many important agricultural commodities including maize, wheat, sorghum, pineapple, sugarcane, banana, chickpea, cowpea, pigeon pea, soybean, roselle, mandarin, sweet potato, mango, pearl millet, sugar beet, tobacco, sesame seed, white mulberry, asparagus, garlic, peppers, rhubarb, fig and oil palm as well as ornamental plants i.e. *Ficus carica*, *Agapanthus* sp., *Aloe arborescens*, *Sensevierna dooneri*, *Dracaena derenensis*, *Gasteria excavata*, *Trachycarpus princeps*, *Cymbidium* sp., *Cattleya hybrid*, *Gossypium* sp., *Laelia* sp., *Crotolaria juncea*, *Sporobolus nitens*, *Paris polyphylla*

var. *chinensis*, *Andropogon gerardii*, *Coix gasteenii*, *Begonia hybrid*, *Striga asiatica*, *Schkuhria pinnata*, *Haemanthus hybrid*, *Striga hermonthica*, *Succisa pratensis*, *Pinus* sp., *Solanum* sp. (see table 1).

Moreover, under favorable environmental conditions, FFSC species are able to produce various secondary metabolites during pre-harvest stages and storage, many of which survive food production processes and can be found in food products. They represent a risk for both human and animal health (Escrivá et al., 2015). After the high incidence of esophageal cancer in certain villages of South Africa was associated with the consumption of beer brewed from *F. verticillioides* contaminated corn (Marasas, 2001), also animal diseases in horses and swine were connected to the ingestion of fumonisins (Colvin and Harrison, 1992; Marasas et al., 1988). Liver and kidneys are the organs, where most of the absorbed fumonisin accumulated in the studied animals. Renal tubular adenoma, anorexia, cardiovascular toxicity, pulmonary edema, hepatotoxicity, carcinogenicity, atherosclerosis and cardiac thrombosis are some of the diagnostics when animals consumed feed contaminated with fumonisins (Ahangarkani et al., 2014). Further, many species in this complex are known as producers of mycotoxins with fumonisins being the most important group of compounds (Pitt, 2009). Fumonisin contaminations in a variety of crops are mainly due the presence of FFSC species (Stepien et al., 2011a).

So far 22 species within the FFSC are considered as fumonisin producers: *F. acutatum*, *F. ananatum*, *F. andiyazi*, *F. anthophilum*, *F. begoniae*, *F. brevicatenulatum*, *F. concentricum*, *F. dlaminii*, *F. fujikuroi*, *F. globosum*, *F. konzum*, *F. lactis*, *F. napiforme*, *F. nygamai*, *F. phylophilum*, *F. proliferatum*, *F. pseudocircinatum*, *F. ramigenum*, *F. subglutinans*, *F. temperatum*, *F. thapsinum* and *F. verticillioides* (Table 1). Only limited research has been published about emerging mycotoxins (fusaproliferin, fusaric acid, beauvericin, enniatins and moniliformin) in FFSC species. This is partially due to the fact

that most of these metabolites were only discovered in the last few decades and the diversity of their toxic effects is often difficult to understand. However, antimicrobial, insecticidal, cytotoxic and phytotoxic properties have already been reported in the literature (Jestoi, 2008). According to published research, thirty-two FFSC species have been associated with the production of emerging mycotoxins (Table 1).

Besides crop damage and productivity reduction, it is estimated that one third of the disseminated human fusarioses in the immunocompromised host are caused by FFSC species (Lortholary et al., 2010). Fifteen FFSC species have been associated with infections in humans: *F. acutatum*, *F. andiyazi*, *F. anthophilum*, *F. fujikuroi*, *F. guttiforme*, *F. mundagurra*, *F. musae*, *F. napiforme*, *F. nygamai*, *F. proliferatum*, *F. pseudocircinatum*, *F. ramigenum*, *F. sacchari*, *F. subglutinans*, *F. thapsinum* and *F. verticillioides* (Table 1). Their imminent role in human pathology is particularly due to their ability to disseminate in the bloodstream (Migheli et al., 2010), propagate over long distances in the atmosphere, being present in abundance in hospital environments (Al-Hatmi et al., 2016b) as well as the intrinsic resistance against different types of antifungals in many FFSC species (Al-Hatmi et al., 2014).

Table 1 Economic relevance and health impact of species belonging to Fusarium fujikuroi complex used in this study. Information about the production of fumonisins (FUM), moniliformin (MON), beauvericin (BEA), enniatins (ENN), fusaproliferin (FP) and fusaric acid (FA) is related to all strains (P), some strains (S) and no strain (N) produced the respective secondary metabolite according to the references. *na means no data available in literature.

| ***Fusarium* spp.** | **Agricultural pathogen** | **Production of secondary metabolites** | | | | | | **Human pathogen** |
|---|---|---|---|---|---|---|---|---|
| | | **FUM** | **MON** | **BEA** | **ENN** | **FP** | **FA** | |
| *F. acutatum* | [15]*Chickpea* plants<br>[38]*Cajanus cajan*<br>[38]Homoptera | [13,25]P | [34,45]P | [13,32,34]P | [32,34]S | [32]N | *na | [52]Foot infection<br>[43]Nail infection<br>[8]Deep infections<br>[2]Hospital environment<br>[49]Presence of lectins |
| *F. agapanthi* | [11]*Agapanthus praecox*<br>[17]*Agapanthus africanus* | [11]N | [11]N | na | na | na | na | na |
| *F. ananatum* | [6, 22, 60]*Ananas comosus* | [6,60]S | [60]N | [6,60,50]S | na | na | na | [2]Hospital environment |
| *F. andiyazi* | [61]Rice<br>[28,62] Maize<br>[63]Sugarcane | [34,41,48]S | [41]P | [41]P | na | na | na | [8]Haemato/oncology<br>[2]Hospital environment<br>[54]Fungal keratitis |
| *F. anthophilum* | [42]Cowpea<br>[38]*Hippeastrum hybrid*<br>[38]*Euphorbia pulcherrima*<br>[17]*Dasylirion longissimum* | [34,25]P | [34, 45, 37]P | [32, 34]S | [32, 34, 37 ]S | [32, 34]P | na | [8]Gangrenous infections<br>[8]Haemato/oncology<br>[2]Hospital environment |
| *F. bactridioides* | [4] *Laelia autumnalis*<br>[4] *Laelia speciosa*<br>[38]*Cronartium conigenum* | na | [45]N | na | na | na | na | na |
| *F. begoniae* | [37]Rice<br>[38]*Begonia hybrid* | [13, 25, 34, 37]S | [13,34,39,45]S | [32, 37]S | [32, 37]N | [32, 34]S | na | na |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *F. brevicatenulatum* | [38]*Striga asiatica* | [13,25]P | [45]N | [29]N | [29]N | [29]N | na | na |
| *F. bulbicola* | [39] Soybean<br>[36] *Nerine bowdenii*<br>[36] *Haemanthus hybrid* | [12,23]N | [12]N | [32, 13]S | [32]N | [32, 13]S | na | na |
| *F. circinatum* | [38]*Pinus radiata,*<br>[38] *P. taeda*<br>[38] *P. patula* | [13,25]N | [13, 45]N | [13, 32, 34]S | [32]N | [13, 32, 34]S | na | na |
| *F. coicis* | [26] *Coix gasteenii* | na | na | na | na | na | na | na |
| *F. concentricum* | [50] *Ananas comosus* var *comosus*<br>[10] Maize<br>[19] *Citrus reticulata*<br>[38] *Musa sapientum*<br>[38] *Nilaparvata lugens*<br>[62] *Paris polyphylla var chinensis*<br>[21] *Hibiscus sabdariffa* | [25,50] S | [13, 34, 45, 60,50]S | [14, 29, 32, 58,50]S | [32, 34]S | [13, 32, 34]S | na | na |
| *F. denticulatum* | [38] *Ipomoea batatas* | [13,25]N | [13, 34, 45]P | [13,32, 34]S | [32, 34]S | [13, 32]N | na | na |
| *F. dlaminii* | [38] Soil | [34, 25] P | [34, 45]S | [32, 34]S | na | na | na | na |
| *F. fracticaudum* | [19] *Pinus maximinoi*<br>[19] *P. tecunumanii*<br>[19] *P. maximinoi* | na | na | na | na | na | na | na |
| *F. fractiflexum* | [5]*Cymbidium* spp. | na | na | na | na | na | na | na |
| *F. fujikuroi* | [60,61] Rice<br>[38] *Oryza sativa*<br>[17]*Trachycarpus princeps* | [34, 37, 41, 60,25]P | [34, 37,41, 45, 60]S | [32, 34, 37, 41, 60]P | [32, 37]S | [32]N | [34]P | [2] Human skin<br>[2] Hospital environment |
| *F. globosum* | [38] *Triticum aestivum* | [34,25]P | [45]N | [32, 34]S | [32]N | [32, 34]S | na | [49]Presence of lectins |
| *F. guttiforme* | [38] *Ananas comosus* | [13,25] N | [13, 45]N | [13, 32, 34]S | [32]N | [13, 32, | na | [30]Finger infection |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | [34]P | | |
| *F. konzum* | [20] *Andropogon gerardii* | [25, 27, 34]S | na | [27, 34]P | na | [27, 34]S | na | na |
| *F. lactis* | [38] *Ficus carica*<br>[60] Peppers | [6,13, 25]S | [13, 45, 34]S | [13, 32]S | [32]N | [13, 32]N | na | [49] Presence of lectins |
| *F. mangiferae* | [20] *Mangifera indica* | na | na | na | na | na | na | na |
| *F. marasasianum* | [20] *Pinus patula,*<br>[20] *P. tecunumanii* | na | na | na | na | na | na | na |
| *F. mexicanum* | [20] *Mangifera indica* | na | na | na | na | na | na | na |
| *F. mundagurra* | [26] Soil | na | na | na | na | na | na | [3]Endobronchial fusariosis |
| *F. musae* | [20,23] Banana | [48,55]N | [29]P | na | na | na | na | [56] Skin infection<br>[53] Keratitis<br>[53] Sinusitis |
| *F. napiforme* | [38] *Pennisetum typhoides* | [34,25]P | [34, 45]S | [32]N | [32]N | [32]N | [34]P | [8] Deep infection<br>[8] Haemato/oncology<br>[2] Hospital environment<br>[2] Keratitis |
| *F. nygamai* | [7] Sugar beet<br>[38,28] Maize<br>[38] *Oryza sativa*<br>[38] *Striga hermonthica*<br>[38] *Nicotiana tabacum*<br>[38] *Sorghum bicolor*<br>[16] *Sporobolus nitens*<br>[16] *Schkuhria pinnata* | [34,25]P | [34,45]S | [32,34]S | [32]N | [32]N | [34]P | [8] Onychomycosis<br>[8]Deep localized infections |
| *F. parvisorum* | [20]*P. patula* | na | na | na | na | na | na | na |
| *F. pininemorale* | [20] *Pinus tecunumanii*<br>[20] *P. maximinoi* | na | na | na | na | na | na | na |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *F. phylophilum* | [24]*Aloe* sp.<br>[38] *Sansvierna dooneri*<br>[38] *Dracaena deremensis*<br>[38] *Gasteria excavata* | [13, 34,25] P | [13, 34, 45]S | [13,32, 34]S | [32]N | [13, 32]N | na | [2] Hospital environment |
| *F. proliferatum* | [39] *Gycine max L.*<br>[7] Sugar beet<br>[61] Rice<br>[38] *Triticum aestivum*<br>[36] Sesame seeds<br>[58] *Asparagus* sp.<br>[58] Garlic<br>[58] Pineapple<br>[58] Maize<br>[58] Wheat<br>[4] *Laelia rubescens*<br>[4] *L. autumnalis*<br>[4] *L. speciosa*<br>[38] *Cymbidium* sp.<br>[38] *Cattleya hybrid*<br>[38] *Morus alba*<br>[17]*Trachycarpus princeps*<br>[38] *Gossypium* sp.<br>[17]*Pogonarthria squarrosa*<br>[41] Soil | [6,25,34,37,41,60]P | [34,37, 41,45, 60]S | [6, 32, 34, 37, 41,60]S | [32,34,37,60]S | [32, 34]S | [34]P | [43]Wound and tissue from patients in the burn unit<br>[8] Onychmycosis<br>[8] Keratitis<br>[8] Endophthalmitis<br>[8]Deep localized infection Haemato/oncology<br>[2] Hospital environment<br>[49] Presence of lectins |
| *F. pseudoanthophilum* | [38] *Zea mays* | [13,25] N | [13, 45]S | [13,32] S | [32] N | [13, 32] N | na | [49] Presence of lectins |
| *F. pseudocircinatum* | [37] Rice<br>[38] Homoptera | [13, 25, 37]P | [13,37,45] S | [13, 32, 37]S | [32, 37] S | [13, 32]S | na | [54] Keratitis |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | [38] *Solanum* sp.<br>[38] *Pinus kesiya*<br>[38]Textile<br>[38] Dead leaves | | | | | | | |
| *F. pseudonygamai* | [38] *Pennisetum typhoides* | [13,25]N | [13, 45]P | [13, 32]N | [32] N | [13, 32]S | na | na |
| *F. ramigenum* | [38] *Ficus carica* | [13,25, 33]S | [13, 45]P | [13, 32]N | [32] N | [13, 32] N | na | [9] Keratitis |
| *F. sacchari* | [63] Sugarcane<br>[1] Banana fruit<br>[38]*Saccharum officinarum*<br>[32] *Musa sapientum*<br>[59] Wheat | [25]N | [34,45] S | [32, 34]S | [32] N | [32] N | [34] P | [2] Corneal ulcers<br>[8, 54]Onychomycosis<br>[8] Skin infection<br>[8,54] Keratitis<br>[8] Endophthalmitis<br>[8] Deep infections |
| *F. secorum* | [46] *Beta vulgaris L.* | [46]N | na | [46] P | na | na | na | na |
| *F. sororula* | [20] *Pinus patula*<br>[20] *P. tecumanii* | na | na | na | na | na | na | na |
| *F. sterilihyphosum* | [37] Rice<br>[20] *Mangifera indica* | [37,18] N | [37] N | [37] P | [37] S | na | na | na |
| *F. subglutinans* | [38] *Zea mays* | [14, 25, 27, 34]S | [34, 45]S | [14, 27, 32, 34]S | [32]N | [27,32, 34]P | [34]P | [8] Keratitis<br>[8] Endophthalmitis<br>[8] Deep infections<br>[2] Hospital environment |
| *F. succisae* | [38] *Succisa pratensis* | [25]N | [45]N | [32]S | [32]N | [32]P | na | na |
| *F. temperatum* | [50]*Ananas comosus* var *comosus*<br>[44]*Zea mays* | [50, 44] S | [50] S | [50,44] P | [44] S | na | na | na |
| *F. thapsinum* | [28,38] Maize<br>[38] *Sorghum* sp. | [27, 34, 25] P | [34, 45]S | [27, 32]N | [32]N | [27, 32]N | [34]P | [8, 54] Keratitis<br>[8] Endophthalmitis<br>[8] Haemato/oncology |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | | | [2] Hospital environment |
| *F. tjaetaba* | [26] *Sorghum interjectum* | na | na | na | na | na | na | na |
| *F. tupiense* | [29, 47] Mango | na | na | na | na | na | na | na |
| *F. udum* | [38] *Cajanus cajan*<br>[38] *Crotalaria juncea* | na | na | [32] N | [32] N | [32]S | na | na |
| *F. verticillioides* | [7] Sugar beet<br>[37,61] Rice<br>[38, 60,28] Maize<br>[60] Pineapple<br>[38] *Zea mays*<br>[38] *Oryza sativa*<br>[38] *Ficus carica*<br>[38] *Striga asiatica*<br>[33]Banana | [25, 34, 37, 41,60] P | [34, 37, 41, 45, 60]S | [27, 32, 34, 37, 41,60]S | [32, 37]N | [27, 32]N | [34]P | [2, 8] Onychomycosis<br>[8] Skin infection<br>[8, 54] Keratitis<br>[8] Endophthalmitis<br>[2, 8] Deep infections<br>[8] Haemato/oncology<br>[2] Hospital environment<br>[2] Leg ulcers<br>[2] Abdominal drainage<br>[2] Urine samples |
| *F. werrikimbe* | [58] *Sorghum leiocladum* | na | na | na | na | na | na | na |
| *F. xylarioides* | [35] *Coffea canephora* | na | na | na | na | na | na | na |

According to: [1]Abd Murad et al. (2017); [2]Al-Hatmi et al. (2016b); [3]Al Yazidi et al. (2019); [4]Almanza-Álvarez et al. (2017); [5]Aoki et al. (2001); [6]Barral et al. (2020); [7]Cao et al. (2018); [8]Diepeningen et al. (2014); [9]Dos Santos et al. (2019); [10]Du et al. (2020); [11]Edwards et al. (2016); [12]Elvira-Recuenco et al. (2020); [13]Fotso et al. (2002); [14]Fumero et al. (2020); [15]Gopalakrishnan et al. (2005); [16]Gryzenhout et al. (2020); [17]Guarnaccia et al. (2019); [18]Haggag et al. (2011); [19]Hasan et al. (2020); [20]Herron et al. (2015); [21]Huda-Shakirah et al. (2020); [22]Jacobs et al. (2010); [23]Kamel et al. (2016); [24]Kishi et al. (1999); [25]Kvas et al. (2009); [26]Laurence et al. (2016); [27]Leslie et al. (2004); [28]Leyva-Madrigal et al., 2015; [29]Lima et al. (2012); [30]Migheli et al. (2010); [31]Molnàr et al. (2015); [32]Moretti et al. (2007); [33]Moretti et al. (2010); [34]Munkvold et al. (2017); [35]Musoli et al. (2008); [36]Nayyar et al. (2018); [37]Nicolli et al. (2020); [38]Nirenberg and O'Donnell (1998); [39]Okello et al. (2020); [40]Oufensou et al. (2019); [41]Qiu et al. (2020); [42]Rodrigues and Menezes (2005); [43]Salah et al. (2015); [44]Scauflaire et al. (2012); [45]Schütt et al. (1998); [46]Secor et al. (2014); [47]Senghor et al. (2012); [48]Shi et al. (2017); [49]Singh and Thakur (2019); [50]Stepien et al. (2013); [51]Summerell et al. (1995); [52]Taj-Aldeen et al. (2006); [53]Triest et al. (2015a); [54]Tupaki-Sreepurna et al. (2018); [55]Van Hove et al. (2011); [56]Verbeke et al. (2020); [57]Vettraino et al. (2018); [58]Walsh et al. (2010); [59]Wang et al. (2015); [60]Waskiewicz and Stepien (2012); [61]Wulff et al. (2010); [62]Xiao et al. (2019); [63]Yao et al. (2020); [64]Zhang et al. (2014)

*na = no information available in literature.

Nowadays, the number of species belonging to the FFSC has been increasing based on molecular phylogenetic and morphologic traits that differentiate new taxa from related known species in the complex. In 2015, Al-Hatmi et al. (2016a) described for the first time *F. ficicrescens* collected from contaminated fig fruits in Iran. Morphologically, *F. ficicrescens* is similar to *F. andiyazi* and phylogenetic analysis showed a close relation with *F. lactis, F. ramigenum*, and *F. napiforme*. Also, the ecosystem surveillance for host-fungus associations in Australia has resulted in the discovery of quite a few novel FFSC taxa including *F. babinda, F. coicis, F. mundagurra, F. newnesense, F. nygamai, F. tjaetaba, F. werrikimbe*, *F. agapanthi* (Laurence et al., 2016; Edwards et al., 2016) and, more recently, *F. terricola* and *F. sudanense* from soil and plant debris in Australia and Sudan, respectively (Moussa et al., 2017). *F. fredkrugeri* was isolated from rhizosphere soils of a native African shrub, *M. acuminata*, in a protected savannah ecosystem deep inside the Kruger National Park (Sandoval-Denis et al., 2018). As a potential clinical pathogen, *F. volatile* was characterized by Al-Hatmi et al. (2019) from a patient with pulmonary infections through a respiratory sample (bronchoalveolar lavage, BAL). The species was demonstrated to be highly resistant to severals antifungals.

Recently, two new species were recovered from Indonesian banana plants, *F. lumajangense* and *F. desaboruense* (Maryani et al., 2019). In contrast to *F. oxysporum* f.sp. *cubense* tropical race 4 as the most devastating pathogen of banana plants of the Cavendish variety, no pathogenicity of the new FFSC species was observed against this banana variety. After a mycological investigation in Nigeria, *F. mandaense* was newly described from groundnut and sorghum (Ezekiel et al., 2020). So far, the last newly described FFSC species are *F. caapi* originating from seeds of *Brachiaria brizantha*, *Oryza sativa* and indoor air in a clinical environment and *F. brachiariae* from *Brachiaria decumbens*. Both new species were isolated from different brazilian regions (Costa et al., 2021).

It has clearly been shown that species within the FFSC are closely associated to plant hosts and with this to a geographical region. Even in cases in which the plant host is of economic importance and therefore distributed over wider areas such as *Sorghum* or even worldwide such as rice, maize or wheat, the associated FFSC species can still be related with their region of origin as was shown by O´Donnell et al. (1998). This author presented a phylogeographic analysis based on gene trees inferred from the ß-tubulin gene exons and introns, mitochondrial small subunit (mtSSU) rDNA, and 5' portion of the nuclear 28S rDNA were in a combined analysis statistically supported for the species division in South America, Africa, and Asia, being the Africa clade the most phylogenetically diverse area.

In order to avoid economic loss in agriculture as well as the entry of mycotoxins into the food chain and the dissemination of human infections, it is pertinent to detect the presence of contaminations with FFSC species as well as to identify them to species level in a timely manner. Efficient systems for monitoring of commodities, from harvest to final product and in patients, need to be implemented into services and concepts that help to improve food quality and human health.

## 1.2 Toxicity of fumonisins produced by FFSC species

Fumonisins comprise a group of structurally similar polyketide mycotoxins. They were found to be produced by strains of several of the species within the FFSC (Kvas et al., 2009; Sandoval-Denis et al., 2018). The most frequently isolated fumonisin producers are *F. fujikuroi*, *F. globosum*, *F. proliferatum*, *F. nygamai*, *F. subglutinans*, *F. temperatum* and *F. verticillioides* (Proctor et al., 2004). Outside this group of closely related species, fumonisins were found to be produced in some strains of *F. oxysporum* and, surprisingly, in *Aspergillus*

*niger* (Frisvad et al., 2007) and in strains in biotechnologically important species within the fungal genus *Tolypocladium* (Mogensen et al., 2011). Studies showed that whilst the most common fumonisin produced by *Fusarium* spp. was fumonisin $B_1$, *A. niger* strains produced fumonisins $B_2$, $B_4$, and $B_6$ (Mansson et al., 2009; Mogensen et al., 2009). *T. cylindrocarpon* produced fumonisins $B_1$ and $B_2$ (Zabalgogeazcoa et al., 2018). Fumonisins are usually divided into 4 groups as A, B, C and G, with the B-group fumonisins being the most toxic (Musser and Plattner, 1997). It was shown to be neurotoxic, hepatotoxic, and nephrotoxic in animals (Stockmann-Juvala and Savolainen, 2008) and it was classified as a possible carcinogen in humans, since there is sufficient evidence of experimental animals for the carcinogenicity of fumonisin $B_1$, but not in humans (Ostry et al., 2017).

Major mechanisms of toxicity involve the strong structural similarity of the alcohol part of polyhydric acid with the amino alcohol complex in sphinganine as the backbone precursor of sphingolipids (Marin et al., 2013). Among the B series of fumonisins, $FB_1$ has been categorized as the most toxic compound in animal studies. Mechanistic studies with animal cell cultures suggest that $FB_1$ strongly inhibits the enzyme ceramide synthase, which results in an imbalance of intracellular acylation of sphinganine and sphingosine (Marin et al., 2013). An overview of the sphingolipid biosynthesis in animal cells and the inhibition of the enzymatic pathway by fumonisin $B_1$ is highlighted in Figure 1 published by Soriano et al. (2005).

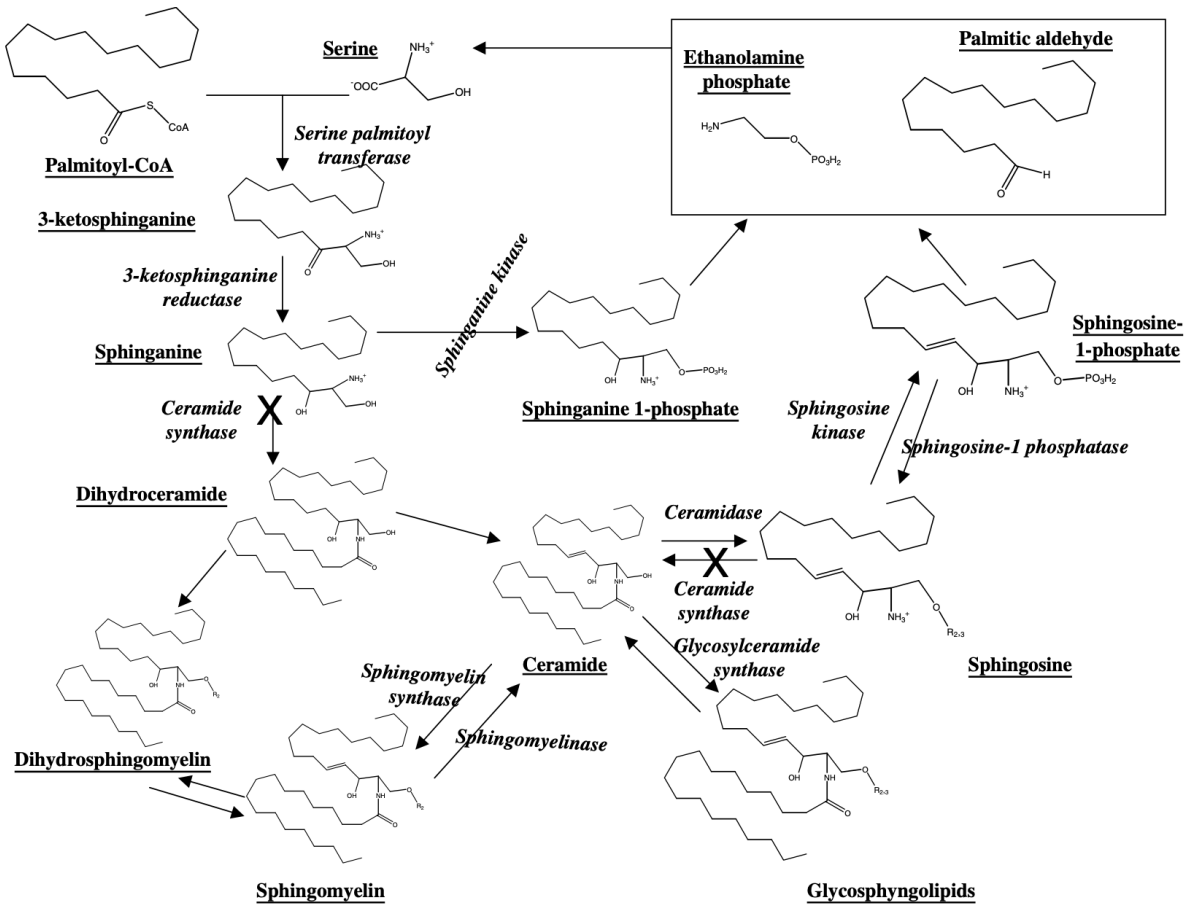


Figure 1 Pathway of the *de novo* sphingolipid biosynthesis in an animal cell and the inhibition by fumonisins $B_1$ (Soriano et al., 2005).

Among the fumonisins, $FB_1$ is the most frequently found compound in naturally contaminated foods and feeds. The toxins have been classified as a category 2B carcinogen by the International Agency for Research on Cancer (IARC, 2002). Moreover, nerve system disorders in horses, pulmonary edema in swine as well as neural tube and esophageal cancer in humans have been associated with ingestion of fumonisins (Harrison et al., 1990; Marasas et al., 1988; Rheeder et al., 1992; Ueno et al., 1997; Wilson et al., 1990). Although the function of some of the genes associated with fumonisin biosynthesis is barely known, others have been characterized experimentally (Alexander et al., 2009). Among them, the *fum1* gene codes for a polyketide synthase (PKS) that is involved at an early step in the assembly of the

fumonisin backbone (Figure 2). In addition, the *fum1* gene is one of the 10 FUM genes that *F. verticillioides* and *Aspergillus niger*, the most important fungus used in biotechnology, have in common (Khaldi and Wolfe, 2011).

The fumonisin structure consists of a linear, 20-carbon backbone with an amine function at carbon atom 2 (C-2), methyl functions at C-12 and C-16, and tricarballylic esters at C-14 and C-15. The fumonisins differ in structure by the presence or absence of hydroxyl functions at C-4, C-5, and C-10 (Alexander et al., 2009). Genes involved in the biosynthesis of fumonisins are organized into a gene cluster. The pathway of fumonisin biosynthesis starts with the formation of a linear dimethylated polyketide and condensation of the polyketide with alanine, followed by a carbonyl reduction, oxygenations, and esterification with two propane-1,2,3- tricarboxylic acids (Desjardins and Proctor, 2007). Currently, it is known that 17 genes are responsible for fumonisin B biosynthesis designated as *fum1*, *fum2*, *fum3*, *fum6*, *fum7*, *fum8*, *fum10*, *fum11*, *fum13*, *fum14*, *fum15*, *fum16*, *fum17*, *fum18*, *fum19*, *fum20* and *fum21* (Deepa and Screenivasa, 2019). The fumonisin biosynthetic cluster is a useful tool but some marker genes seem to be very selective because of the variability of toxin levels in isolates with the same marker profile (Stepien et al., 2011a). The production of fumonisin strongly dependends on *fum1*, which encodes an enzyme complex known as PKS that synthesizes the toxin backbone which is subsequently modified by other enzymes (Alexander et al., 2009) to result in the final $FB_1$ product. Considering the major enzymatic activities predicted to be essential for formation of the predominant fumonisins produced by *F. verticillioides*, the FUM gene functions are illustrated in Figure 2 according to Alexander et al. (2009). The function of *fum15* - *fum21* resulted in indirect roles in fumonisin biosynthesis.

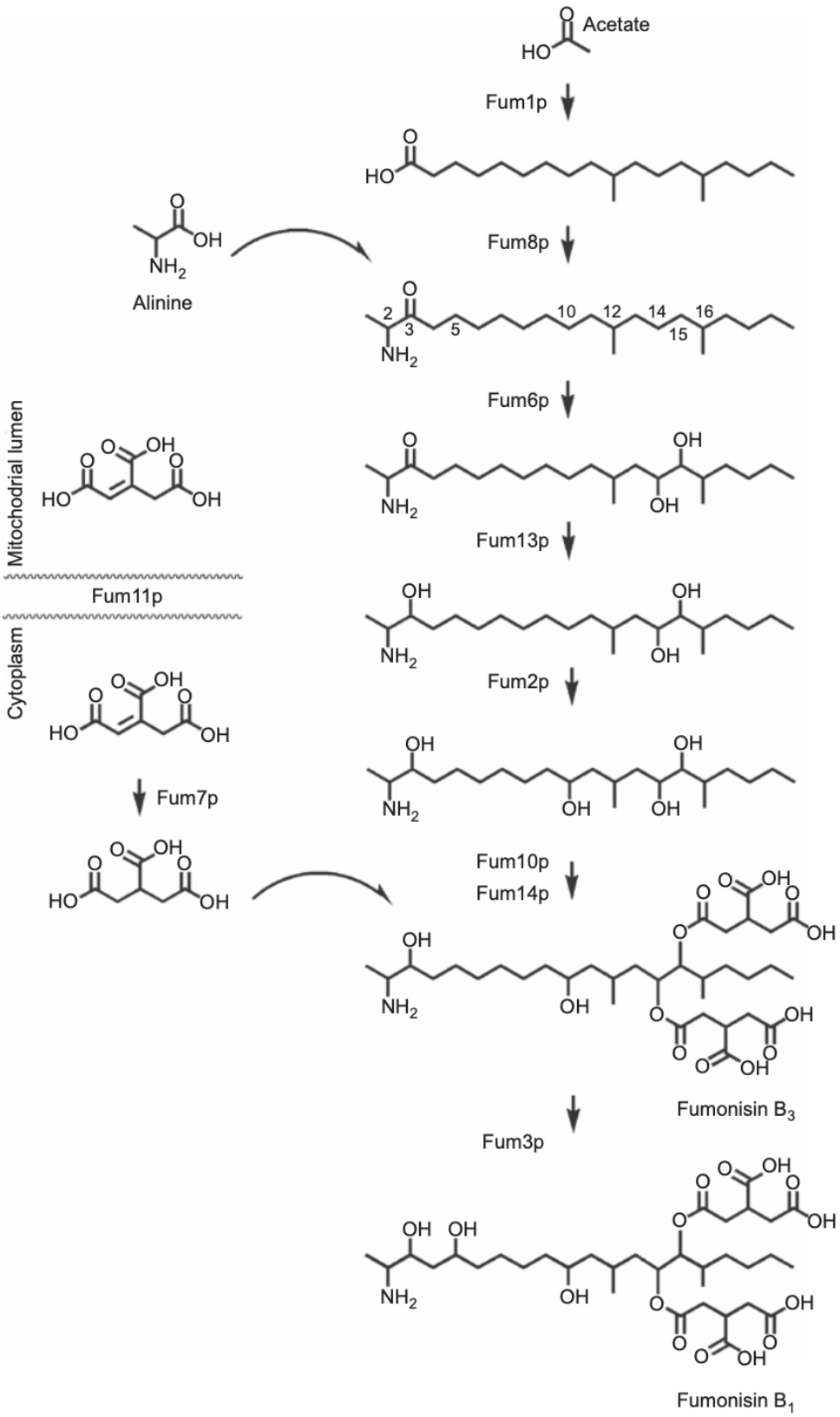


Figure 2 Biosynthetic pathway for fumoinisin $B_1$ showing functions of FUM gene/proteins proposed by Alexander et al. (2009).

Maize and maize-based products have been identified as the major source of fumonisin contamination in food and feed (Ahangarkani et al., 2014). Maize is the main ingredient for manufacturing several products like tortillas, corn flakes, popcorn, grits, flour and oils. Besides the contamination by fumonisins decrease during the food processing, they are stable in nature and do not eliminate (Kamle et al., 2019). In order to minimize the risk of fumonisin consumption from contaminated maize, the European Commission has established legal limits for fumonsins in unprocessed maize and maize-based products ranging from 200 µg/kg to 2000 µg/kg for the sum of $FB_1$ and $FB_2$ (European Commission, 2006). Many countries, which take reference to the EU legislation, such as Turkey, Bosnia and Herzegovina, Norway and Switzerland, have fixed similar maximum limits for fumonisin in food. However, no specific legal limits for fumonisins have been established yet in major markets including China, Japan, India, Gulf Cooperation Council (GCC), Russia, Canada and many Latin American countries. As a result of the frequent contamination of grapes with *A. niger*, fumonisins $B_2$ and $B_4$ were found to contaminate raisins (Knudsen et al., 2010) and grapes as well as juices and wines made thereof (Logrieco et al., 2010; Mogensen et al., 2010). Fumonisins were also detected as contaminants of barley (Gil-Serna et al., 2013; Piacentini et al., 2015) or wheat (Cendoya et al., 2014; Stankovic et al., 2012). Furthermore, they can be detected on a regular basis in barley and wheat derived beers (Bertuzzi et al., 2011; Soriano and Dragacci, 2004), to which they are introduced by maize-based brewing adjuncts. Moreover, fumonisins can be regularly found in traditional African beers that are brewed from sorghum and maize as the single ingredients (Nkwe et al., 2005; Shephard, 2008).

Many studies involving fumonisin biosynthesis and the nutrient supply have demonstrated the complexity of this subject. However, the prediction involving food safety issues is only possible if more information is collected describing the influence of different

environmental conditions on the level of fumonisin biosynthesis. Most of the existing studies are focused on water activity, temperature, growth, preservatives and biotic factors and their effect on *F. verticillioides* and *F. proliferatum* as model organisms (Marín et al., 2004). However, there is limited information in the literature about the influence that selected carbon sources have on fumonisin production (Jiménez et al., 2003; Achimón et al., 2019). Fungal development depends on carbon sources, especially carbohydrates. The nutrition sources of a grain can determine not only which fungi will be able to grow but also their lifespan and production of secondary metabolites on a given substrate (Marín et al., 2004). In order to understand the influence of different substrate components on the fumonisin biosynthesis, understanding the role of each component is essential for agricultural research and breeding to select hybrids that may suppress high fumonisin levels due to optimized substrate composition.

### 1.3 Identification methods for FFSC species

The taxonomy of *Fusarium* spp. described first by Link in 1809 and officially approved in 1821 by Fries was based solely on morphological species recognition in terms of the International Botanical Code. For many years, microscopic analyses involving detailed measurements of conidia and the visual inspection of agar cultures in Petri dishes was the unique technique to differentiate and discover new *Fusarium* species. Unfortunately, the method required highly experienced professionals who were able to identify an isolate correctly and who were able to establish whether a given culture belonged to a new species. Lack of expertise resulted in regularly occurring misidentifications.

Some changes were historically important to establish the taxonomy of species belonging the *Fusarium fujikuroi* species complex (FFSC) as it is used nowadays. The first was the discontinuation of the use of a dual naming system for fungi, in which the anamorphic and teleomorphic forms of a fungus had different names. This allowed to define mating populations A-L of the teleomorphic fungus *Gibberella fujikuroi* as separate species because only *F. fujikuroi* is conspecific with this teleomorph (Aoki et al., 2014). The other important change was a result of the combined use of morphological and molecular phylogenetic analyses that allowed the discrimination of over 50 phylogenetically distinct species (O'Donnell et al., 1998a) within the FFSC. In addition, the nomenclature of one FFSC was changed, *F. moniliforme* was updated to *F. verticillioides*, since many unreliable recognitions are linked to the conservative name (Seifert et al., 2003).

Differentiation of taxa within the FFSC is a major challenge considering their morphological similarity and overlapping morphological traits that underestimate the true diversity of the complex. For most of the known species in the FFSC, no sufficient discriminatory morphological markers are available. Multilocus sequencing technologies (MLST) are nowadays applied to clearly differentiate the 60 species described so far (Sandoval-Denis et al., 2018). On the other hand, the method has considerable disadvantages such as time consumption, high costs, and the necessity of in-depth knowledge of molecular biological data analysis as well as fungal systematics and taxonomy.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of subproteomic mass spectra has been shown to be a promising tool for species identification and differentiation in fungi (Chalupová et al., 2014). Besides medically important fungi, reliable species identification by MALDI-TOF MS has been reported for food spoilage fungi (Quéro et al., 2018), foodborne yeasts (Quintilla et al., 2018), environmental fungi (Sanitá-Lima et al., 2019), *Stachybotrys* spp. (Ulrich et al., 2016),

and indoor wood decay fungi (Schmidt and Kallow, 2005). However, the reference spectra available as an on-pack coming with the MALDI-TOF MS equipment comprise only medically relevant fungal species, including few from the FFSC.

MALDI-TOF mass spectrometry is a combination of two technologies: MALDI source and the time of flight (TOF) mass together with a mass spectrometry that identifies and quantifies molecules by analyzing the mass-to-charge ratios (m/z) of any ionized susceptible biological molecule (Hou et al., 2019). Figure 3 illustrates the workflow of MALDI-TOF mass spectrometry. Briefly, a short summary is given about the functionality of MALDI-TOF mass spectrometry published by Chalupová et al. (2014), Singhal et al. (2015) and Lima and Santos (2017). Before the measurement starts, it is important to mix or coat the sample with an appropriate matrix solution, which is an organic compound and energy-absorbent. The solvents of the matrix penetrate the cell wall of microorganisms and extract the intracellular proteins. Both sample and matrix will dry on the steel MALDI target and co-crystallize. Since the sample is inside the MALDI equipment, the desorption and ionization are started in an automated mode with a laser beam. Protonated ions are generated from analytes in the sample and accelerated at a fixed potential, through which these separate from each other on the basis of their mass-to-charge ratio (m/z). The detection of the charged analytes occurs and is measured by using time of flight (TOF) analyzers, which consists of determining the time required for the ion (m/z ratio) to travel the length of the flight tube. Finally, the TOF information is transferred into a characteristic peptide mass fingerprint (PMF) or spectrum. The identification occurs by comparing the PMF of an unknown fungus with the PMFs of reference fungal isolates contained in a database. The typically used mass range m/z is 2-20 kDa, which represents mainly ribosomal proteins along with a few housekeeping proteins.

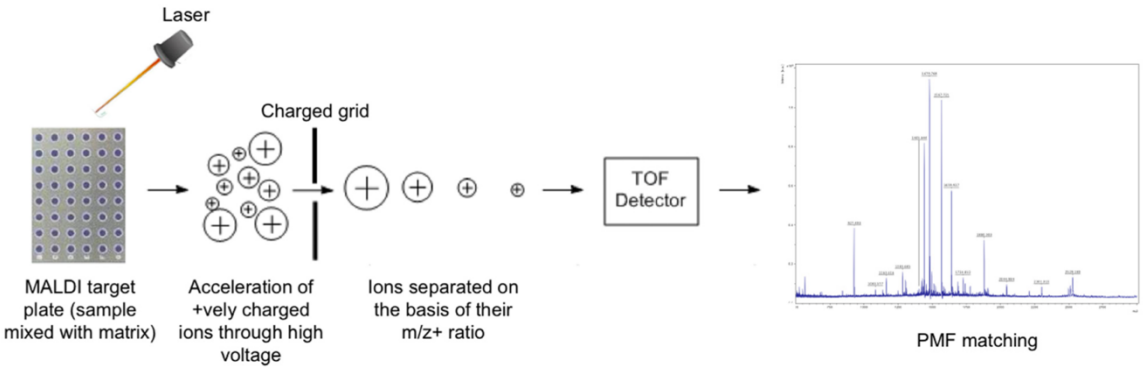


Figure 3 Schematic diagram showing the workflow in MALDI-TOF mass spectrometry (Singhal et al., 2015).

### 1.4 Detection of fumonisin producing fungi

Detection and identification of typical fumonisin producing *Fusarium* species can be performed using traditional microbiological techniques such as dilution plating of samples to SNA medium and microscopical analysis of fungal structures involving morphological characteristics for species determination. As an alternative, molecular biological identification of mycelia can be performed using the nucleotide sequence of the EF1α gene (Geiser et al., 2004) or by PCR using species- or group specific primers (Baird et al., 2008; Niessen, 2007; Ramana et al., 2012). Although PCR is a rather rapid diagnostic technology, it needs sophisticated laboratory equipment to be performed. Loop-mediated isothermal amplification (LAMP) has been established as an alternative method for enzyme-based in vitro DNA amplification (Notomi et al., 2000). With this technology, reactions run under isothermal conditions at 65 °C and can therefore be performed with very basic lab equipment, e.g., a heating block or water bath. Moreover, LAMP is less prone to DNA-impurities than PCR, which permits less elaborate protocols for sample preparation. Finally, its short reaction

time and the use of indicators that provide a visible color change in positive reactions make LAMP an attractive tool for point of analysis applications. LAMP-based assays for the species-specific detection of *F. temperatum* (Shan et al., 2019), *F. proliferatum* (Wang et al., 2020) and the presence of *fum1* gene in many FFSC species (Wigmann et al., 2020) were very recently published.

The mechanism of the LAMP reaction was discussed in detail by Notomi et al. (2000) and is depicted in Figure 4. In total, 4 primers are necessary to specifically recognize 6 binding sites within the target DNA sequence: 2 inner primers (forward inner primer, FIP and backward inner primer, BIP) and 2 outer primers (B3 and F3c, F3 complementary to F3c). Both inner primers contain two distinct sequences corresponding to the sense and antisense sequences of the target DNA. In the first step, one sequence is for priming and in later steps the other sequence is for self-priming. Two steps lead the LAMP reaction by DNA polymerase with strand displacement activity: the initial steps are performed with all 4 primers starting structure producing step and cycling amplification step. The structure producing step starts initiating the replicative DNA synthesis by Bst DNA polymerase. The outer primer F3 displace the newly synthesized DNA strand and release the target DNA. The similar performance by BIP (or FIP) and B3 (or F3) result in a single-stranded dumbbell-like starting structure with loops at both ends. Next, the starting structure is converted to stem-loop DNA by self-primed DNA synthesis, and then FIP (or BIP) hybridizes and primes strand displacement DNA synthesis. Since the last two steps are established, the elongated products are also produced by the elongation and recycling step reactions (Tomita et al., 2008).

The detection of successful DNA amplification in a LAMP assay is one of the biggest advantages of the method. Two different phenomena can be used for indirect signal generation, both of which commonly occur when DNA is enzymatically generated *in vitro*. One phenomenon is the generation of pyrophosphate as a by-product of DNA synthesis.

Pyrophosphate forms a complex with bivalent cations such as magnesium, calcium or manganese. Precipitation of a pyrophosphate-magnesium complex results in the formation of turbidity in positive LAMP reactions as an indirect measure for DNA synthesis (Mori et al., 2001). Visual indicators such as calcein or hydroxy naphthol blue (HNB) change their fluorescence (calcein) or color (HNB) upon formation of a complex with bivalent cations. Pyrophosphate as a strong complexing agent can outcomplex such cations from the dyes and induce a color change in positive LAMP reactions which can be observed under UV (calcein) or day light (HNB) conditions (Tomita et al., 2008). The process of the incorporation of DNA polymerase and deoxynucleoside triphosphate into the nascent DNA results in by-products as pyrophosphate and also hydrogen ions. The release of this proton during the reaction allows for a second mode of signal detection that is based on the drop of pH during positive LAMP reactions. This pH drop can be monitored by the addition of pH- sensitive indicator dyes to a weakly buffered master mix that allows the pH to drop when protons are released during DNA synthesis (Tanner et al., 2015). The use of such colorimetric indicators enables easy visual detection of DNA amplification by color change without any further technical requirements.

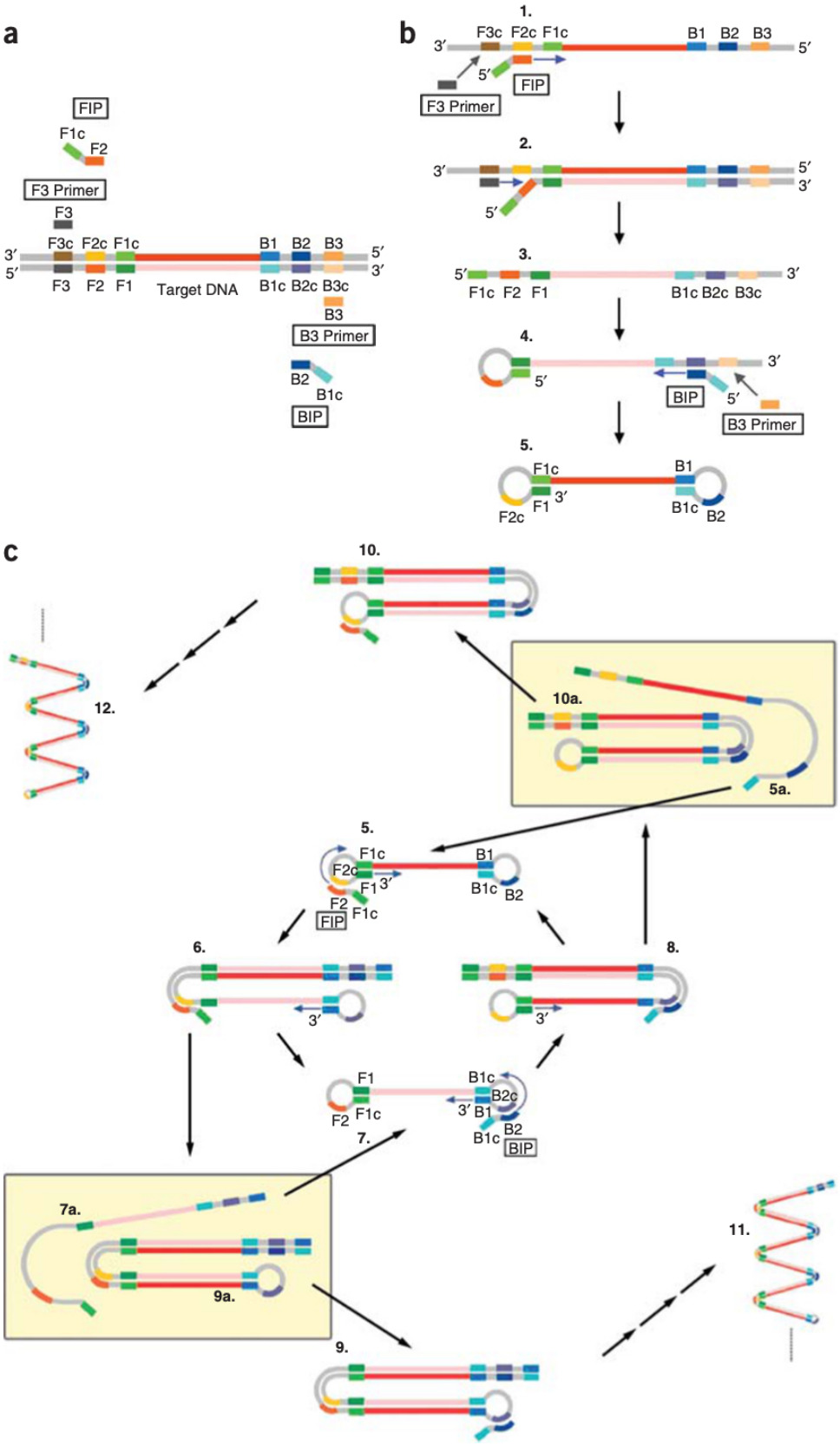


Figure 4 Principle of loop-mediated isothermal amplification (LAMP) method (Tomita et al., 2008).

# 2 Hypotheses

Traditional methods for species differentiation and the detection of relevant genes involved on the production of fumonisins of FFSC species demand high professional experience, high investments in technical equipment, and are time consuming. Nowadays, MALDI-TOF MS analysis and the LAMP assay technologies offer easy-to-use, rapid and accurate alternatives to distinguish FFSC species and detect genes as well as gene expression, respectively. The development of methods using these technologies can help to add new information about the ever-growing number of species in the *Fusarium fujikuroi* species complex.

The aim of this study was the development of methods for the taxonomic differentiation of the species within the *Fusarium fujikuroi* species complex. This should be the basis to provide a new option to avoid diseases on plants and humans caused by FFSC species as it enables targeted detection of typical fumonisin-producing *Fusarium* spp.. The characterization of the expression of *fum1*, a gene of imminent importance in the biosynthesis of fumonisins improves measures that can help to prevent the entry of fumonisins into the food chain. The work accomplished in this Ph.D. thesis should therefore address three sections:

(I) **MALDI-TOF mass spectrometry as a high-throughput technology for species level differentiation of the FFSC**

The reference spectra available as an on-pack coming with the MALDI-TOF MS equipment comprise only medically relevant fungal species, including few from the FFSC. In order to enable the identification of a special set of species, an in-house spectra database should be constructed and validated that should be combined with the firmware database to

enable species - level identifications in the FFSC. Therefore, the purpose of the first part of the study was to set up a supplementary database containing reference mass spectra of a maximum number of the currently described FFSC species in order to provide an alternative tool for the identification of fungal pure cultures. Moreover, the database should be validated with FFSC isolates that are external to the database.

Working hypotheses:

- MALDI-TOF MS produces spectra with sufficient quality to differentiate FFSC species
- A supplementary database can be established, which enables MALDI-TOF MS for identification of FFSC species with high accuracy and discriminatory power
- MALDI-TOF MS can be demonstrated and established as a reliable tool for the identification of FFSC strains that are external to the supplementary database
- Modifications to the standard MALDI-TOF MS sample preparation protocol enable differentiation of otherwise indiscernible fungal species.

(II) **Rapid and sensitive group-specific detection of fumonisin-producing *Fusarium* spp.**

Fumonisin production in genus *Fusarium* occurs exclusively in the FFSC and many of the species can produce it. The aim of this part of the thesis was to design a set of LAMP primers, including loop primers, based on the *fum1* gene sequence present in typical fumonisin producing *Fusarium* spp. and to establish a LAMP-based assay for the detection of this group of species in pure cultures as well as in contaminated sample materials.

Working hypotheses:

- Primers can be designed for a group-specific LAMP assay, which enable reliable detection of the presence of the *fum1* gene in thoses FFSC species, which typically produce fumonisins
- Results of the LAMP assay can be correlated with the fumonisin production potential of strains

- The LAMP assay enables detection of the presence of the *fum1* gene in naturally contaminated samples as an indication for the presence of fumonisin producers.

(III) **Insight into fumonisin expression and regulation**

This part of the thesis was focused on the development of a rapid, sensitive and specific RT-LAMP assay to study of *fum1* gene expression upon growth of *F. verticillioides* strains with different host origin, and to analyze the influence of different carbohydrates as nutrient factors on *fum1* gene expression and production of fumonisin $B_1$.

Working hypotheses:

- RT-LAMP primers can be designed to detect exclusively cDNA of the *fum1* gene
- Primers shall not amplify genomic DNA that contains the *fum1* gene
- Carbon sources like fructose, glucose, maltose and sucrose differ in their impact on *fum1* gene expression, biomass and fumonisin production in *F. verticillioides* strains with different host origin.

# 3 Material and Methods

## 3.1 Fungal strains

The strains were collected from public culture collections as well as directly from authors of the publications describing new FFSC species. A list of the analyzed fungal isolates is shown in Table 3, 7 and 8. Glycerol stocks were prepared from all strains as described by Niessen and Vogel (1997). All strains were maintained at – 80 °C in the culture collection of the Chair of Technical Microbiology at Technical University of Munich (TMW, Freising, Germany). Fungal cultures that are not belonging to FFSC species were grown on MEA (3.2.2).

## 3.2 Media

Media prepared for experiments presented in this thesis were sterilized at 121 °C for 15 minutes.

### 3.2.1 Synthetic nutrient agar (SNA)

FFSC strains were maintained as working cultures on SNA ($KH_2PO_4$ 1 g; $KNO_3$ 1 g; KCL 0.5 g; $ZnSO_4 \cdot 7H_2O$ 0.01 g; $CuSO_4 \cdot 5H_2O$ 0.05 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; glucose 0.4 g; sucrose 0.4 g; agar 20 g; distilled water 1000 ml) (Nirenberg, 1981) and stored at 4 °C.

### 3.2.2 Malt extract agar (MEA)

Fungal cultures were grown on 2% malt extract agar (malt extract 20 g; soy peptone 2 g; agar 15 g (Difco, Heidelberg, Germany); distilled water 1000 ml, adjust to pH 5.4).

### 3.2.3 Sabouraud dextrose medium (SDB and SDA)

Young fungal material was obtained using Sabouraud dextrose broth (SDB, tryptone/peptone from casein 5 g; peptone from meat 5 g; glucose 20 g; distilled water 1000 ml) in sterile flasks and were rotated during two days at ambient temperature (AT, 22 ± 1 °C).

Mature fungal material was prepared using Sabouraud dextrose agar (SDA), which contain the same constituints mentioned for SDB but with additional 20 g/L of agar. Petri plates were inoculated with 1 agar disc (8 mm) from 7d old SNA cultures (3.2.1) and incubated for 10 days at AT.

### 3.2.4 Fumonisin-inducing liquid medium (FUMB)

The strains were cultured in 500 mL Erlenmeyer flasks containing 110 mL of the following fumonisin-inducing liquid medium (López-Errasquín et al., 2007): malt extract 0.5 g; yeast extract 1 g; peptone from soya 1 g; $KH_2PO_4$ 1 g; $MgSO_4.7H_2O$ 0.3 g; KCL 0.3 g; $ZnSO_4 \cdot 7H_2O$ 0.05 g; $CuSO_4 \cdot 5H_2O$ 0.01 g; fructose 20 g; distilled water 1000 ml. Cultures were inoculated with 3 agar disks from 5 days-old SNA cultures (section 3.2.1) and incubated

at 25 °C under static conditions for 3, 5 and 7 days in the dark. From each culture, 10 mL of the medium were retained for HPLC analyses and 100 mL were used for RNA isolation in duplicates.

### 3.2.5 Czapek's broth medium

In order to observe the influence of different carbon sources as nutrient factors using RT-LAMP analyses, cultures were incubated with 3 agar disks from 5-days-old SNA cultures (section 3.2.1) at 25 °C on a rotary shaker at 200 rpm in 500 mL Erlenmeyer flasks containing 110 mL of Czapek's broth medium as described by Jian et al. (2019): $NaNO_3$ 3 g; $K_2HPO_4$ 1 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; KCL 0.5 g; $FeSO_4$ 0.01 g; respective carbon source 30 g; distilled water 1000 ml. For each strain, four different carbon sources were tested: fructose, glucose, maltose and sucrose. The medium was sterilized at 121 °C for 15 min and then cooled down to room temperature before adding the respective carbon source, which was filter sterilized through a 0.2 μm membrane (Sarstedt AG & Co. KG, Nümbrecht, Germany).

## 3.3 Fungal biomass production

Cultures were growth in FUMB (section 3.2.4) or Czapek's broth medium (section 3.2.5) and mycelial mass was weighted in duplicate to assess the growth rate before RT-LAMP and LC-MS/MS analyses were performed. Each culture was vacuum filtrated through folded filters (pore size < 2 μm, Whatman GmbH, Dassel, Germany). Ten milliliters of the filtrate were retained for fumonisin analysis before the mycelial pellet was washed 3 times

with 50 mL of sterile tap water. Washed and vacuum-dried mycelia were weighted before freezing in liquid nitrogen. Frozen mycelia were kept at – 80 °C for RNA isolation.

## 3.4 Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

### 3.4.1 Sample preparation for MALDI-TOF MS analysis

Table 3 shows the strains used for MALDI-TOF MS identifications as well as the origin of each isolate. In total, 3 different sample preparations were applied using MALDI-TOF MS analysis. The first sample preparation (section 3.4.1.1) was applied for all 49 *Fusarium* spp. listed in Table 3, otherwise the other 2 extra sample preparations (3.4.1.2) were applied only for the closely related species: *F. musae* (TMW 4.2594, 4.2595 and 4.2596) and *F. verticillioides* (TMW 4.0709, 4.0943, 4.1003 and 4.2444).

#### 3.4.1.1 Sample preparation for 49 *Fusarium* spp. using MALDI-TOF MS

The 49 species of *Fusarium* sp. (Table 3) were precultivated on SNA plates (section 3.2.1) that were centrally inoculated by transferring a 1-2 cm agar disc cut from a culture plate with the mycelial side facing the agar medium. Pre-cultures were incubated at AT at daylight with a natural day/night rhythm. After 48 h of growth, two agar discs (8 mm) were cut out from using sterile scissors with visible fungal growth and transferred to 6 ml of SDB (section 3.2.3) in sterile 15 ml conical tubes (Sarstedt, Nümbrech, Germany). Tubes were rotated for 48 h in an overhead tube rotator (Heidolph, Instruments GmbH, Schwabach,

Germany) at 10 rpm at AT. For sample preparation, 1 ml of culture broth was collected with a pipette tip and transferred to a sterile 1.5-ml reaction tube (Sarstedt, Nümbrecht, Germany). The sample was centrifuged (Andreas Hettich GmbH, Tuttlingen, Germany) for 10 min at 4 °C, 21,380 x g. Following centrifugation and discard of supernatants, 1 ml of sterile deionized water was added to the supernatant, vortexed and samples were centrifuged as described above. If the pellet was still not formed, a final centrifugation for 20 min under the above conditions was needed prior to further analyses.

Live fungal cells were inactivated by applying 900 µl of ethanol (VWR International, Fontenay-sous-Bois, France), vortexing, and subsequent addition of 300 µl of sterile deionized water before samples were vortexed and centrifuged for 10 min under the same centrifugation conditions described previously. The pellet was stored open in a vaccum desiccator at 5 mbar until completely dry (ca. 10 min). Proteins were extracted by application of 50 µl of formic acid (70%) (Sigma-Aldrich Chemie, Steinheim, Germany) to the dry pellet, followed by the same volume of acetonitrile (100 %) (Sigma-Aldrich Chemie, Steinheim, Germany) and suspended by vortexing for 5 min at maximum amplitude. Samples were centrifuged for 10 min at 21 °C, 21,380 xg for precipitation of solid cell debris. One microliter of the cell-free supernatant was transferred onto a MALDI 96 polished steel target plate (Bruker Daltonics, Bremen, Germany) and air-dried, overlaid with 1 µl of matrix solution containing 10 mg/ml α-cyano-4-hydroxy-cinnamic acid (Sigma-Aldrich Chemie, Steinheim, Germany) in acetonitrile, deionized water, and trifluoroacetic acid (50:47.5:2.5, v/v/v) (Sigma-Aldrich Chemie, Steinheim, Germany), and air- dried again. All samples were deposited in triplicate on the MALDI target plate.

### 3.4.1.2 Sample preparation for closely related FFSC species using MALDI-TOF MS

The two additional sample preparations applied for *F. musae* and *F. verticillioides* strains are characterized as young fungal material and mature fungal material. The young fungal material consisted of five agar discs (1-2 cm of diameter) that were transferred from an SNA grown culture to 250 mL Erlenmeyer flasks containing 50 mL of SDB (section 3.2.3) and agitated at 130 rpm for 2 days at 25 °C in the dark. After incubation, young fungal material was harvested by vacuum filtration through folded filters (pore size < 2µm, Whatman GmbH, Dassel, Germany), washed 3 times with 50 mL of sterile tap water, and ground to a fine powder in liquid nitrogen with a mortar and pestle. Samples were prepared in biological triplicates. The mature fungal material preparation consisted of Petri plates containing the SDA (section 3.2.3). The Petri plates were inoculated with 1 agar disc (8 mm) from 7d old SNA cultures. Inoculum was placed on a sterile cyclopore membrane (0.1 µm, Whatman GmbH, Dassel, Germany) and incubated for 10 d at 25 °C in the dark. Following incubation, the fungal material was carefully scraped from the filter with a sterile scalpel and ground to a fine powder in liquid nitrogen with a mortar and pestle. Samples were prepared in biological triplicates.

The protein extraction for both, young and mature fungal material, was performed as described previously for the supplementary database of 49 *Fusarium* spp (section 3.4.1.1) with some modifications. Ethanol (900 µL) was added to the finely ground mycelia in a sterile 2 mL Eppendorf tube and vortexed for 5 minutes at maximum amplitude. Subsequently, 300 µL of sterile deionized water was added before samples were vortexed again (5 minutes) and centrifuged (Andreas Hettich GmbH, Tuttlingen, Germany) for 10 minutes at ambient temperature, 21,380 x g. The pellet was evaporated in a vacuum desiccator at 5 mbar until completely dry. Proteins were extracted by suspending the dry

pellet with 50 µL of formic acid (70 %, Sigma-Aldrich Chemie, Steinheim, Germany) and vortexing for 5 minutes at maximum amplitude. The same volume of acetonitrile was added (100 %, Sigma-Aldrich Chemie, Steinheim, Germany), followed by vortexing and centrifugation the conditions previously mentioned. The supernatant (20 µL) was mixed with 20 µL of matrix solution containing 10 mg/mL of α-cyano-4-hydroxy-cinnamic acid (Sigma-Aldrich Chemie, Steinheim, Germany) in acetonitrile, deionized water, and trifluoroacetic acid (50:47.5:2.5, v/v/v) (Sigma-Aldrich Chemie, Steinheim, Germany) and vortexed for 20 minutes. One microliter of the mixture was transferred onto a MALDI 96 polished steel target plate (Bruker Daltonics, Bremen, Germany), and air-dried at ambient temperature. For the last step, the two-layer volume technique published by Dong et al. (2009) was applied: 0.5 µL of additional matrix solution was deposited onto the already dried 1:1 sample/matrix spot. All samples were deposited in triplicate on the MALDI target plate.

### 3.4.2 Reproducibility test for MALDI-TOF MS method

The sample preparation mentioned in section 3.4.1.1 was applied for FFSC species to verify the reliability of the MALDI-TOF MS method. Before supplementary database implementation of 49 *Fusarium* species, nine species were selected randomly to execute preliminary analysis in order to determine the spectra similarity from biological and technical replicates. Among the FFSC species randomly chosen are 2 strains of *F. acutatum* (TMW 4.0852 and TMW 4.0835), *F. andiyazi* (TMW 4.2661 and 4.2662) and *F. anthophilium* (TMW 4.0490 and 4.0891) and only 1 strain of *F. mexicanum* (TMW 4.2587), *F. proliferatum* (TMW 4.0952) and *F. verticillioides* (TMW 4.0702). Biological replicates were considered spectra originating from five independent parallel pre-culture SNA plates (section

3.2.1) inoculated from the same working culture plate. Moreover, five liquid cultures in SDB (3.2.3) inoculated in parallel from the same SNA pre-culture plate were chosen as technical replications. In total, 10 tubes for each strain (5 from biological replicates and 5 from technical replicates) were inoculated and incubated as described previously (3.4.1.1). Each sample was spotted in triplicate and measured twice by MALDI-TOF MS.

### 3.4.3 MALDI-TOF MS parameters

Mass spectra were automatically acquired with a Microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser (lambda = 337 nm) at a laser frequency of 60 Hz operating in linear positive ion detection mode under MALDI Biotyper 3.0 Realtime classification (RTC) (Bruker Daltonics, Bremen, Germany) and FlexControl 3.4 (Bruker Daltonics, Bremen, Germany) included in the package of Bruker Compass 1.4 (Bruker Daltonics, Bremen, Germany). Mass spectra were recorded in a range from 2,000 to 20,000 Da for each sample analyzed. The intensity of the laser was adjusted to 35–40% with an offset of 48%. One main MALDI-TOF MS spectrum was summarized from 240 single spectra that were generated by 40-laser-shot steps from random positions of the target spot. The system was calibrated using *Escherichia coli* ribosomal protein standard (Bruker Daltonics, Bremen, Germany).

### 3.4.4 MALDI-TOF MS data processing and statistical analysis

For identification, the acquired spectrum was loaded into the MALDI Biotyper Software package (Bruker Daltonics, Bremen, Germany) and analyzed by using the standard pattern-matching algorithm, which compares the acquired spectrum with spectra deposited in a reference library as main spectrum profile (MSP). FlexAnalysis Software (Bruker Daltonics, Bremen, Germany) was used for visual inspection and mass spectra processing such as baseline subtraction, smoothing, and peak picking. Each raw spectrum was converted to a text file by FlexAnalysis software listing intensities versus m/z data points spaced 0.25 Da from each other. After a preprocessing step where the single mass spectra were smoothed and baseline-subtracted, the noise level for each m/z value was used to calculate the signal-to-noise-ratio (SNR). Subsequently, peak picking was performed by finding all local maxima and eliminating those with intensities lower than a non-uniform threshold proportional to the noise level (Currie, 1999; Yasui et al., 2003). Since the mass spectra might have been inaccurately aligned after the calibration procedure, a maximum tolerance distance equal to 600 ppm of the m/z value was considered acceptable (Fushiki et al., 2006; Wang et al., 2006). Peak classification based on the peak detection rate (pdr) was carried out, which was expressed by the ratio between the number of spectra containing the considered peak and the total number of analyzed spectra (Mantini et al., 2007).

The reliability of the MALDI-TOF MS method is visualized by Multidimensional scaling (MDS), which conduct the database comparison to prearrange the blind-coded isolates in groups. MDS graphical representation was done with 30 MALDI- TOF MS measurements per strain for illustrating the similarity calculations. The distance between the symbols represents the correlation of the respective variables. Considering the variables are

non-negatively intercorrelated, the interpretation is simple: the closer two symbols, the higher the correlation of the variables (Borg et al., 2013).

MSPs were exported using FlexAnalysis and the mass spectrometry profiles were matches into clusters based on Euclidean distance matrices and visualized using RStudio 3.0.3 (RStudio, Boston, MA, USA) in order to distinguish the spectra among the included species. The calculation was based on the distance matrix using the method “manhattan_bc.” Hierarchical clustering was performed with the method “complete linkage” and visualized via dendrogram. In order to construct the supplementary database, five biological replicates were used. All of such independent samples were spotted in triplicate onto the MALDI stainless polished steel target for technical replication and measured twice to obtain 30 spectra per strain. Identification of test spectra was performed with the Biotyper version 3.0 software using the MSP library previously set up.

Results were judged as correct identifications by MALDI-TOF MS if 2 out of 3 spots tested for one strain matched the MSP of the same species and had a score value equal or greater than 2.0 (at the species level). The manufacturer recommends the follow interpretation of MALDI-TOF MS scores: score equal or greater than 2.0 indicates species level identification, a score of 1.99 to 1.7 indicates identification only to the genus level, and a score lower than 1.7 is interpretated as no identification. For outcomes that did not fulfill the criteria, the strain was checked by DNA sequencing. It was also checked for each specific identification (each spot) whether the best match with the highest score correspond to the DNA sequence-based identification of a sample in order to ensure correct interpretation of the result.

Differentiation of MALDI-TOF mass spectra from FFSC was accomplished by discriminant analysis of principal component (DAPC) using the “adegenet” package (2.0.1) included in the RStudio software package (Jombart, 2008). Details about the analysis were

described by Usbeck et al., (2014). DAPC is characterized by optimizing the variance between groups while neglecting within-group variation using MALDI-TOF mass spectra. As a result, DAPC provides a barplot of eigenvalues, a scatterplot representing individuals as dots, a histogram and main peaks responsible for the separation in a loading plot. All single spectra (n=30 of each strain) were analyzed by DAPC analysis to obtain a scatterplot to visualize the MALDI-TOF MS spectra forming groups.

### 3.5 Molecular detection methods

#### 3.5.1 Sample preparations for molecular detection methods

##### 3.5.1.1 Genomic DNA template preparation

Genomic DNA (gDNA) extraction from fungal mycelia was obtained after incubating each fungal isolate for 5–7 days at AT on a rotary shaker (100 rpm) in sterile 15 ml conical plastic tubes containing 7 ml of malt extract broth (section 3.2.2 but without agar). Removal of liquid culture medium was done by vacuum filtration followed by three rinses with sterile tap water. Total DNA of each isolate was extracted using the PeqGOLD Fungal DNA Mini Kit (VWR International, Erlangen, Germany) according to the manufacturer's recommendation. Testing of species other than FSSC was done using gDNA from highly purified stock that is stored at −20 °C in high concentration aliquots. For these cultures, DNA was purified from freeze dried pulverized mycelia using three sequential extraction steps with phenol, phenol-CIA and CIA. DNA was ethanol precipitated and then reprecipitated after treatment with protease and RNAse as described in Niessen and Vogel (1997).

Alternatively, the total fungal DNA was also isolated using the following CTAB-based protocol that consisted in mixing the mycelium with 3 ml lysis buffer (1.21 g/l Tris-

base, 47.8 g/l guanidine-HCl, 14.6 g/l NaCl, 10 g/l Triton X-100, 20 g/l polyvinylpyrrolidone (PVP 360), 20 mM EDTA, pH 7.9), 0.5 g sterile sea sand, and 1 g sterile glass beads (Ø 1.25–1.65 mm) in a sterile 15-ml reaction tube. Tubes were treated in a FastPrep®-24 cell disruptor (M.P. Biomedicals, Eschwege, Germany) at 6.5 m/s for 60 s at ambient temperature and before centrifugation for 5 min at 18,000×g. One volume of cetyltrimethylammonium bromide (CTAB) precipitation buffer (5 g/l CTAB, 1.21 g/l Tris-base, pH 7.9) was added to the supernatant and incubated for 60 min at ambient temperature. After centrifugation for 15 min at 18,000×g, the resulting pellet was washed three times each with 250 µl of 70% ethanol. The washed pellet was suspended in 250 µl 5 M ammonium acetate by gentle shaking before centrifugation (5 min at 18,000×g). The supernatant was transferred to a sterile 1.5-ml reaction vessel and mixed with 250 µl of ultra-pure water. The sample was combined carefully with one volume of isopropanol and left overnight at ambient temperature. Next day, the sample was centrifuged (5 min at 18,000×g), washed with 200 µl of 70% ethanol, and dried in a vacuum desiccator. The dry pellet was suspended in 50 µl of ultra-pure water. DNA concentrations were measured with a NanoDrop 1000 spectrophotometer (Peqlab Biotechnology, Erlangen, Germany).

#### 3.5.1.2 Complementary DNA template preparation

Total RNA was isolated from approximately 50 to 100 mg of the filtered mycelia using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and stored at -80 °C. Following the manufacturer's instructions, the extracted mRNA was digested with RNase-free DNase using the Ambion DNA-free DNA Removal Kit (ThermoFisher Scientific, Waltham, Massachussetts, USA). First strand cDNA was

generated from mRNA using M-MLV reverse transcriptase (RNase H minus, Promega GmbH) and Oligo(dt)15 primer (Promega GmbH) according to the manufacturer's standard protocol. RNA concentration of samples was measured with a NanoDrop 1000 spectrophotometer (Peqlab Biotechnology, Erlangen, Germany).

The concentration of RNA samples was adjusted to 2 µg of mRNA in 10 µL of nuclease-free water and 2 µL of Oligo(dT)$_{15}$ primer were added. After incubation at 70 °C for 5 min and cooling down on ice for 5 min, 11 µL were added to the reverse transcription reaction mixture, which consisted of M-MLV 5x reaction buffer (5 µL), 200 units of M-MLV Reverse Transcriptase, RNase H (-), Point Mutant (1 µL), 10 mM each of dNTPs (1.25 µL), 25 units Recombinant RNasin® Ribonuclease Inhibitor (0.63 µL) and nuclease-free water to a final reaction volume of 25 µL. Reverse transcription was performed at 40 °C for 10 minutes and subsequently at 45 °C for 50 minutes. Complementary DNA (cDNA) samples were stored at -20 ° C for further analyses.

#### 3.5.1.3 Conidial suspension template preparation

Conidial suspensions were prepared from *Fusarium*-cultures by suspending 1 mL sterile tap water on SNA plates (section 3.2.1) and harvested after homogenization of the culture surface with a sterile Drigalski spatula. The collected suspensions were centrifuged twice (6000 ×g, 5 min, AT) and washed with 1 mL of sterile deionized water. After a serial dilution, the total conidial numbers were counted in a hemocytometer (Thoma type, 0.1 mm chamber depth, Brand GmbH, Wertheim, Germany). Conidial suspensions were stored at 4°C.

#### 3.5.1.4 Naturally contaminated samples

Samples of maize grains and maize based products from Argentina (n = 10) and Germany (n = 11), including 2 uncontaminated samples confirmed by HPLC analysis, were examined using the new LAMP assay (Table 9). Sample template was prepared by adding 2.5 mL of sterile tap water containing 1% (v/v) Tween 20 (Gerbu Biotechnik GmbH, Heidelberg, Germany) to 1 g of sample followed by vigorous hand mixing. Tortilla flour was prepared in 100 mM $(NH_4)_2SO_4$ instead of water, without Tween 20 and centrifuged at 11,000 ×g following shaking. Subsequently, supernatants (around 2 mL) were transferred to 2 mL reaction vessels and centrifuged for 2 min at 11,000 ×g. Tween 20 was removed by washing the resulting pellet 3 times each with 1 mL sterile deionized water with intermediate centrifugation as described previously. The pellet was suspended in 500 µL of sterile deionized water and 5 µL of the suspension was used as template in LAMP reactions. Each sample was analyzed in triplicate.

### 3.5.2 Loop mediated isothermal amplification (LAMP)

The LAMP primers were designed using the PrimerExplorer V5 Software tool (Freeware at http://primerexplorer.jp/e/, Eiken Chemical Co., Ltd. (Tokyo, Japan)) using gDNA (LAMP assay) or cDNA sequences and intron bridging in order to exclude gDNA from being amplified (RT-LAMP assay).

The LAMP master mix for neutral red-based detection and for calcein-based detection was set up containing the following components per 25 µL of reaction volume: 1.9 µL 10×

ammonium sulfate buffer for neutral red-based detection (100 mM $(NH_4)_2SO_4$ (Gerbu, Heidelberg, Germany), 100 mM KCl (Carl Roth, Karlsruhe, Germany), pH 8.7) or 2.5 µL 10× MOPS buffer for calcein-based detection (200 mM MOPS (Sigma Aldrich, Taufkirchen, Germany), 100 mM KCl, 100 mM $(NH_4)_2SO_4$, pH 8.8), 1 µL $MgCl_2$ (200 mM stock, Carl Roth, Karlsruhe, Germany), 3.5 µL dNTP mix (10 mM each GATC, MP Biomedicals, Eschwege, Germany), 2.6 µL primer mix (Eurofins, Ebersberg, Germany) resulting in end concentrations of 1.6 µM each FIP and BIP, 0.8 µM each LF and LB, 0.2 µM each F3 and B3. One microliter of calcein reagent (according to Niessen and Vogel (2010) or 1.5 µL of 2.5 mM neutral red were added as indicator dyes for visual signal detection. One microliter of Bst DNA polymerase (New England Biolabs, Frankfurt, Germany) and 5 µL of gDNA or cDNA template were added per reaction and filter sterilized and UV-treated deionized water was added to a final reaction volume of 25 µL.

Table 6 and 10 show the sequences of LAMP and reverse transcription LAMP primers used during the current study. Bst polymerase was stored at −20 °C. All other reagents were stored a 4 °C. Master mix preparation and addition of DNA template into the LAMP reaction were performed in separate rooms using two separate sets of pipettes in order to avoid cross contamination. Sterile pipet tips with filters were used for all pipetting. Incubation of LAMP reactions was performed in a Mastercycler® Gradient thermal cycler (Eppendorf, Hamburg, Germany) at 65 °C for 60 min (neutral red) and 90 min (calcein), respectively. LAMP results were documented with a handheld digital camera under visible light (neutral red) or under a MiniUvis UV366 nm light source (Desaga, Heidelberg, Germany) for calcein fluorescence detection in front of a black background. For sequencing of LAMP fragments, DNA was excised from dimidium bromide-stained agarose gels after electrophoresis and extracted using the GeneJET Gel Extraction Kit (Thermo Fischer Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions. DNA

was Sanger-sequenced in both directions by a commercial service (Eurofins, Ebersberg, Germany) using primers that were identical with the F2 and B2 portions of primers FIP and BIP, respectively. Sequences and chromatograms were made available as electronic data.

### 3.5.3 Polymerase chain reaction (PCR)

The identity of FFSC strains was confirmed by sequencing a part of the translation elongation factor 1 α (TEF1α) gene (Table 2) using PCR analysis (O'Donnell et al., 1998b). For reverse transcription PCR, primers binding to the ß-tubulin gene were designed as a positive control for the amplification of cDNA templates and another primer set for the *fum1* gene was designed in order to differentiate the gDNA from cDNA samples (Table 2).

Table 2 List of PCR primers used in the current study.

| **Primer name** | **Sequence 5' > 3'** |
|---|---|
| EF – 1* | ATG GGT AAG GA(A/G) GAC AAG AC |
| EF – 2* | GGA(G/A) GTAC CAG T(G/C)AT CAT GTT |
| ß - Tub - F | GCT GCT TTC TGG CAA ACC AT |
| ß - Tub - R | ATG GTA CCA GGC TCG AGG T |
| *fum1*- F | GCA TTG CAG CTC TGA ACC TG |
| *fum1*- R | AAG CTT CTG TTG GCC TCG AA |

*O'Donnell et al., 1998b.

Amplification was executed in 25 µl of reaction mixture containing 2.5 µl of 10× buffer (Taq polymerase incubation mix, including $MgCl_2$), 0.25 µl of each primer (50 pmol/µl), 0.50 µl of dNTPs (10 mM dNTP), 0.25 µl of Taq DNA polymerase (5 U/µl, MP Biomedicals, Heidelberg, Germany), 20.25 µl of sterile deionized water, and 1 µl of template DNA. PCR was performed in a Mastercycler gradient thermal cycler (Eppendorf, Wesseling-Berzdorf, Germany) under the following conditions: initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. PCR products were forward sequenced by a commercial sequencing service (Eurofins-GATC, Konstanz, Germany). A list showing the accession numbers for each sequenced clone can be retrieved as Table 3.

For analysis PCR based DNA sequencing analysis, each sequence was compared with reference sequences of the respective species using the pairwise alignment tool available at the homepage of the Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands) (http://www.westerdijkinstitute.nl/ Collections/DefaultInfo.aspx?Page=Home) as well as using the BLAST algorithm on the GenBank data base available on the NCBI homepage (https://blast.ncbi.nlm.nih.gov/Blast. cgi) (Altschul et al., 1990). For phylogenetic analysis, sequences were aligned using the Clustal W multiple alignment tool (Thompson et al., 1994) available in the BioEdit version 7. 2.5 software package (Hall, 1999). Alignments were used to calculate Maximum Likelihood phylogenetic trees using the MEGA version 10.0.4 software package (Kumar et al., 2018). Information about program settings is given in the legend to Fig. 9.

### 3.5.4 Analytical agarose gel electrophoresis

Agarose gel electrophoresis was generally carried out in 1× TBE buffer (2.0 M Tris, 1.0 M acetic acid, 0.1 M EDTA, pH 8.2) at 100 V/90 mA. Gels were prepared with 1% agarose (Biozym Scientific, Hessisch Olendorf, Germany) in 1× TBE buffer. DNA samples (7.5 µL) were mixed with 1.5 µL 6× DNA loading buffer (Thermo Fischer Scientific, Waltham, Massachusetts, USA). The DNA fragment length (bp) was identified using GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, Waltham, Massachusetts, USA). For DNA staining, gels were submerged in a dimidium bromide solution for 10 min (Karl Roth, Karlsruhe, Germany) followed by washing in deionized water for 10 min. Gels were inspected in an UV cabinet at a wavelength of 365 nm and documented using a video imaging system (Intas Science Imaging Instruments, Göttingen, Germany).

## 3.6 Determination of fumonisin

Fumonisin analyses in liquid cultures were performed in collaboration with Dr. Karsten Meyer from the Chair of Animal Hygiene (TUM, Germany). Analyses for maize and maize based products were conducted in collaboration with Dr. Eugenia Cendoya from the Research Institute on Mycology and Mycotoxicology (IMICO, Argentina) and Dr. Ronald Maul from Unit Plant Toxins and Mycotoxins (BfR – Federal Institute for Risk Assessment, Germany).

### 3.6.1 Fumonisin detection in liquid cultures

The content of fumonisin $B_1$ ($FB_1$) in fungal strains by LC-MS/MS analysis was performed using 10 mL of liquid medium (3.2.4 or 3.2.5). For sample preparation, 500 µL of the cell free liquid medium were diluted with 2.5 mL of methanol (Baker HPLC-Grade, Fisher Scientific, Schwerte, Germany) and shaken vigorously for 30 s. SPE-Tubes Strata-SAX (55 µm, Phenomenex, California, USA) were preconditioned with two washes with 1 mL methanol/water (3:1). Extracts were filtered through Chromafil syringe filters (PTFE, 0,45 µm, 15 mm, Macherey- Nagel) and transferred to SPE-tubes. Tubes were washed twice with 1 mL methanol/water (3:1) and once with 1 mL methanol. Tubes were vacuum dried before elution of fumonisins with 3 mL methanol/acetic acid (95:5). Eluates were reduced to 200 µL by vacuum centrifugation for 30–40 min. Extracts were transferred to Crimp-Cap (BGB Analytik) HPLC vials with an additional rinse with 200 µL methanol before total evaporation in a N2-evaporator (Barkey, Leopoldshöhe, Germany). Dry extracts were dissolved in 150 µL water/acetonitrile (1:1) and loaded onto a Gemini 5 µ C18 110A, 150 × 2 mm HPLC column (Phenomenex, California, USA).

The HPLC system (Perkin Elmer Series 200) with automatic sample injection, degasser and column oven was connected to a triple-quadruple mass spectrometer (API 3200, Applied Biosystems, California, USA) with a Turbo-V ionization source. Solvent A consisted of ultrapure water, 5 mM $NH_4$-formate and 0.1% formic acid and solvent B of methanol, 5 mM $NH_4$-formate and 0,1% formic acid. The gradient started at 40% B (4 min), increased linearly to 100% B (10 min) and was held for 10–12 min at a flow rate of 400 µL/min. Identification of fumonisin $B_1$ in liquid medium samples was based on retention time of selected ions: m/z 722,3/722,1 and 722,3/334,3. The limit of detection for fumonisin $B_1$ (LOD) was 5.25 ng/mL of growth medium.

### 3.6.2 Fumonisins detection in naturally contaminated samples

Fumonisin analysis of maize grain samples from Argentina was done as described in Palacios et al. (2011). Cleanup for samples of German maize grains and Tortilla flour was carried out according to the method described in Kunz et al. (2020). The LOD was derived from the tenfold analysis of spiked blank samples according to Wenzl et al. (2016). Detection limits for both methods are given in Table 9.

# 4 Results

## 4.1 Differentiation of FFSC species by MALDI-TOF mass spectrometry

### 4.1.1 Reliability of the MALDI-TOF MS method applied for 49 *Fusarium* spp

Preliminary studies using the sample preparation described in section 3.4.2 showed that reproducible mass spectra were obtained using the established MALDI-TOF MS method with nine randomly chosen strains representing six different FFSC species. Figure 5 shows both experiments (technical and biological replicates) in a MDS graphic (section 3.4.4) in which each data point represents a replicate mass spectrum. According to Fig. 5, high reliability of the method was observed suggesting that there is no difference between biological and technical replicates originating from the same strain analyzed by MALDI-TOF MS. Also, the majority of spectra grouped together strain-wise with the six species forming well-separated clusters. In cases where two strains of the same species were analyzed, i.e., *F. andiyazi* (TMW 4.2661 and 4.2662), *F. acutatum* (TMW 4.0852 and 4.0835), and *F. anthophilum* (TMW 4.0490 and 4.0891), the species clusters segregated into well-separated subclusters showing that there is variation of spectra within a species. Whether or not this variation can be used for strain-level identification can be further elucidated but was not required within the scope of this study.

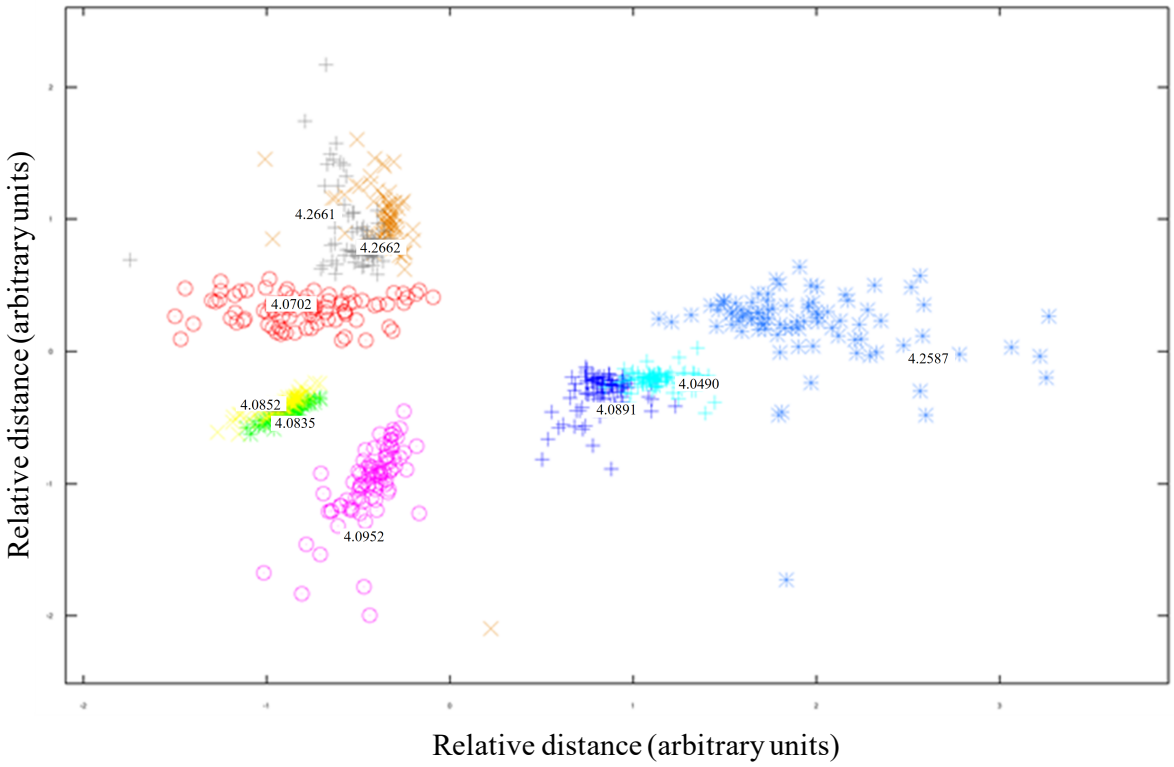


Figure 5 Multidimensional scaling (MDS) graphic of single spectra produced by MALDI-TOF MS analysis according to biological and technical replicates from nine FFSC strains representing six different species (*F. acutatum* TMW 4.0852 and 4.0835, *F. andiyazi* TMW 4.2661 and 4.2662, *F. anthophilum* TMW 4.0490 and 4.0891, *F. mexicanum* TMW 4.2587, *F. proliferatum* TMW 4.0952, and *F. verticillioides* TMW 4.0702).

### 4.1.2 Development of a MALDI supplementary database with 49 *Fusarium* species

After investigating the accuracy of the generated spectra (section 4.1.1), an in-house database was set up including MSP of 87 strains that represented 49 *Fusarium* species (47 belonging to the FFSC) to allow species-level identification by MALDI-TOF MS. A strain of *F. culmorum* was added that was intended as outgroup for cluster analysis. The MALDI-TOF MS method used is described in section 3.4.1.1. All isolates used to set up the database were verified by sequencing of their TEF1α genes by PCR analysis (section 3.5.3) before generation of the MSP for the database. MSP of two strains per species were generated

wherever possible. However, only one strain each was available to this study to set up reference spectra in *F. ananatum*, *F. babinda*, *F. begoniae*, *F. fracticaudum*, *F. mangiferae*, *F. marasasianum*, *F. pininemorale*, *F. sororula*, *F. succisae*, *F. sterilihyphosum*, and *F. werrikimbe*.

In order to explore the ability of MALDI- TOF MS to discriminate species within the FFSC, a dendrogram was established that was based on the MALDI-TOF MS spectra included in the supplementary database. Results presented in Figure 6 showed that in 96.41% of the species that were represented by two database entries, both analyzed isolates clustered together in groups that were well separated from all other species. As the only exception, the two available isolates of *F. fractiflexum* formed separate branches within the cluster analysis. Analysis of partial TEF1α sequences of the two isolates, one of which was the type strain of *F. fractiflexum* (TMW 4.2639 = NNRL 28852), showed that there were five single nucleotide polymorphisms (SNP). This result confirmed a certain degree of genetic inhomogeneity within *F. fractiflexum* that was reflected by the cluster analysis of MALDI-TOF MS spectra. Comparison of the TEF1 α sequences of two more *F. fractiflexum* isolates showed that the number of polymorphisms was even higher.

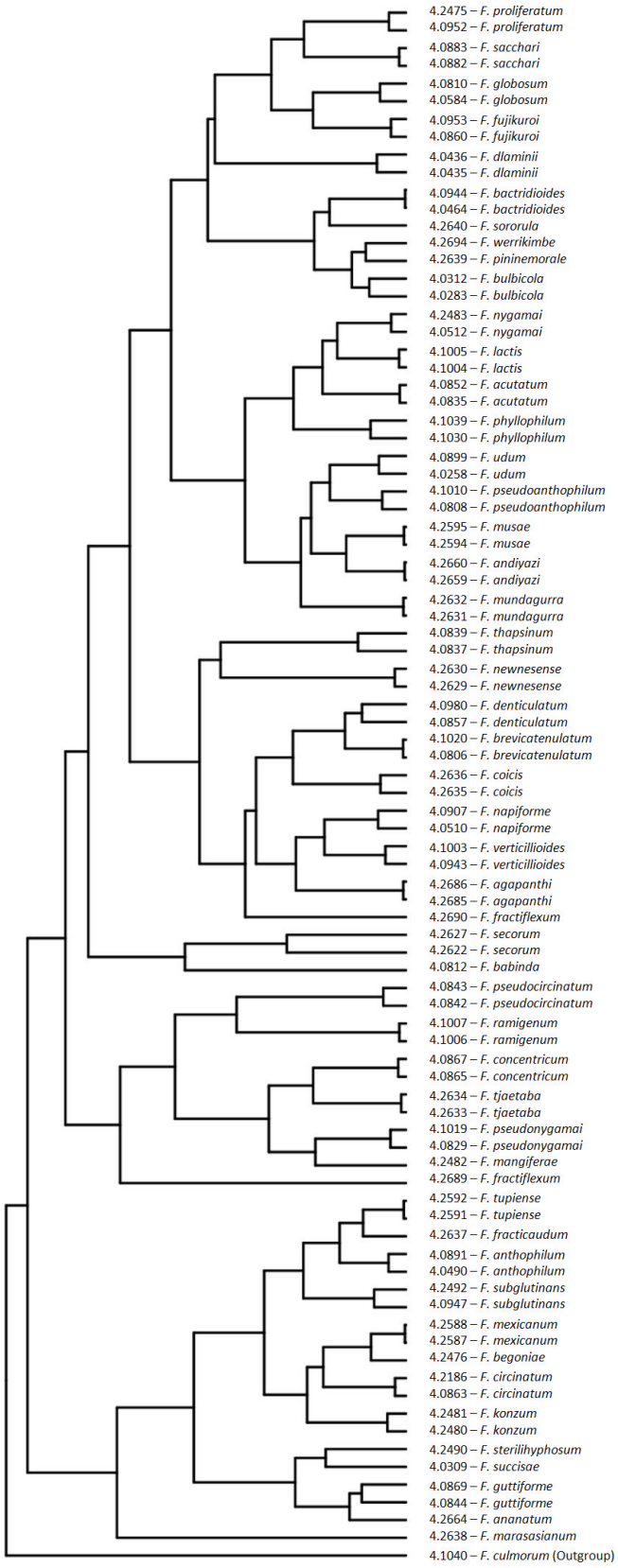


Figure 6 Cluster analysis of MALDI-TOF MS spectra representing 47 species of *Fusarium fujikuroi* species complex as well as *F. babinda* and *F. newnesense* included in the supplementary database. Spectra of *F. culmorum* TMW 4.1040 were used as outgroup.

### 4.1.3 Discriminatory power of MALDI-TOF MS

For testing the integrity of the database, MALDI-TOF MS - based species-level identification was performed with each individual isolate. Protein extracts were freshly prepared (section 3.4.1.1) and the spectra were identified using the newly established supplementary database (section 4.1.2). Table 3 shows the identification for each isolate as well as the MALDI Biotyper top score out of three parallel samples in the two rightmost columns. Of the 167 tested isolates, 161 (96.41%) were correctly identified with a top score > 2.0. Whereas the majority of isolates were correctly identified in most species with unambiguous results, only one out of three isolates was correctly identified in *F. musae*. The two incorrectly identified isolates were identified as *F. verticillioides* with a high score value indicating that they might have been misidentified. Moreover, TEF1α sequence by PCR analysis (section 3.5.3) of the two isolates of *F. musae* showed 100% homology with the sequence of *F. musae*, but also with that of *F. verticillioides*, suggesting a very close relatedness of both species.

Data were used to calculate identification rates (%) for each species represented in the supplementary database. The identification rate was defined as the number of correct identifications relative to the total number of samples measured for a given species. Calculations revealed that 43 out of the 47 FFSC species included in the supplementary database had an identification rate of 100%, meaning that all analyzed spots were correctly identified. Table 4 shows an example for the identification rates of 15 selected species of which *F. anthophilum*, *F. begoniae*, *F. guttiforme*, *F. napiforme*, *F. nygamai*, *F. tupiense*, *F. udum*, and *F. verticillioides* had an identification rate of 100%. In addition, Table 4 shows those seven species, for which identification rates lower than 100% were detected. The lowest identification rate was observed for *F. musae* (22%), followed by *F. ananatum*, *F.*

*circinatum*, *F. ramigenum*, *F. bulbicola*, *F. fractiflexum* and *F. thapsinum* with identification rates ranging from 66 to 95%. Only one out of three tested *F. musae* isolates was identified as *F. musae* by MALDI-TOF MS even though the incorrect identified isolates were the ones used to set up the reference spectra for the species. As mentioned previously, they were identified as *F. verticillioides*, a species that is very closely related to *F. musae*. Interestingly, an isolate originally obtained as *F. udum* was identified as *F. musae* by MALDI- TOF MS.

Table 3 Identification of FFSC strains used in the current study by DNA sequencing and MALDI-TOF MS analysis.

| **Information of strains** | | | **Idenfication of strains** | | |
|---|---|---|---|---|---|
| **Strain number** | **Clone** | **Source and place of isolation** | **DNA-Identification (TEF-1α)** | **MALDI-TOF Identification** | **MALDI-TOF Top score** |
| [a]BBA 69580 | 4.0835* | Unknown source - India | *F. acutatum* | *F.acutatum* | 2.474 |
| BBA 69719 | 4.0852* | Insect - Pakistan | *F. acutatum* | *F.acutatum* | 2.301 |
| BBA 69718 | 4.0851 | Unknown source | | *F. acutatum* | 2.378 |
| [b]IMI 205517 | 4.2515 | *Cajanus indicus*, India | *F. acutatum* | *F. acutatum* | 2.475 |
| [c]NRRL 54463 | 4.2685* | *Agapanthus* sp. - Australia | *F. agapanthi* | *F. agapanthi* | 2.510 |
| NRRL 54465 | 4.2686* | *Agapanthus* sp. - Australia | *F. agapanthi* | *F. agapanthi* | 2.439 |
| NRRL 54465 | 4.2687 | *Agapanthus* sp. - Australia | | *F. agapanthi* | 2.456 |
| NRRL 54466 | 4.2688 | *Agapanthus* sp. - Australia | | *F. agapanthi* | 2.432 |
| [d]MRC 8165 | 4.2664* | Pineapple – South Africa | *F. ananatum* | *F. ananatum* | 2.130 |
| [e]ITEM 6031 | 4.2659* | *Sorghum* sp. - South Africa | *F. andiyazi* | *F. andiyazi* | 2.319 |
| ITEM 6034 | 4.2660* | *Sorghum* sp. soil debris - South Africa | *F. andiyazi* | *F. andiyazi* | 2.181 |
| ITEM 6036 | 4.2661 | *Sorghum* grain – Colorado, USA | | *F. andiyazi* | 2.137 |
| ITEM 6057 | 4.2662 | *Sorghum* grain - Ethiopia | | *F. andiyazi* | 2.289 |
| ITEM 6057 | 4.2663 | *Sorghum* grain - Nigeria | | *F. andiyazi* | 2.173 |
| [f]CML 2756 | 4.2751 | *Sorghum bicolor* – Minas Gerais, Brazil | | *F. andiyazi* | 2.270 |
| [g]CBS 222.76 | 4.0490* | Stem of *Euphorbia pulcherrima* - Germany | *F. anthophilum* | *F. anthophilum* | 2.525 |
| BBA 62266 | 4.0891* | *Hippeastrum* hybrid - Germany | *F. anthophilum* | *F. anthophilum* | 2.460 |
| BBA 63270 | 4.0880 | Unknown source | | *F. anthophilum* | 2.439 |
| BBA 70278 | 4.0812* | Unknown source | *F. babinda* | *F. babinda* | 2.529 |
| CBS 100057 | 4.0464* | *Cronartium conigerum* on Pinus leiophylla - USA | *F. bactridioides* | *F. bactridioides* | 2.549 |
| BBA 63602 | 4.0944* | *Cronartium conigerum* - USA | *F. bactridioides* | *F. bactridioides* | 2.176 |
| ITEM 3505 | 4.2476* | *Begonia* hybrid - Germany | *F. begoniae* | *F. begoniae* | 2.376 |
| BBA 69198 | 4.0806* | *Striga asiatica* - Madagascar | *F. brevicatenulatum* | *F. brevicatenulatum* | 2.525 |

| | | | | | |
|---|---|---|---|---|---|
| BBA 69197 | 4.1020* | *Striga asiatica* - Madagascar | *F. brevicatenulatum* | *F. brevicatenulatum* | 2.471 |
| [h]DSM 62273 | 4.0283* | *Vallota speciosa*, rotting bulb - Germany | *F. bulbicola* | *F. bulbicola* | 2.511 |
| DSM 62270 | 4.0312* | *Nerine bowdenii*, rotting bulb - Germany | *F. bulbicola* | *F. bulbicola* | 2.463 |
| BBA 63620 | 4.0946 | Unknown source | *F. bulbicola* | Incorrect identification | |
| BBA 63622 | 4.0955 | Unknown source | | *F. bulbicola* | 2.006 |
| BBA 63628 | 4.0951 | Unkonwn source | | *F. bulbicola* | 2.435 |
| ITEM 3515 | 4.0863* | *Pinus patula* - South Africa | *F. circinatum* | *F. circinatum* | 2.538 |
| CBS 122161 | 4.2186* | *Pinus radiata* - Spain | *F. circinatum* | *F. circinatum* | 2.357 |
| BBA 69721 | 4.0854 | Unknown source | *F. circinatum* | Incorrect identification | |
| NRRL66233 | 4.2635* | *Coix gasteenii* - Australia | *F. coicis* | *F. coicis* | 2.481 |
| NRRL66234 | 4.2636* | *Coix gasteenii* - Australia | *F. coicis* | *F. coicis* | 2.380 |
| ITEM 3519 | 4.0865* | *Musa sapientum* - Guatemala | *F. concentricum* | *F. concentricum* | 2.452 |
| BBA 69858 | 4.0867* | *Musa sapientum* - Guatemala | *F. concentricum* | *F. concentricum* | 2.449 |
| BBA 69855 | 4.0864 | Unknown source | | *F. concentricum* | 2.457 |
| BBA 69857 | 4.0866 | Unknown source | | *F. concentricum* | 2.325 |
| [i]TMW 4.1926 | 4.1926 | Soil debris - USA | | *F. concentricum* | 2.102 |
| BBA 69021 | 4.0822 | Unknown source | | *F. concentricum* | 2.157 |
| BBA 69012 | 4.1013 | Unknown source | | *F. concentricum* | 2.144 |
| DSM 62191 | 4.1040 | *Triticum aestivum* | *F. culmorum* | | |
| BBA 69725 | 4.0857* | *Ipomoea batatas* - Peru | *F. denticulatum* | *F. denticulatum* | 2.238 |
| BBA 65244 | 4.0980* | *Ipomoea batatas* - Cuba | *F. denticulatum* | *F. denticulatum* | 2.469 |
| BBA 67771 | 4.0801 | Unknown source | | *F. denticulatum* | 2.169 |
| CBS 175.88 | 4.0435* | Soil debris - South Africa | *F. dlaminii* | *F. dlaminii* | 2.738 |
| CBS 258.52 | 4.0436* | ex trunk of *Coffea* sp. - Ivory Coast | *F. dlaminii* | *F. dlaminii* | 2.564 |
| BBA 69859 | 4.0868 | Unknown source | | *F. dlaminii* | 2.145 |
| [j]CMW 25237 | 4.2637* | *Pinus tecunumanii* - Colombia | *F. fracticaudum* | *F. fracticaudum* | 2.424 |
| NRRL 28852 | 4.2689* | *Cymbidium* sp. | *F. fractiflexum* | *F. fractiflexum* | 2.310 |

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|---|---|---|---|---|---|
| NRRL 26794 | 4.2690* | *Cymbidium* sp. | *F. fractiflexum* | *F. fractiflexum* | 2.548 |
| NRRL 28853 | 4.2691 | *Cymbisium* sp. - Japan | *F. fractiflexum* | Incorrect identification | |
| NRRL 28854 | 4.2692 | *Cymbisium* sp. - Japan | | *F. fractiflexum* | 2.306 |
| BBA 69741 | 4.0860* | *Oryza sativa* - China | *F. fujikuroi* | *F. fujikuroi* | 2.690 |
| BBA 63630 | 4.0953* | *Oryza sativa* - Taiwan | *F. fujikuroi* | *F. fujikuroi* | 2.642 |
| ITEM 3155 | 4.2478* | Unknown source | *F. fujikuroi* | *F. fujikuroi* | 1.929 |
| MRC 6646 | 4.0584* | Maize - South Africa | *F. globosum* | *F. globosum* | 2.545 |
| BBA 70241 | 4.0810* | *Zea mays* - South Africa | *F. globosum* | *F. globosum* | 2.525 |
| MRC 6650 | 4.0588 | Unknown source | | *F. globosum* | 2.348 |
| MRC 6649 | 4.0587 | Unknown source | | *F. globosum* | 2.408 |
| BBA 69661 | 4.0844* | *Ananas comosus* - Brazil | *F. guttiforme* | *F. guttiforme* | 2.394 |
| BBA 69860 | 4.0869* | *Ananas comosus* - Brazil | *F. guttiforme* | *F. guttiforme* | 2.536 |
| BBA 69665 | 4.0847 | Unknown source | | *F. guttiforme* | 2.136 |
| ITEM 3538 | 4.2479 | Unknown source | | *F. guttiforme* | 2.217 |
| BBA 69664 | 4.0846 | Unknown source | | *F. guttiforme* | 1.847 |
| BBA 65668 | 4.0985 | Unknown source | | *F. guttiforme* | 2.167 |
| ITEM 3106 | 4.2480* | *Andropogon gerardii* plant - USA | *F. konzum* | *F. konzum* | 2.598 |
| ITEM 3141 | 4.2481* | *Andropogon gerardii* plant - USA | *F. konzum* | *F. konzum* | 2.590 |
| ITEM 3541 | 4.1004* | *Ficus carica* - USA | *F. lactis* | *F. lactis* | 2.512 |
| BBA 68591 | 4.1005* | *Ficus carica* - USA | *F. lactis* | *F. lactis* | 2.581 |
| BBA 69723 | 4.0856 | Unknown source | | *F. lactis* | 2.043 |
| ITEM 7646 | 4.2482* | Unknown source | *F. mangiferae* | *F. mangiferae* | 2.400 |
| BBA 69662 | 4.0845 | Unknown source | | *F. mangiferae* | 2.490 |
| CMW 25253 | 4.2638* | *Pinus tecunumanii* - Colombia | *F. marasasianum* | *F. marasasianum* | 2.603 |
| CML 2582 | 4.2587* | *Mangifera indica* – Michoacan, Mexico | *F. mexicanum* | *F. mexicanum* | 2.401 |
| CML 2585 | 4.2588* | *Mangifera indica* - Michoacan, Mexico | *F. mexicanum* | *F. mexicanum* | 2.257 |
| CML 0411 | 4.2589 | Unknown source | | *F. mexicanum* | 2.248 |

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|---|---|---|---|---|---|
| NRRL 66235 | 4.2631* | Soil , Qeensland, Australia | *F.mundagurra* | *F. mundagurra* | 2.578 |
| NRRL 66236 | 4.2632* | Mango - Australia | *F. mundagurra* | *F. mundagurra* | 2.546 |
| CML 3662 | 4.2766 | *Brachiaria ruziziensis* – Sao Paulo, Brazil | | *F. mundagurra* | 1.992 |
| ITEM 1113 | 4.2594* | *Musa* sp., fruit - Panama | *F. musae* | Incorrect identification | |
| ITEM 1129 | 4.2595* | *Musa* sp., fruit - Panama | *F. musae* | Incorrect identification | |
| IMI 255452 | 4.2516 | *Cajanus cajan* - Malawi | | *F. musae* | 2.398 |
| CBS 693.94 | 4.0510* | Unknown source | *F. napiforme* | *F. napiforme* | 2.421 |
| ITEM 3544 | 4.0907* | *Pennisetum typhoides* - South Africa | *F. napiforme* | *F. napiforme* | 2.527 |
| BBA 69861 | 4.0870 | Unknown source | | *F. napiforme* | 2.421 |
| NRRL 66238 | 4.2629* | Soil - Tasmania, Australia | *F. newnesense* | *F. newnesense* | 2.628 |
| NRRL 66239 | 4.2630* | Soil - Tasmania, Australia | *F. newnesense* | *F. newnesense* | 2.428 |
| CBS 572.94 | 4.0512* | *Cajanus cajan* - India | *F. nygamai* | *F. nygamai* | 2.512 |
| ITEM 7600 | 4.2483* | Maize - California | *F. nygamai* | *F. nygamai* | 2.553 |
| CBS 137240 | 4.2639* | *Pinus tecunumanii* - Colombia | *F. pininemorale* | *F. pininemorale* | 2.163 |
| BBA 63175 | 4.0879 | Unknown source | | *F. nygamai* | 2.512 |
| ITEM 7601 | 4.2484 | Unknown source | | *F. nygamai* | 2.505 |
| BBA 70695 | 4.1030* | *Sansevieria trifasciata* - Germany | *F. phyllophilum* | *F. phyllophilum* | 2.434 |
| BBA 70990 | 4.1039* | Unknown source | *F. phyllophilum* | *F. phyllophilum* | 2.395 |
| BBA71921 | 4.1032 | Unknown source | | *F. phyllophilum* | 2.409 |
| BBA 70988 | 4.1038 | Unkwown source | | *F. phyllophilum* | 2.385 |
| BBA 63629 | 4.0952* | *Oryza sativa* - Vietnam | *F. proliferatum* | *F. proliferatum* | 2.395 |
| ITEM 7588 | 4.2475* | Unknown source | *F. proliferatum* | *F. proliferatum* | 2.456 |
| DSM 62261 | 4.0236 | *Cymbidium* hybrid, leaf spot - Germany | | *F. proliferatum* | 2.469 |
| BBA 69540 | 4.0827 | Unknown source | | *F. proliferatum* | 2.535 |
| ITEM 3269 | 4.2485 | Unknown source | | *F. proliferatum* | 2.361 |
| ITEM 3272 | 4.2486 | Unknown source | | *F. proliferatum* | 2.430 |
| CBS 258.54 | 4.0461 | *Oryza sativa* - Japan | | *F. proliferatum* | 2.239 |

| | | | | | |
|---|---|---|---|---|---|
| ITEM 2625 | 4.2491 | Unknown source | | *F. proliferatum* | 2.244 |
| TMW 4.2699 | 4.2699 | Grape - USA | | *F. proliferatum* | 2.341 |
| TMW 4.0628 | 4.0628 | Unknown source | | *F. proliferatum* | 2.508 |
| TMW 4.2096 | 4.2096 | Unknown source | | *F. proliferatum* | 2.392 |
| TMW 4.2098 | 4.2098 | Cereal malt - Europe | | *F. proliferatum* | 2.363 |
| NRRL72532 | 4.2693 | Unknown source | *F. proliferatum* | *F. proliferatum* | 2.306 |
| BBA 70129 | 4.0808* | *Zea mays* - Zimbabwe | *F. pseudoanthophilum* | *F. pseudoanthophilum* | 2.637 |
| BBA 69003 | 4.1010* | *Zea mays* - Zimbabwe | *F. pseudoanthophilum* | *F. pseudoanthophilum* | 2.434 |
| BBA 69598 | 4.0842* | Insect - Papua New Guinea | *F. pseudocircinatum* | *F. pseudocircinatum* | 2.263 |
| BBA 69636 | 4.0843* | *Solanum* sp. - Ghana | *F. pseudocircinatum* | *F. pseudocircinatum* | 2.454 |
| ITEM 3564 | 4.2651 | *Ficus carica* - Italy | | *F. pseudocircinatum* | 2.229 |
| ITEM 3565 | 4.2652 | *Solanum* sp. - Ghana | | *F. pseudocircinatum* | 2.565 |
| BBA69723 | 4.0856 | Unknown source | | *F. pseudocircinatum* | 2.272 |
| BBA 69551 | 4.0829* | *Pennisetum typhoides* - Nigeria | *F. pseudonygamai* | *F. pseudonygamai* | 2.422 |
| BBA 69131 | 4.1019* | *Begonia* hybrid - Germany | *F. pseudonygamai* | *F. pseudonygamai* | 2.232 |
| BBA 68592 | 4.1006* | *Ficus carica* - USA | *F. ramigenum* | *F. ramigenum* | 2.290 |
| BBA 68593 | 4.1007* | *Ficus carica* - USA | *F. ramigenum* | *F. ramigenum* | 2.311 |
| ITEM 7607 | 4.2488 | Unknown source | | *F. ramigenum* | 2.542 |
| ITEM 2963 | 4.2489 | Unknown source | | *F. ramigenum* | 2.248 |
| ITEM 3570 | 4.2654 | *Ficus carica* – California, USA | *F. ramigenum* | Incorrect identification | |
| BBA 63320 | 4.0882* | *Saccharum officinarum* - India | *F. sacchari* | *F. sacchari* | 2.531 |
| BBA 63340 | 4.0883* | *Saccharum officinarum* - India | *F. sacchari* | *F. sacchari* | 2.589 |
| CML 3570 | 4.2758 | *Saccharum officinarum* – Maranhao, Brazil | | *F. sacchari* | 2.492 |
| CML 3557 | 4.2761 | *Saccharum officinarum* – Piaui, Brazil | | *F. sacchari* | 2.511 |
| 1089-10 | 4.2622* | Sugarbeet - USA | *F. secorum* | *F. secorum* | 2.494 |
| [k]FS12-4a | 4.2627* | Sugarbeet – USA | *F. secorum* | *F. secorum* | 2.514 |
| [k]938 – 6 | 4.2625 | Sugarbeet – Minnesota, USA | | *F. secorum* | 2.487 |

| | | | | | |
|---|---|---|---|---|---|
| [k]757 – 1 | 4.2628 | Sugarbeet – Minnesota, USA | | *F. secorum* | 2.506 |
| CMW 25517 | 4.2640* | *Pinus patula* - Colombia | *F. sororula* | *F. sororula* | 2.680 |
| ITEM 7647 | 4.2490* | Mango – South Africa | *F. sterilihyphosum* | *F. sterilihyphosum* | 2.023 |
| BBA 63621 | 4.0947* | *Zea mays* - Germany | *F. subglutinans* | *F. subglutinans* | 2.537 |
| ITEM 2860 | 4.2492* | *Zea mays* - Austria | *F. subglutinans* | *F. subglutinans* | 2.433 |
| CBS 215.76 | 4.0408 | *Zea mays* – Germany | | *F. subglutinans* | 2.335 |
| BBA 62275 | 4.0892 | Unknown source | | *F. subglutinans* | 2.369 |
| BBA 65621 | 4.0988 | Unknown source | | *F. subglutinans* | 2.408 |
| TMW 4.1972 | 4.1972 | Maize - Romania | | *F. subglutinans* | 2.177 |
| TMW 4.2087 | 4.2087 | Cereal malt grain - Germany | | *F. subglutinans* | 2.134 |
| MRC 8167 | 4.2666 | Pineapple – South Africa | *F. subglutinans* | *F. subglutinans* | 2.179 |
| MRC 8168 | 4.2667 | Pineapple – South Africa | *F. subglutinans* | *F. subglutinans* | 2.178 |
| DSM 63162 | 4.0309* | *Succisa pratensis* - Germany | *F. succisae* | *F. succisae* | 2.256 |
| BBA 69582 | 4.0837* | *Sorghum* sp. | *F. thapsinum* | *F. thapsinum* | 2.549 |
| BBA 69584 | 4.0839* | *Sorghum* sp. - USA | *F. thapsinum* | *F. thapsinum* | 2.612 |
| BBA 69581 | 4.0836 | Unknown source | | *F. thapsinum* | 2.399 |
| ITEM 3580 | 4.2655 | *Sorghum* sp. - Philippines | | *F. thapsinum* | 2.459 |
| ITEM 3582 | 4.2656 | *Sorghum* sp. | | *F. thapsinum* | 2.398 |
| ITEM 3583 | 4.2657 | *Sorghum* sp. – Mississippi, USA | | *F. thapsinum* | 2.435 |
| ITEM 3924 | 4.2658 | *Sorghum vulgare* – Texas, USA | | *F. thapsinum* | 2.410 |
| NRRL 66244 | 4.2633* | *Sorghum interjectum* - Australia | *F. tjaetaba* | *F. tjaetaba* | 2.483 |
| NRRL 66245 | 4.2634* | *Sorghum interjectum* - Australia | *F. tjaetaba* | *F. tjaetaba* | 2.603 |
| CML 0389 | 4.2591* | *Mangifera indica* – Juazeiro, BA - Brazil | *F. tupiense* | *F. tupiense* | 2.487 |
| CML 0263 | 4.2592* | *Mangifera indica* – Juazeiro, BA - Brazil | *F. tupiense* | *F. tupiense* | 2.438 |
| CML 0262 | 4.2590 | Unknown source | | *F. tupiense* | 2.284 |
| CBS 747.79 | 4.0258* | *Cayanus indica* -India | *F. udum* | *F. udum* | 2.290 |
| BBA 65058 | 4.0899* | *Cajanus cajan* - India | *F. udum* | *F. udum* | 2.527 |

| | | | | | |
|---|---|---|---|---|---|
| BBA 62451 | 4.0893 | Unknown source | | *F. udum* | 2.493 |
| TMW 4.0488 | 4.0488 | Unknown source | | *F. udum* | 2.532 |
| BBA 62188 | 4.0943* | *Zea mays* - Germany | *F. verticillioides* | *F. verticillioides* | 2.594 |
| BBA 68588 | 4.1003* | *Ficus carica* - USA | *F. verticillioides* | *F. verticillioides* | 2.564 |
| CBS 218.76 | 4.0409 | *Zea mays* - Germany | | *F. verticillioides* | 2.486 |
| [l]BFE 314 | 4.0702 | Unknown source | | *F. verticillioides* | 2.567 |
| BFE 315 | 4.0703 | Unknown source | | *F. verticillioides* | 2.546 |
| BBA 69704 | 4.0850 | Unknown source | | *F. verticillioides* | 2.467 |
| BFE 313 | 4.0709 | Unknown source | | *F. verticillioides* | 2.428 |
| NRRL 54250[T] | 4.2694* | Unknown host – Australia | *F. werrikimbe* | *F. werrikimbe* | 2.462 |

*mass spectra included in the supplementary database
[T]type strain
[a]BBA = Julius Kühn Institut, Bundesforschungsinstitut für Kulturpflanzen, Berlin, DE;
[b]IMI = Institute of Microbiology and Infection, Birmingham, UK;
[c]NRRL = Northern Regional Research Laboratory, Peoria (Illinois), USA;
[d]MRC = South African Medical Research Council, Tygerberg, SA;
[e]ITEM = Istituto Tossine e Micotossine da Parassiti Vegetali, CNR, Bari, IT;
[f]CML = Coleção micológica de Lavras, Minas Gerais, BR;
[g]CBS = Westerdijik Fungal Biodiversity Institute Utrecht, NL;
[h]DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen, Darmstadt, DE;
[i]TMW= Lehrstuhl für Technische Mikrobiologie Weihenstephan, Technical University of Munich, Freising, DE;
[j]CMW= Collection oft he Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa;
[k] Collection of Melvin Bolten, Sugarbeet and Potato Research, USDA, ARS, NCSL, Fargo, USA;
[l]BFE = Max Rubner Institut, Bundeserforschungsanstalt für Ernährung, Karlsruhe, DE.

Table 4 Identification rates of 15 species belonging to the *Fusarium fujikuroi* species complex analyzed by MALDI-TOF mass spectrometry. Identifcation rates (%) represent the number of identifications for database entries given in the left column relative to the number of spots analyzed for each species in the top row of the table. The other 34 species analyzed in this study (49 species in total) resulted in 100 % of correct identifications.

| *Fusarium* sp. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 strain | 3 strains | 1 strain | 5 strains | 3 strains | 4 strains | 6 strains | 3 strains | 3 strains | 4 strains | 5 strains | 7 strains | 3 strains | 4 strains | 7 strains |
| 1-*F.ananatum* | 66.67 % | | | | | | | | | | | | | | |
| 2- *F.anthophilum* | | 100 % | | 6.66 % | | | | | | | | | | | |
| 3- *F.begoniae* | | | 100 % | 6.66% | | | | | | | | | | | |
| 4- *F.bulbicola* | | | | 80 % | 33.33% | | | | | | | | | | |
| 5- *F.circinatum* | | | | | 66.67 % | | | | | | | | | | |
| 6- *F.fractiflexum* | | | | | | 83.33 % | | | | | | | | | |
| 7- *F.guttiforme* | 33.33 % | | | | | | 100 % | | | | | | | | |
| 8 - *F.musae* | | | | | | | | 22.22 % | | | 20 % | | | | |
| 9- *F.napiforme* | | | | | | 16.67 % | | | 100 % | | | | | | |
| 10- *F.nygamai* | | | | | | | | | | 100 % | | 4.76 % | | | |
| 11-*F.ramigenum* | | | | | | | | | | | 73.33 % | | | | |
| 12- *F.thapsinum* | | | | | | | | | | | | 95.24 % | | | |
| 13- *F.tupiense* | | | | 6.66 % | | | | | | | | | 100 % | | |
| 14- *F.udum* | | | | | | | | 11.11 % | | | | | | 100 % | |
| 15- *F.verticillioides* | | | | | | | | 66.67 % | | | 6.67 % | | | | 100 % |

### 4.1.4 Discriminant analysis of principal components (DAPC) of 49 *Fusarium* spp.

Mass spectra of 49 *Fusarium* spp. generated by MALDI-TOF MS were compared with each other and grouped according to similarity using discriminant analysis of principal components (DAPC) as described in section 3.4.4 using the MALDI method mentioned in section 3.4.1.1. Figure 7 shows the distribution of MSP spectra of 87 reference isolates present in the supplementary database (representing 49 *Fusarium* species included in the supplementary database, excluding *F. culmorum* as outgroup species) into similarity groups. Species included in the three DAPC groups are listed in Table 5. *F. circinatum*, *F. guttiforme*, *F. pseudonygamai*, and *F. secorum* could not be clearly attributed to one of the three groups by DAPC because more than 30% of spectra fell into more than one DAPC group, respectively. DAPC results were further analyzed to identify the masses that led to the separation of the three groups of spectra. As shown in Figure 8, comparison of each group with the respective other two groups resulted in individual loading plots with three (Fig. 8), five (Fig. 8a), or seven (Fig. 8c) peaks that characterize each of the three groups. Each group had a major peak of 6447.6, 3373.1, and 6445.3 Da that clearly differentiated group 1 from 2, group 1 from 3, and group 2 from 3, respectively.

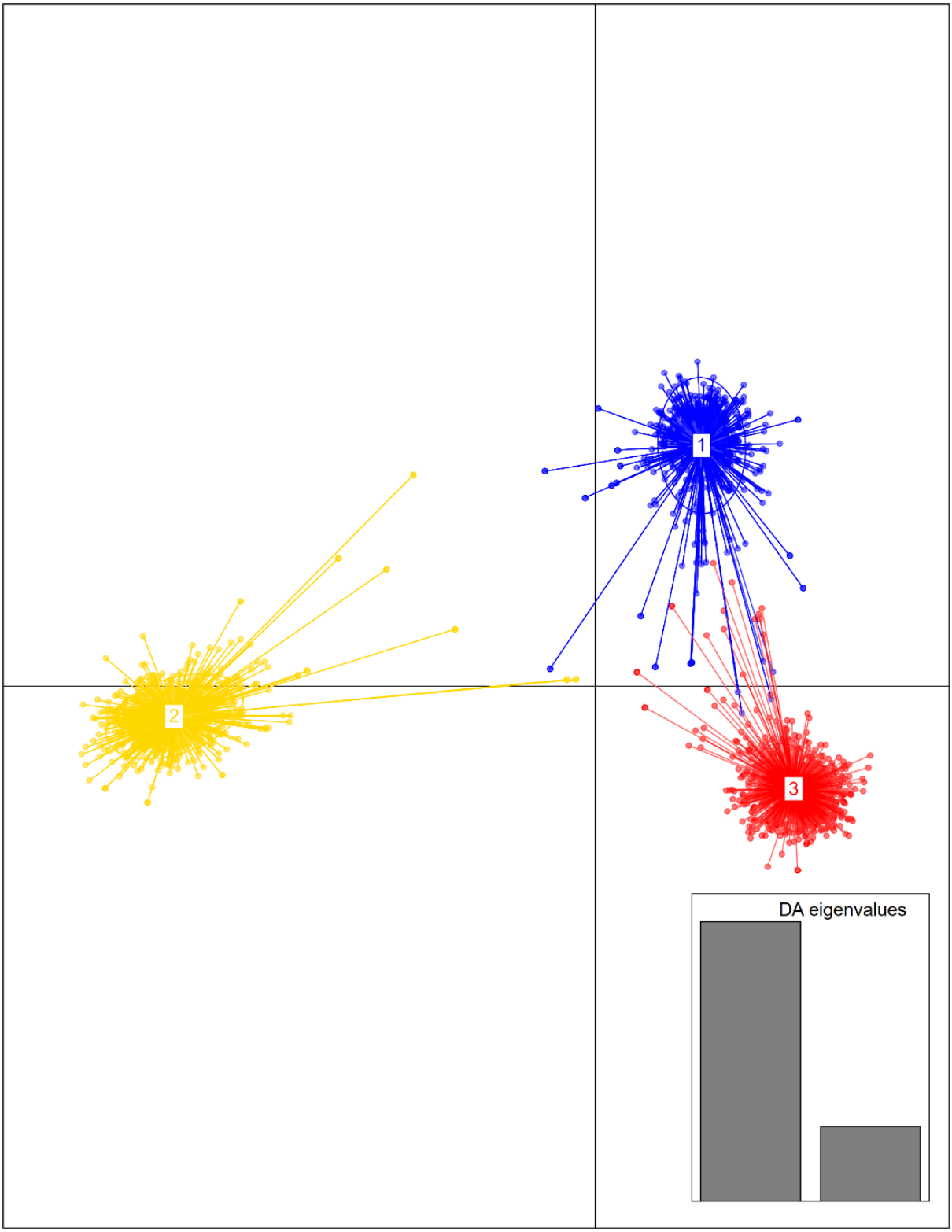


Figure 7 Discriminant analysis of principal component (DAPC) performed with MALDI-TOF mass spectra from 49 *Fusarium* species included in the supplementary database (see Table 5 for assignment of species to DAPC groups)

Table 5 PCA-based grouping of 49 *Fusarium* spp.

| **Group 1** | **Group 2** | **Group 3** |
|---|---|---|
| *F. babinda* | *F. ananatum* | *F. acutatum* |
| *F. concentricum* | *F. anthophilum* | *F. agapanthi* |
| *F. dlaminii* | *F. bactridioides* | *F. andiyazi* |
| *F. fractiflexum* | *F. begonia* | *F. brevicatenulatum* |
| *F. mangiferae* | *F. bulbicola* | *F. coicis* |
| *F. newnesense* | *F. circinatum* | *F. denticulatum* |
| *F. pseudocircinatum* | *F. fracticaudum* | *F. fujikuroi* |
| *F. ramigenum* | *F. guttiforme* | *F. globosum* |
| *F. sacchari* | *F. konzum* | *F. lactis* |
| *F. tjaetaba* | *F. marasassianum* | *F. mundagurra* |
| | *F. mexicanum* | *F. musae* |
| | *F. pininemorale* | *F. napiforme* |
| | *F. sororula* | *F. nygamai* |
| | *F. sterilihyphosum* | *F. phyllophilum* |
| | *F. succisae* | *F. proliferatum* |
| | *F. subglutinans* | *F. pseudoanthophilum* |
| | *F. tupiense* | *F. pseudonygamai* |
| | *F. werrikimbe* | *F. secorum* |
| | | *F. thapsinum* |
| | | *F. udum* |
| | | *F. verticillioides* |

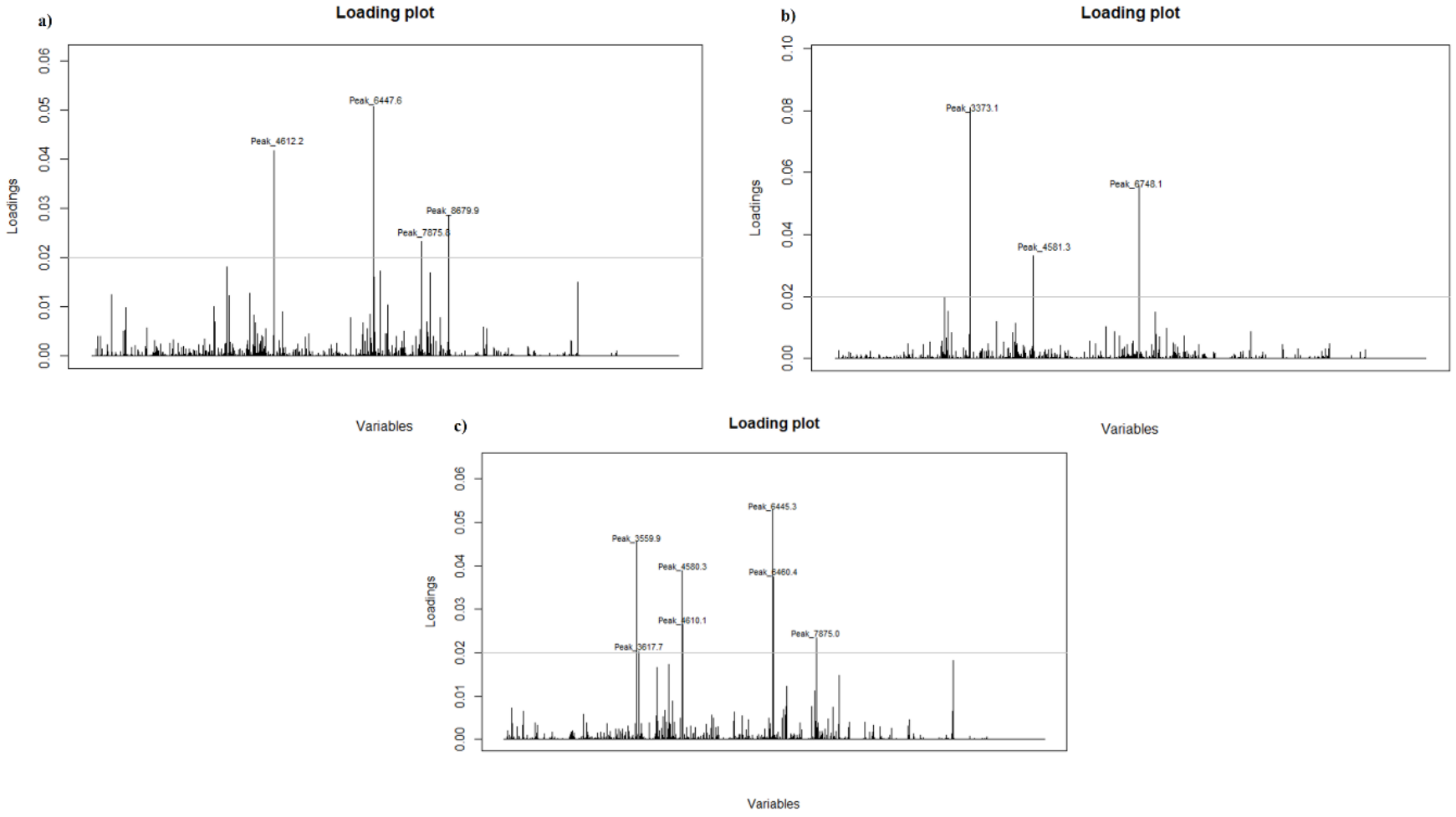


Figure 8 Illlustration of major peaks responsible for the separation of mass spectra of *Fusarium* species obtained by MALDI-TOF mass spectrometry. a) Separation of groups 1 and 2; b) separation of groups 1 and 3; c) separation of groups 2 and 3.

### 4.1.5 Phylogenetic data based on DNA sequencing

The Maximum Likelihood (ML) phylogeny shown in Fig. 9 inferred from the TEF1α sequences obtained from *Fusarium* sp. isolates during the current study (see Table 3 for accession numbers). The sample preparation was performed as described in section 3.5.1.1 and PCR analysis as in section 3.5.3. In order to check the quality of the TEF1α sequences used, another ML phylogenetic tree was calculated that contained two to three additional reference sequences (NCBI) for each analyzed species (Figure 9). Results showed that sequences clustered closely with sequences of reference strains in the majority of analyzed species. Only in *F. werrikimbe*, the sequence clustered together with *F. bulbicola* reference strains and sequences of *F. ananatum* formed two distinct clusters with references sequences of *F. guttiforme*. One such cluster contained the two *F. guttiforme* sequences obtained in the current study.

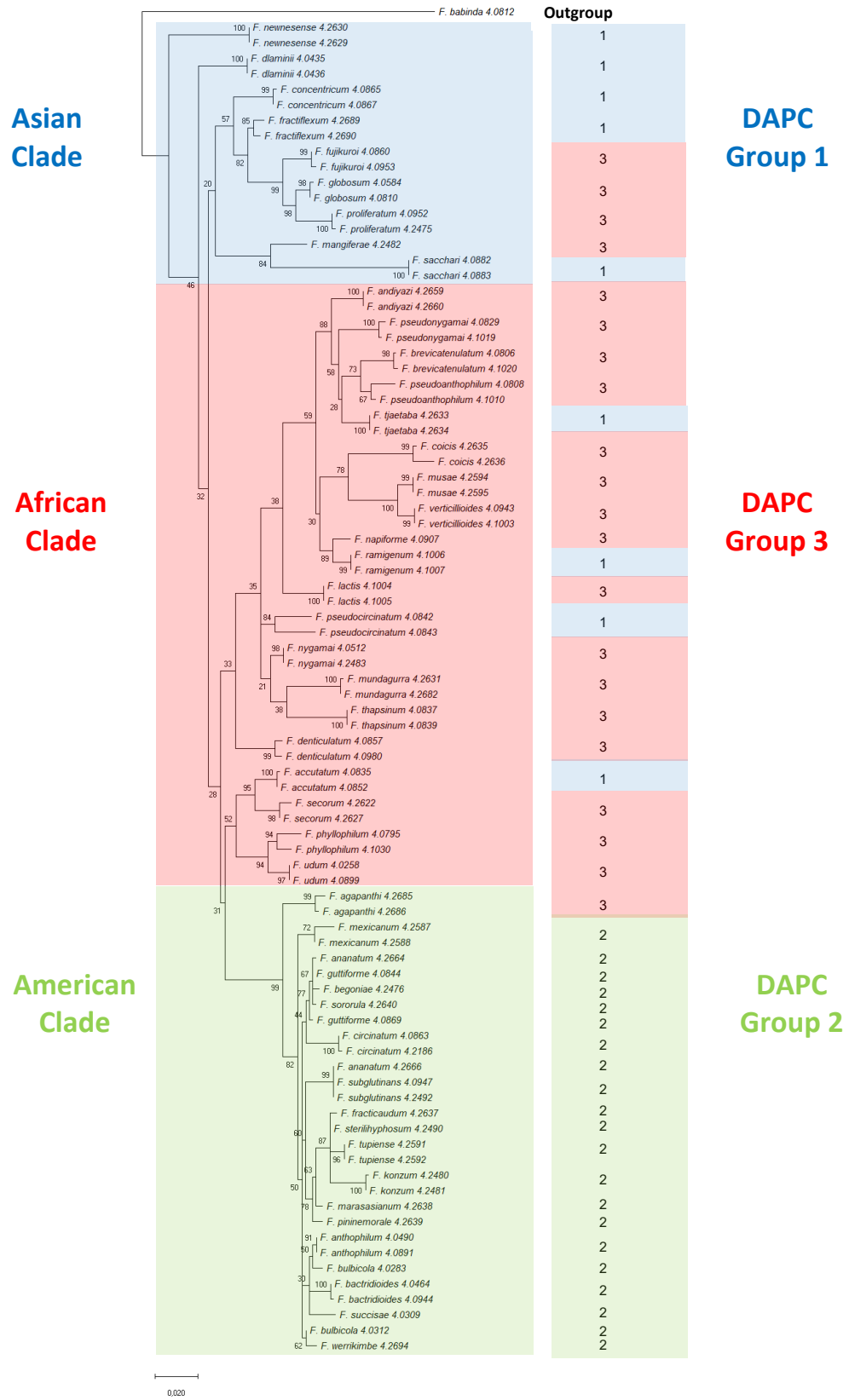


Figure 9 Maximum Likelihood phylogeny of partial TEF1α sequences of 47 FFSC isolates and *F. newnesense* used to set up a supplementary MALDI-TOF MS database. *Fusarium babinda* was used as outgroup. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) with 500

bootstrap simulations. The tree with the highest log likelihood (− 4987.52) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 87 nucleotide sequences. There was a total of 688 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

### 4.1.6 Comparison of two additional MALDI-TOF MS methods for *F. musae* differentiation

The MALDI method mentioned in section 3.4.1.1 to construct the supplementary database was not able to differentiate *F. musae* within the 49 *Fusarium* spp. For this reason, other two methods were applied to *F. musae* and its sister-species, *F. verticillioides,* based on Sabouraud dextrose (section 3.2.3) grown cultures of different age for their potential in MALDI-TOF MS based differentiation (section 3.4.1.2). *F. musae* strains (TMW 4.2594, TMW 4.2595 and TMW 4.2596) and *F. verticillioides* strains (TMW 4.0709, TMW 4.0943, TMW 4.1003 and TMW 4.2444) were selected for this experiment. Figure 10 shows the results of the DAPC analysis (3.4.4) of mass spectra obtained from MALDI-TOF MS of mature fungal material and young fungal material. DAPC based on the spectra of two different *F. musae* and *F. verticillioides* strains showed that the spectra obtained from young fungal material formed one cluster in which none of the strains or species were sufficiently resolved. On the other hand, the use of mature fungal material resulted in a very clear separation between the two species, *F. verticillioides* and *F. musae* (Figure 10).

Moreover, the use of mature fungal material allowed a strict separation of groups of spectra generated from each strain (Figure 10). Results were further analyzed to identify the mass fragments that were responsible for the discrimination of *F. musae* strains from *F.*

*verticillioides* strains. The loading plot shown in Figure 11 revealed seven different fragment masses that where responsible for the discrimination of the two species. The identity of the strains included in the supplementary database were verified by sequencing of their TEF-1α genes (Table 3).

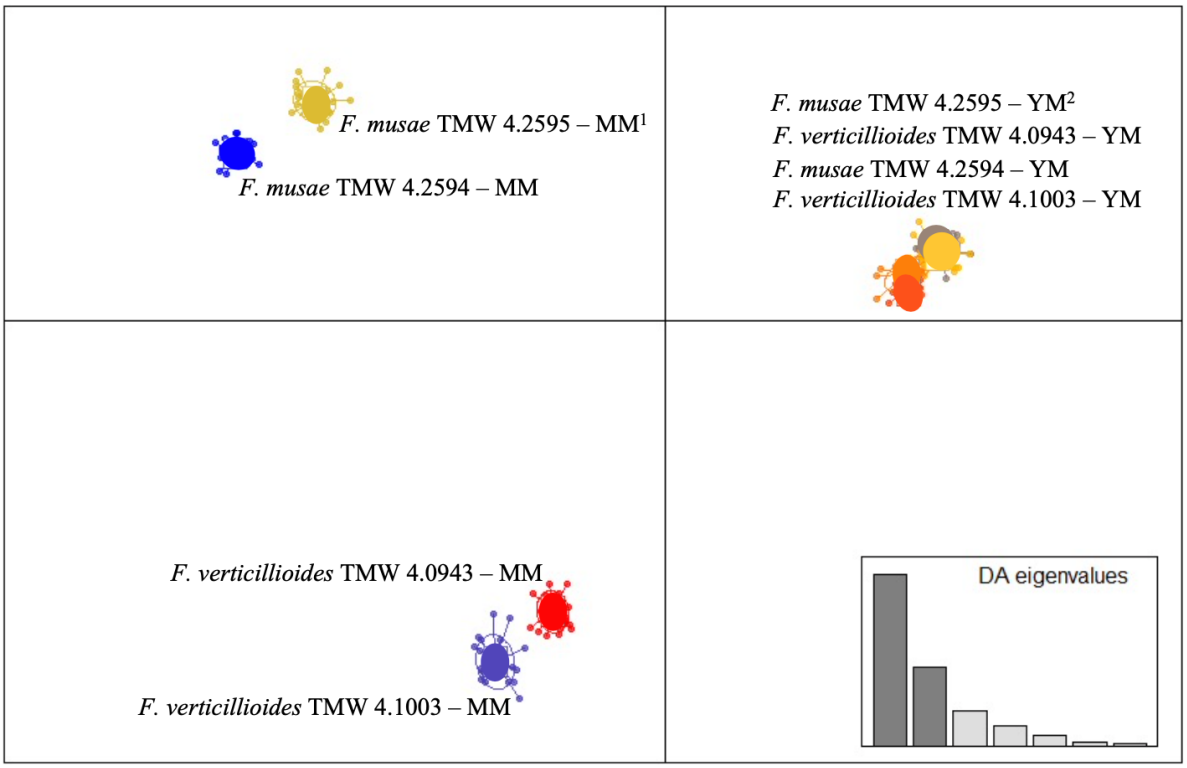


Figure 10 Discriminant analysis of principal component (DAPC) performed with 32 MALDI-TOF mass spectra for each strain based on protein extraction from young and mature fungal material of *F. musae* (TMW 4.2594 and TMW 4.2595) and *F. verticillioides* (TMW 4.0943 and TMW 4.1003). $MM^1$ = Mature fungal material (10 d of incubation); $YM^2$ = Young fungal material (2 d of incubation).

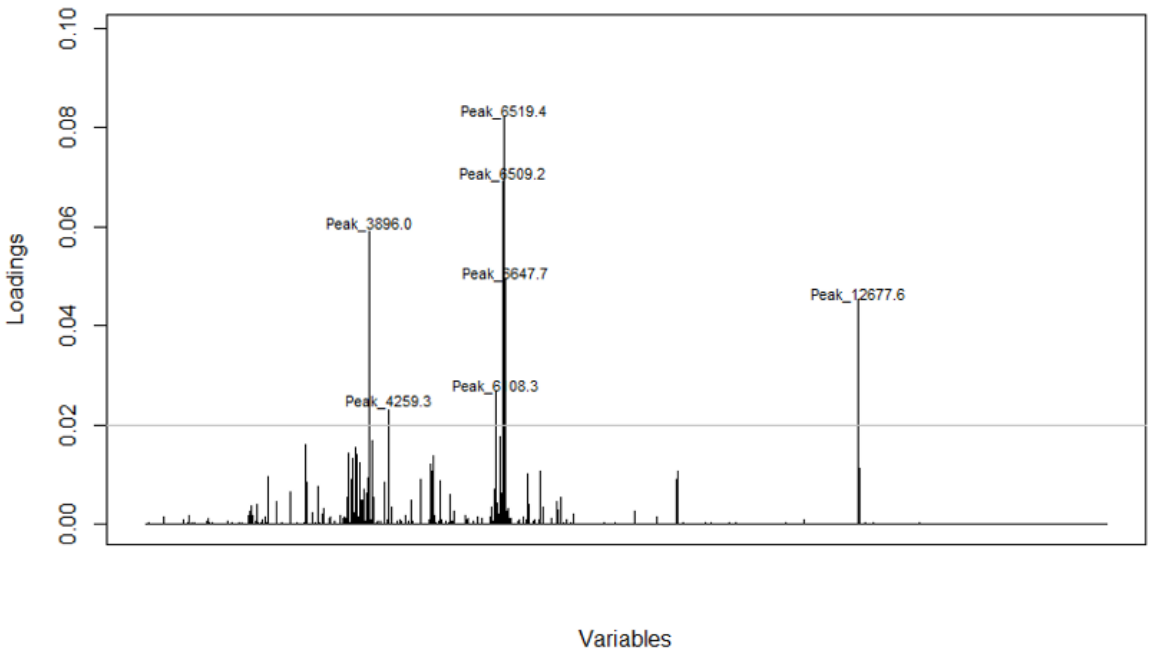


Figure 11 Illlustration of major peaks responsible for the separation of mass spectra of protein extraction from mature fungal material of *F. musae* and *F. verticillioides* obtained by MALDI-TOF MS mass spectrometry.

### 4.1.7 Incorporation of the new method for discrimination of *F. musae* into the supplementary database

For testing the integrity of the supplementary database, MALDI-TOF MS based species-level identification was performed with fresh preparations from four and three strains of *F. verticillioides* and *F. musae*, respectively, using the protocol described previously for mature fungal material (section 3.4.1.2). Each spectrum was analyzed against the newly established supplementary database and also against the oldest supplementary database including spectra of 49 *Fusarium* spp that were prepared from SDB grown mycelia (section 3.4.1.1).

Table 3 exhibits the DNA sequence-based identification for each of the seven isolates. MALDI Biotyper top scores obtained from measuring three technical replications per sample of *F. musae* TMW 4.2594, TMW 4.2595, TMW 4.2596 were 2.543, 2.588 and 2.443, respectively. The top scores for *F. verticillioides* TMW 4.0709, 4.0943, 4.1003 and 4.244 were 2.395, 2.100, 2.688 and 2.171, respectively. Of the 7 tested isolates in triplicate, 100% demonstrated correct identification with a top score between 2.1 - 2.6 for *F. verticillioides* strains and 2.4 - 2.5 for *F. musae* strains. Comparing mass spectra, it was found that for young fungal material, most of the identified peaks were distributed within a mass range of 3 - 8 kDa whereas they were distributed over a wider range of 2 to 13 kDa in mature fungal material. Taken together, the new method using mature fungal material enables unabigous discrimination of *F. musae* from 47 FFSC species, including *F. verticillioides*.

## 4.2 Detection of the presence of the *fum1* gene in *Fusarium* spp. using a LAMP assay

### 4.2.1 LAMP primer design and product verification

The PrimerExplorer V.5 software tool (http://primerexplorer.jp/e) provided by Eiken Chemical Co., Ltd. (Tokyo, Japan) was used for LAMP primer design. LAMP primers resulting from this calculation are listed in Table 6. Fig. 12 illustrates the positions and orientation of the used primers within the *fum1* genes of typical fumonisin producers. Wobble positions were introduced into primers where nucleotides varied within a given position permitting the detection of a broader spectrum of potential fumonisin-producers. The specificity of the designed primers was tested and confirmed in silico by the nucleotide BLAST search tool on the NCBI database (Altschul et al., 1990).

Table 6 List of LAMP primers used in the current study.

| Analysis | Primer name | Sequence 5' > 3' |
|---|---|---|
| LAMP | F3–fum*1* ID3 | NAT WTT CTC ACC GAC CAT GT |
| | B3–fum*1* ID3 | TCR GAG CTT GGC ATS GAC AT |
| | FIP–fum*1* ID3 | [a]TGA TTG CCT CGC CTC TTG CAT–GCA AG GMG TTT TGT CCC CAG |
| | BIP–fum*1* ID3 | [a]ATG CCC TSC GGG AYG GAG ATC–KCC GAT CCG ART TTG AAG |
| | LF–fum*1* ID3 | CCG TTT GCA TTT GCA TCA AAG GT |
| | LB–fum*1* ID3 | ATC AGG GCT CTT GTC AGG GC |
| | F2–fum*1* ID3 | GCA AG GMG TTT TGT CCC CAG |
| | B2–fum*1* ID3 | CTT CAA ART CGG ATC GGK |

[a]Hyphen indicates junction between F1c/B1c (left) and F2/B2 (right) parts of FIB/BIP primers.
N = any base, W = A or T, R = A or G, S = G or C, M = A or C, Y = C or T

LAMP products resulting from a tenfold serial dilution of purified DNA (section 3.5.1.1) of *F. verticillioides* TMW 4.0703 and *F. proliferatum* TMW 4.2485, respectively, were separated on an agarose gel (3.5.4) for confirmation of DNA amplification in positive LAMP reactions. The typical ladder-like pattern of DNA fragments only occurred in reactions that showed a color change of the indicator dye from yellow to pink (neutral red) as shown in Figure 13. To verify whether the amplified DNA corresponds with the *fum1* gene sequence, the smallest amplified DNA fragment was cut out from the gel and sequenced using primers F2-*fum1* and B2-f*um1* (see Table 6) in separate Sanger sequencing reactions. Figure 14 shows a comparison of the obtained consensus sequence with the *fum1* gene sequence of *F. verticillioides* TMW 4.0703 with the corresponding gene area from GenBank accession no. DQ384220.1as reference. The sequences of the LAMP products showed 100% identity with the reference sequences for both *F. verticillioides* TMW 4.0703 and *F.*

*proliferatum* TMW 4.2485 with the respective reference sequences providing that the used primer set binds specifically to the *fum1* gene of typical fumonisin producers.

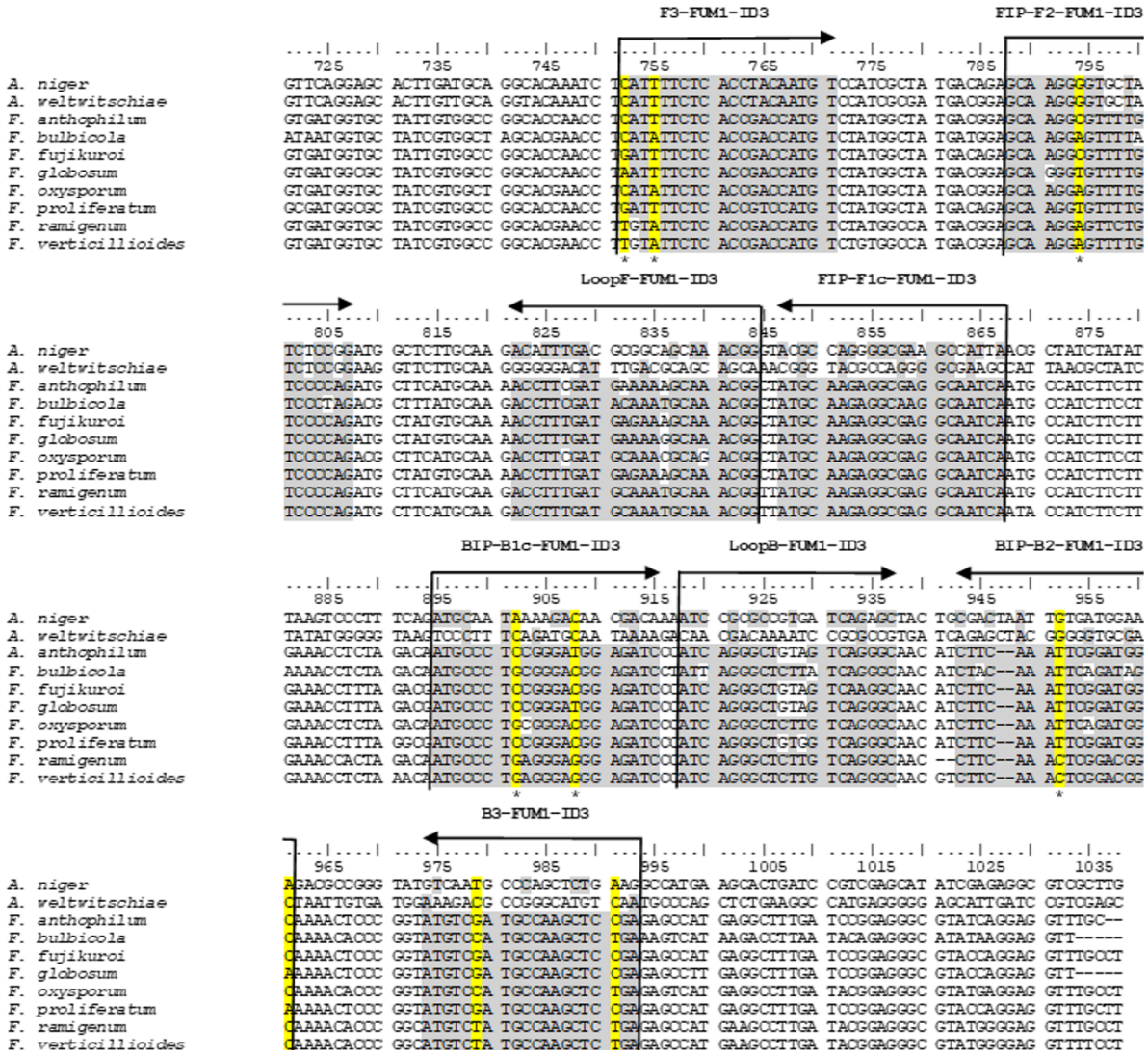


Figure 12 Multiple alignment of a partial sequence of the *fum1* gene of fumonisin producing *Fusarium* spp. and *Aspergillus* spp. Positions of LAMP primers are marked above the sequence with arrowheads indicating orientation of the primers used in the current study (→ = forward primer, ← = reverse primer). Nucleotide positions that correspond with primer sequences are highlighted in grey. Wobble positions are marked in yellow and with an asterisk (*) below the sequence. Nucleotide numbering refers to nucleotide positions in the *fum1* gene of *F. verticillioides* (GenBank accession no. DQ384220.1).

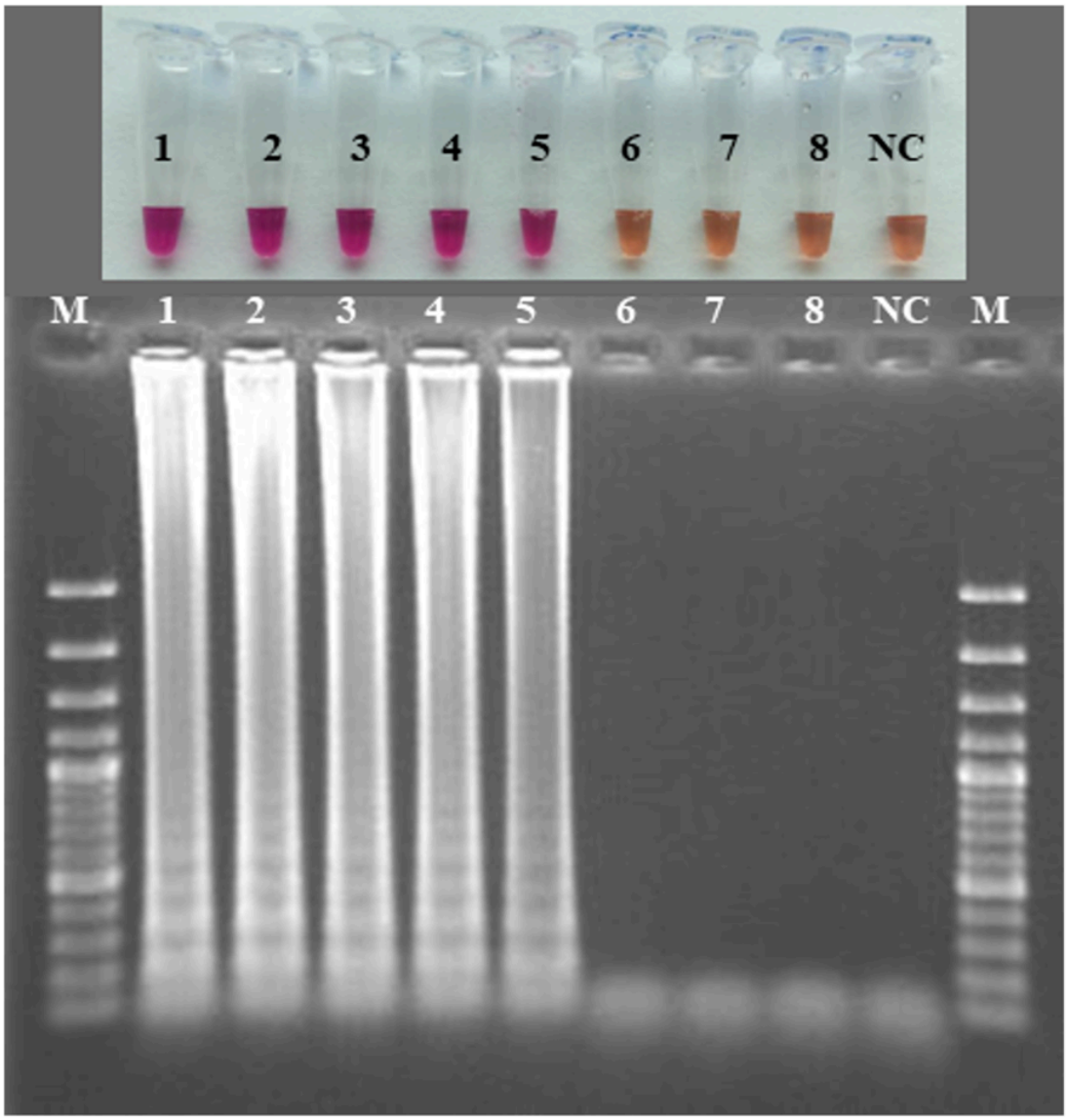


Figure 13 Analysis of LAMP amplification products by gel electrophoresis and comparison to signals in the *fum1* LAMP assay. LAMP assay with a tenfold serial dilution of purified DNA of *F. verticillioides* TMW 4.0703 ranging from 50 ng/reaction to 5 fg/reaction. 1 = 50 ng/reaction, 2 = 5 ng/reaction, 3 = 500 pg/ reaction, 4 = 50 pg/reaction, 5 = 5 pg/reaction, 6 = 500 fg/reaction, 7 = 50 fg/reaction, 8 = 5 fg/ reaction, NC = negative control using water instead of DNA. LAMP reactions were subsequently loaded to agarose gel and separated by electrophoresis. M = size marker (Gene Ruler 100 bp Plus DNA Ladder, Thermo Fisher Scientific Inc., Waltham, USA), bp = base pairs. Pink = positive reaction, orange = negative reaction.

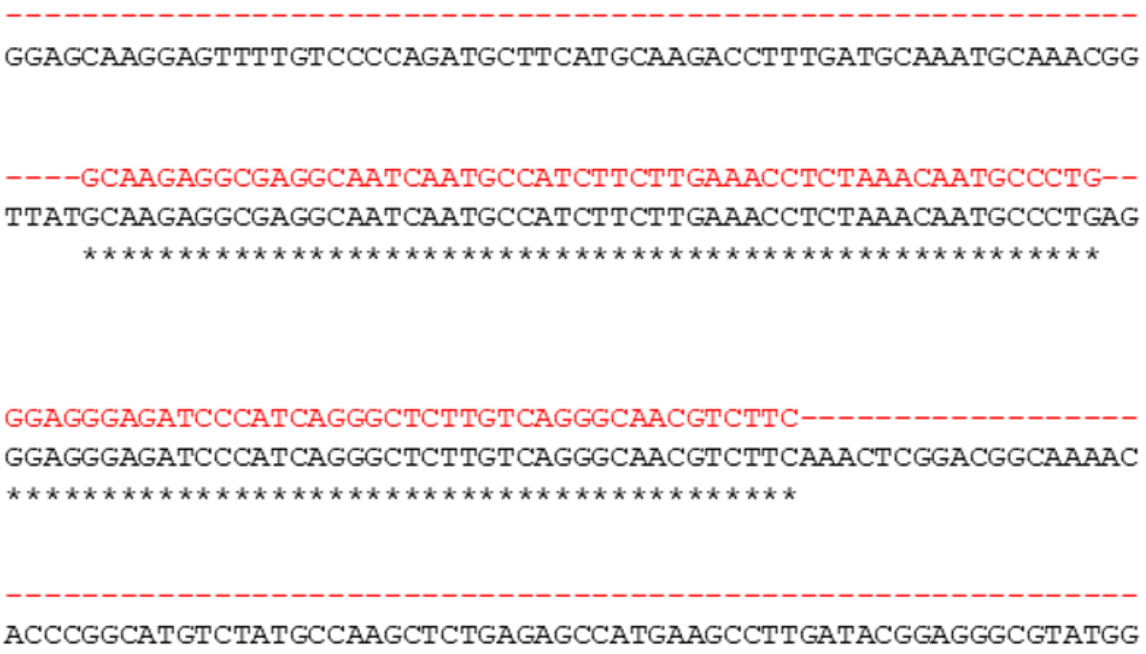


Figure 14 Sequence comparison of the *fum1* LAMP-product consensus sequence (in red) after amplification of *F. verticillioides* TMW 4.0703 gDNA with a previously accessioned *fum1* gene partial sequence from *F. verticillioides* (GenBank accession no. DQ384220.1) (in black) as reference sequence. Asterisk (*) = reference sequence match, dash (−) = no match with reference sequence.

### 4.2.2 Temperature optimization of the LAMP assay

The optimum incubation temperature was determined by submitting LAMP reactions to a temperature gradient for isothermal incubation using calcein and neutral red as indicator dyes, respectively. Purified gDNA (3.5.1.1) from *F. proliferatum* TMW 4.0952 (5 ng per reaction) was used as template and reactions were incubated at individual temperatures ranging from 60 °C to 70.5 °C in 0.86 °C increments. After 1 h (neutral red) and 1 h and 30 min (calcein), respectively, a positive signal was found in all reactions at temperatures between 60 °C and 65.5 °C with both indicators. Higher temperatures inhibited the LAMP

assay completely (Figure 15). To run the LAMP assay under highly specific conditions, 65 °C was selected for isothermal incubation in all further experiments.

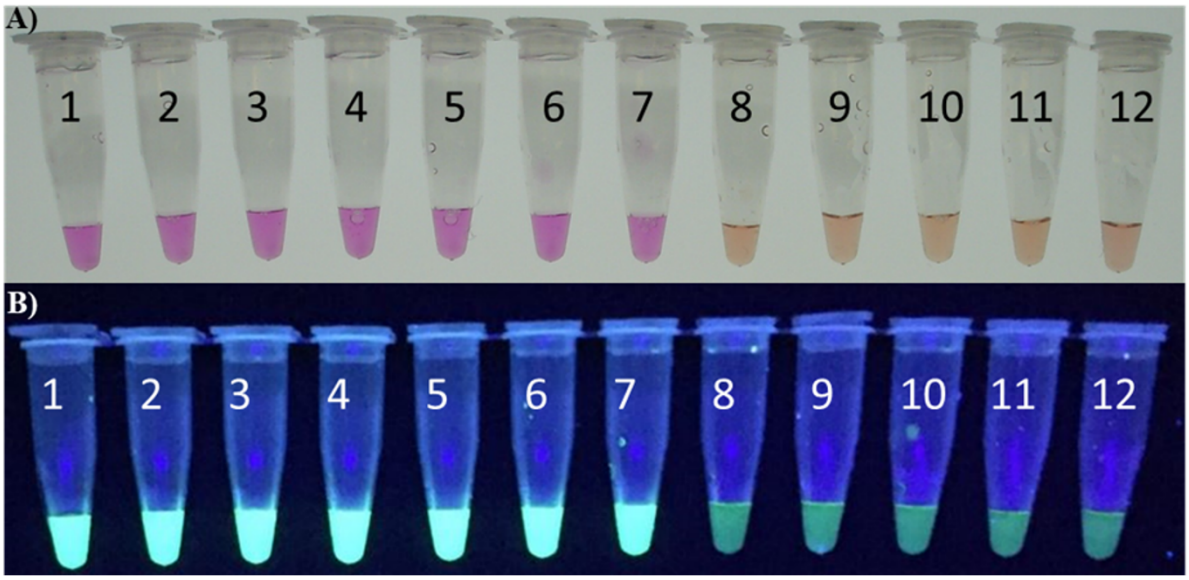


Figure 15 Temperature gradient of LAMP assay from 60 °C to 70.5 °C with purified gDNA of *F. proliferatum* TMW 4.0952 incubated at 1 = 60 °C, 2 = 60.2 °C, 3 = 60.7 °C, 4 = 61.6°C, 5 = 62.8 °C, 6 = 64.1 °C, 7 = 65.5 °C, 8 = 66.9 °C, 9 = 68.1 °C, 10 = 69.2 °C, 11 = 70°C, 12 = 70.5 °C. A) LAMP assay using neutral red as template. Pink = positive reaction, orange = negative reaction. B) LAMP assay using calcein as template under UV 366nm light. Bright green fluorescence = positive reaction.

### 4.2.3 LAMP assay specificity and sensitivity using gDNA as template

The specificity of the assay was determined by testing purified gDNA (500 ng/reaction) of 213 strains representing 158 fungal species in 27 genera using neutral red as indicator dye. A summary of results for all tested isolates can be found in Table 7 and 8. An overview of 100 fungal strains representing 47 FFSC species is given in Table 7. At least one of the tested isolates of 25 species displayed a positive LAMP reaction when analyzed with the *fum1* specific LAMP assay (*F. agapanthi*, *F. ananatum*, *F. anthophilum*, *F. brevicatenulatum*, *F. bulbicola*, *F. coicis*, *F. concentricum*, *F. dlaminii*, *F. fractiflexum*, *F.*

*fujikuroi*, *F. globosum*, *F. konzum*, *F. lactis*, *F. mundagurra*, *F. napiforme*, *F. nygamai*, *F. phyllophilum*, *F. proliferatum*, *F. ramigenum*, *F. sacchari*, *F. subglutinans*, *F. tjaetaba*, *F. tupiense*, *F. udum* and *F. verticillioides*). All FFSC isolates were tested both with calcein and neutral red as indicator dyes and the results were 100% identical between both detection methods.

The literature described 22 species of *Fusarium* sp. (Table 1) as fumonisin producers and a positive signal was detected for all strains, except for *F. acutatum*, *F. pseudocircinatum* and *F. thapsinum* and some strains of *F. anthophilum, F. dlaminii* and *F. napiforme* (Table 7). Otherwise, 12 species of *Fusarium* sp. (Table 1) are known as non-fumonisin producers, which for most of them was associated with the absence of *fum1* gene using LAMP assay (Table 7), excluding some strains of *F. agapanthi*, *F. bulbicola* and *F. sacchari*. Among the species without information in the literature about the possibility of producing fumonisins, the presence of *fum1* gene using LAMP assay was observed in: *F. coicis* (2), *F. marasasianum* (1), F. *mundagurra* (2) and *F. pininemorale* (1) and the absence of *fum1* gene in: *F. bactridioides* (2), *F. fracticaudum* (1), *F. mangiferae* (1), *F. mexicanum* (2), *F. newnesense* (2), *F. sororula* (1) and *F. werrikimbe* (1). For *F. fractiflexum*, *F. tjaetaba*, *F. tupiense* and *F. udum* only half of the strains presented LAMP positive.

The sensitivity of the developed assay in the different species was assessed by testing tenfold serial dilutions of purified gDNA from all isolates with a positive reaction in the *fum1* LAMP assay as depicted in Figure 16A. Limits of detection (LOD) ranged from 0.005 ng/reaction in isolates of *F. fujikuroi*, *F. nygamai* and *F. verticillioides* to 500 ng/reaction in *F. concentricum* (Table 7). In species, in which all isolates were tested positive, LODs often varied by a factor of 10 (*F. proliferatum*) to 100 (*F. fujikuroi*, *F. verticillioides*) between isolates. In others such as *F. coicis*, *F. globosum*, *F. konzum*, *F. mundagurra, F. phylophilum* and *F. ramigenum* the LOD was identical between tested isolates.

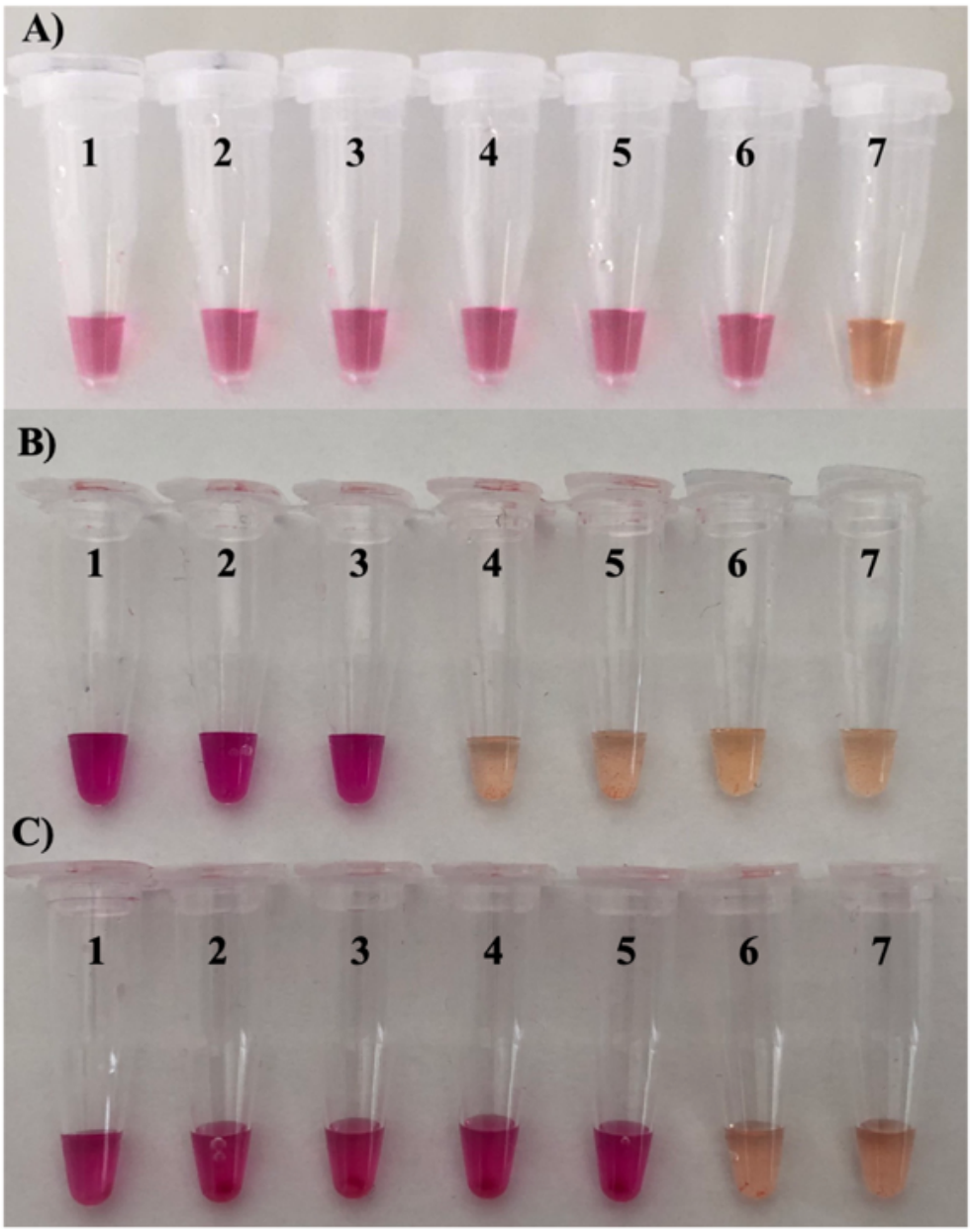


Figure 16 LAMP assay with a tenfold serial dilution of *F. verticillioides* TMW 4.0850. A) Using purified gDNA as template: 1 = 500 ng/reaction, 2 = 50 ng/reaction, 3 = 5 ng/reaction, 4 = 500 pg/reaction, 5 = 50 pg/reaction, 6 = 5 pg/reaction, 7 = 0.5 pg/ reaction. B) Using spore suspension as template: 1 = $10^7$ spores/reaction, 2 = $10^6$ spores/reaction, 3 = $10^5$ spores/reaction, 4 = $10^4$ spores/reaction, 5 = $10^3$ spores/reaction, 6 = $10^2$ spores/reaction, 7 = $10^1$ spores/reaction. C) Mechanical cell disruption treatment applied to spore suspensions as template: 1 = $10^7$ spores/reaction, 2 = $10^6$ spores/ reaction, 3 = $10^5$ spores/reaction, 4 = $10^4$ spores/ reaction, 5 = $10^3$ spores/reaction, 6 = $10^2$ spores/ reaction, 7 = $10^1$ spores/reaction.

Table 7 Strains from the *Fusarium fujikuroi* species complex (FSSC) used in the current study, their reactions in the LAMP assay with purified genomic DNA (100 ng/reaction) as template using two different indicator dyes, limits of detection and concentrations of fumonisin $B_1$ ($FB_1$) as determined by LC-MS/MS analysis. + = positive reaction, - = negative reaction, LOD = limit of detection.

| **Species** | **Strain** | **Clone** | **LAMP reaction** | | | **LC-MS/MS analysis** |
|---|---|---|---|---|---|---|
| | | | **Calcein** | **Neutral red** | **LOD [ng gDNA/reaction]** | **$FB_1$ [ng/mL]** |
| *F. agapanthi* | [a]NRRL 54463 | [b]TMW 4.2685 | + | + | 0.5 | +(5.72) |
| *F. ananatum* | [c]MRC 8167 | TMW 4.2666 | + | + | | +(6.19) |
| *F. anthophilum* | [d]BBA 62266 | TMW 4.0891 | + | + | 0.05 | |
| *F. begoniae* | [e]ITEM 3505 | TMW 4.2476 | + | + | | +(54.2) |
| *F. brevicatenulatum* | BBA 69198 | TMW 4.0806 | + | + | | +(9.50) |
| | BBA 69197 | TMW 4.1020 | + | + | 50 | |
| *F. bulbicola* | [f]DSM 62270 | TMW 4.0312 | + | + | 0.05 | +(1.73) |
| | BBA 63620 | TMW4.0946 | + | + | 50 | +(31.5) |
| | BBA 63628 | TMW 4.0951 | + | + | 5 | +(8.07) |
| *F. coicis* | [g]RBG 5368 | TMW 4.2635 | + | + | 0.5 | +(42.1) |
| | RBG 5369 | TMW 4.2636 | + | + | 0.5 | +(2.22) |
| *F. concentricum* | BBA 69856 | TMW 4.0865 | + | + | 500 | +(1.07) |
| *F. dlaminii* | [h]CBS 175.88 | TMW 4.0435 | + | + | | +(1.77) |
| | ITEM 3527 | TMW 4.1015 | + | + | 50 | |
| *F. fractiflexum* | NRRL 26794 | TMW 4.2690 | + | + | | +(3.0) |
| *F. fujikuroi* | BBA 69741 | TMW 4.0860 | + | + | 0.005 | |
| | ITEM 3155 | TMW 4.2478 | + | + | 0.5 | |
| *F. globosum* | MRC 6646 | TMW 4.0584 | + | + | 0.05 | |
| | BBA 70241 | TMW 4.0810 | + | + | 0.05 | |
| *F. konzum* | ITEM 3106 | TMW 4.2480 | + | + | 50 | |
| | ITEM 3141 | TMW 4.2481 | + | + | 50 | |
| *F. lactis* | BBA 68591 | TMW 4.1005 | + | + | 50 | +(1.39) |
| *F. marasasianum* | [i]CMW 25353 | TMW 4.2638 | + | + | | +(1.83) |
| *F. mundagurra* | RBG 5717 | TMW 4.2631 | + | + | 0.5 | +(909) |
| | RBG 5599 | TMW 4.2632 | + | + | 0.5 | +(1940) |
| *F. napiforme* | CBS 693.94 | TMW 4.0510 | + | + | 50 | |

| Species | Strain | Clone | LAMP reaction | | | LC-MS/MS analysis |
|---|---|---|---|---|---|---|
| | | | Calcein | Neutral red | LOD [ng gDNA/reaction] | $FB_1$ [ng/mL] |
| *F. nygamai* | ITEM 7600 | TMW 4.2483 | + | + | 0.05 | |
| | ITEM 7601 | TMW 4.2484 | + | + | 0.005 | |
| *F. phyllophillum* | BBA 63639 | TMW 4.0795 | + | + | 5 | |
| | BBA 70695 | TMW 4.1030 | + | + | 5 | |
| *F. pininemorale* | CMW 25243 | TMW 4.2639 | + | + | 50 | +(7.38) |
| *F. proliferatum* | BBA 63629 | TMW 4.0952 | + | + | 0.5 | |
| | ITEM 7588 | TMW 4.2475 | + | + | 0.05 | |
| | ITEM 3269 | TMW 4.2485 | + | + | 0.5 | |
| | ITEM 3272 | TMW 4.2486 | + | + | 0.05 | |
| *F. ramigenum* | BBA 68592 | TMW 4.1006 | + | + | 0.5 | |
| | BBA 68593 | TMW 4.1007 | + | + | 0.5 | |
| *F. sacchari* | BBA 63320 | TMW 4.0882 | + | + | 0.05 | +(0.99) |
| *F. subglutinans* | BBA 65921 | TMW 4.0988 | + | + | 5 | |
| | ITEM 2625 | TMW 4.2491 | + | + | 0.5 | |
| | ITEM 2860 | TMW 4.2492 | + | + | 5 | |
| *F. tjaetaba* | RBG 5363 | TMW 4.2633 | + | + | | +(8.08) |
| *F. tupiense* | CML 0263 | TMW 4.2592 | + | + | | +(3.55) |
| *F. udum* | DSM 62451 | TMW 4.0258 | + | + | 50 | +(9.19) |
| *F. verticillioides* | [j]BFE 315 | TMW 4.0703 | + | + | 0.05 | |
| | BBA 69704 | TMW 4.0850 | + | + | 0.005 | |
| | BBA 62188 | TMW 4.0943 | + | + | 0.05 | |
| | BBA 68588 | TMW 4.1003 | + | + | 0.05 | |
| *F. acutatum* | BBA 69580 | [b]TMW 4.0835 | - | - | | -(0) |
| | BBA 69718 | TMW 4.0852 | - | - | | -(0) |
| | [k]IMI 205517 | TMW 4.2515 | - | - | | -(0) |
| *F. agapanthi* | NRRL 54464 | TMW 4.2686 | - | - | | |
| *F. ananatum* | MRC 8185 | TMW 4.2664 | - | - | | +(5.01) |
| *F. andiyazi* | ITEM 6036 | TMW 4.2661 | - | - | | |
| | ITEM 6049 | TMW 4.2662 | - | - | | |
| *F. anthophilum* | CBS 222.76 | TMW 4.0490 | - | - | | -(0) |

| Species | Strain | Clone | LAMP reaction | | | LC-MS/MS analysis |
|---|---|---|---|---|---|---|
| | | | Calcein | Neutral red | LOD [ng gDNA/reaction] | $FB_1$ [ng/mL] |
| *F. bactridioides* | CBS 100057 | TMW 4.0464 | - | - | | -(0) |
| | BBA 63602 | TMW 4.0944 | - | - | | -(0) |
| *F. bulbicola* | DSM 62273 | TMW 4.0283 | - | - | | |
| *F. circinatum* | BBA 69854 | TMW 4.0863 | - | - | | |
| | CBS 122161 | TMW 4.2186 | - | - | | |
| *F. concentricum* | BBA 69855 | TMW 4.0864 | - | - | | |
| | BBA 69858 | TMW 4.0867 | - | - | | |
| *F. denticulatum* | BBA 69725 | TMW 4.0857 | - | - | | |
| | BBA 65244 | TMW 4.0980 | - | - | | |
| *F. dlaminii* | CBS 258.52 | TMW 4.0436 | - | - | | -(0) |
| | BBA 69859 | TMW 4.0868 | - | - | | -(0) |
| *F. fracticaudum* | CMW 25237 | TMW 4.2637 | - | - | | +(15.6) |
| *F. fractiflexum* | NRRL 28852 | TMW 4.2689 | - | - | | +(2.97) |
| *F. guttiforme* | BBA 69661 | TMW 4.0844 | - | - | | |
| | BBA 69665 | TMW 4.0847 | - | - | | |
| *F. lactis* | BBA 68590 | TMW 4.1004 | - | - | | |
| *F. mangiferae* | ITEM 7646 | TMW 4.2482 | - | - | | NA |
| *F. mexicanum* | [l]CML 2585 | TMW 4.2588 | - | - | | -(0) |
| | CML 0411 | TMW 4.2589 | - | - | | -(0) |
| *F. musae* | ITEM 1113 | TMW 4.2594 | - | - | | |
| | ITEM 1245 | TMW 4.2596 | - | - | | |
| *F. napiforme* | BBA 67630 | TMW 4.0907 | - | - | | -(0) |
| *F. newnesense* | RBG 5444 | TMW 4.2629 | - | - | | +(6.9) |
| | RBG 5445 | TMW 4.2630 | - | - | | -(0) |
| *F. pseudoanthophilum* | BBA 70129 | TMW 4.0808 | - | - | | |
| | BBA 69003 | TMW 4.1010 | - | - | | |
| *F. pseudocircinatum* | BBA 69598 | TMW 4.0842 | - | - | | -(0) |
| | BBA 69636 | TMW 4.0843 | - | - | | -(0) |
| *F. pseudonygamai* | BBA 69551 | TMW 4.0829 | - | - | | |

| Species | Strain | Clone | LAMP reaction | | | LC-MS/MS analysis |
|---|---|---|---|---|---|---|
| | | | Calcein | Neutral red | LOD [ng gDNA/reaction] | $FB_1$ [ng/mL] |
| | BBA 69131 | TMW 4.1019 | - | - | | |
| *F. sacchari* | BBA 63340 | TMW 4.0883 | - | - | | |
| *F. sororula* | CMW 25517 | TMW 4.2640 | - | - | | +(23.3) |
| *F. secorum* | [m]1009-3-1 | TMW 4.2626 | - | - | | |
| | [m]FS12-4a | TMW4.2627 | - | - | | |
| *F. sterilihyphosum* | ITEM 7647 | TMW 4.2490 | - | - | | |
| *F. subglutinans* | BBA 62275 | TMW 4.0892 | - | - | | +(7.49) |
| | BBA 63621 | TMW 4.0947 | - | - | | -(0) |
| *F. succisae* | DSM 63162 | TMW 4.0309 | - | - | | |
| *F. thapsinum* | BBA 69582 | TMW 4.0837 | - | - | | -(0) |
| | BBA 69584 | TMW 4.0839 | - | - | | -(1.93) |
| *F. tjaetaba* | RBG 5364 | TMW 4.2634 | - | - | | +(35.8) |
| *F. tupiense* | CML 0389 | TMW 4.2591 | - | - | | -(0) |
| *F. udum* | BBA 65058 | TMW 4.0899 | - | - | | +(6.88) |
| *F. werrikimbe* | NRRL 54250 | TMW 4.2694 | - | - | | +(22.3) |

[a]NRRL = Northern Regional Research Laboratory, Peoria (Illinois), USA
[b]TMW= Lehrstuhl für Technische Mikrobiologie Weihenstephan, Technical University of Munich, Freising, DE
[c]MRC = South African Medical Research Council, Tygerberg, SA
[d]BBA = Julius-Kühn-Institut, Bundesforschungsinstitut für Kulturpflanzen, Berlin, DE
[e]ITEM = Istituto Tossine e Micotossine da Parassiti Vegetali, CNR, Bari, IT
[f]DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen, Darmstadt, DE
[g]RBG = Royal Botanic Gardens Victoria, AUS
[h]CBS = Westerdijik Fungal Biodiversity Institute Utrecht, NL
[i]CMW = Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa
[j]BFE = Max-Rubner-Institut, Bundeserforschungsanstalt für Ernährung, Karlsruhe, DE
[k]IMI = Institute of Microbiology and Infection, Birmingham, UK
[l]CML = Coleção micológica de Lavras, Minas Gerais, BR
[m]Collection of Melvin Bolten, Sugarbeet and Potato Research, USDA, ARS, NCSL, Fargo, USA

Table 8 Fungal strains used in the current study, their reactions in the LAMP assay with purified genomic DNA (100 ng/reaction) as template using two different indicator dyes and sensitivity analyses of positive LAMP reactions. + = positive reaction, - = negative reaction, n.d. = not determined.

| **Genus** | **Species** | **Strain** | **Clone** | **LAMP assay** | |
|---|---|---|---|---|---|
| | | | | **Calcein** | **Neutral red** |
| *Alternaria* spp. | *A. alternata* | [a]CBS 106.24 | TMW 4.1406 | *n.d. | - |
| | *A. mali* | [b]TMW 4.1428 | TMW 4.1428 | n.d. | - |
| *Aspergillus* spp. | *A. aculeatus* | TMW 4.1776 | TMW 4.1776 | n.d. | - |
| | *A. arachidicola* | [c]IBT 27128 | TMW 4.2204 | - | - |
| | *A. auricomus* | CBS 467.65 | TMW 4.1631 | n.d. | - |
| | *A. awamori* | CBS 101704 | TMW 4.1066 | n.d. | - |
| | *A. bombycis* | IBT 23536 | TMW 4.2210 | n.d. | - |
| | *A. bridgeri* | CBS 350.81 | TMW 4.1632 | n.d. | - |
| | *A. caelatus* | IBT 29700 | TMW 4.2209 | n.d. | - |
| | *A. carbonarius* | TMW 4.1512 | TMW 4.1512 | n.d. | - |
| | *A. clavatus* | CBS 513.65 | TMW 4.1086 | n.d. | - |
| | *A. elegans* | CBS 310.80 | TMW 4.1633 | n.d. | - |
| | *A. ellipticus* | CBS 707.79 | TMW 4.1629 | n.d. | - |
| | *A. flavus* | TMW 4.1859 | TMW 4.1859 | n.d. | - |
| | *A. foetidus* | CBS 114.49 | TMW 4.1628 | n.d. | - |
| | *A. fresenii* | CBS 550.65 | TMW 4.1067 | n.d. | - |
| | *A. fumigatus* | CBS 113.55 | TMW 4.0623 | n.d. | - |
| | *A. helicothrix* | CBS 677.79 | TMW 4.1630 | n.d. | - |
| | *A. heteromorphus* | CBS 117.55 | TMW 4.1626 | n.d. | - |
| | *A. insulicola* | CBS 382.75 | TMW 4.1634 | n.d. | - |
| | *A. japonicus* | CBS 114.51 | TMW 4.1627 | n.d. | - |
| | *A. minisclerotigenes* | IBT 27177 | TMW 4.2205 | n.d. | - |
| | *A. niger* | CBS 101698 | TMW 4.1068 | n.d. | - |

| | | | | | |
|---|---|---|---|---|---|
| | *A. nomius* | CBS 260.88 | TMW 4.1960 | n.d. | - |
| | *A. ochraceoroseus* | CBS 101887 | TMW 4.1772 | n.d. | - |
| | *A. ochraceus* | CBS 263.67 | TMW 4.0706 | n.d. | - |
| | *A. oryzae* | IBT 28103 | TMW 4.2208 | n.d. | - |
| | *A. parasiticus* | CBS 126.62 | TMW 4.1768 | n.d. | - |
| | *A. parvisclerotigenes* | IBT 3850 | TMW 4.2205 | n.d. | - |
| | *A. petrakii* | CBS 105.57 | TMW 4.1087 | n.d. | - |
| | *A. pseudotamarii* | IBT 21092 | TMW 4.2212 | n.d. | - |
| | *A. rambelli* | IBT 14580 | TMW 4.2211 | n.d. | - |
| | *A. sclerotiorum* | CBS 549.65 | TMW 4.1089 | n.d. | - |
| | *A. sojae* | IBT 21643 | TMW 4.2207 | n.d. | - |
| | *A. tamarii* | CBS 591.68 | TMW 4.1771 | n.d. | - |
| | *A. terreus* | CBS 377.64 | TMW 4.1060 | n.d. | - |
| | *A. toxicarus* | CBS 822.72 | TMW 4.1766 | n.d. | - |
| | *A. tubingensis* | [d]ITEM 4496 | TMW 4.2008 | n.d. | - |
| | *A. usamii var. shiro-usamii* | CBS 101700 | TMW 4.1072 | n.d. | - |
| *Aureibasidium* spp. | *A. pullulans* | TMW 4.2253 | TMW 4.2253 | n.d. | - |
| *Bipolaris* spp. | *B. sorokiniana* | CBS311.64 | TMW 4.0509 | n.d. | - |
| *Cladobotryum* spp. | *C. dendroides* | [e]NRRL 2903 | TMW 4.0467 | n.d. | - |
| *Cladosporium* spp. | *C. sphaerospermum* | TMW 4.2370 | TMW 4.2370 | n.d. | - |
| *Colletotrichum* spp. | *C. acutatum* | CBS 295.67 | TMW 4.0652 | n.d. | - |
| | *C. fragariae* | CBS 142.31 | TMW 4.0651 | n.d. | - |
| *Cryptomela* spp. | *C. acutispora* | CBS 157.33 | TMW 4.1620 | n.d. | - |
| *Drechslera* spp. | *D. teres* | CBS 378.59 | TMW 4.0558 | n.d. | - |
| | *D. tricici-repentis* | CBS 265.80 | TMW 4.0559 | n.d. | - |
| *Emericella* spp. | *E. astellata* | IBT 21903 | TMW 4.2202 | n.d. | - |
| | *E. olivicola* | IBT 26499 | TMW 4.2201 | n.d. | - |
| | *E. venezuelensis* | IBT 20956 | TMW 4.2203 | n.d. | - |

| | | | | | |
|---|---|---|---|---|---|
| *Epicoccum* spp. | *E. nigrum* | TMW 4.1407 | TMW 4.1407 | n.d. | - |
| *Fusarium* spp. | *F. acuminatum* | CBS 485.94 | TMW 4.0701 | n.d. | - |
| | *F. avenaceum* | [f]DSM 62161 | TMW 4.0140 | n.d. | - |
| | *F. beomiforme* | [g]BBA 69406 | TMW 4.0513 | n.d. | - |
| | *F. cerealis* | CBS 589.93 | TMW 4.0406 | n.d. | - |
| | *F. chlamydosporum* | CBS 145.25 | TMW 4.0404 | n.d. | - |
| | *F. compactum* | CBS 466.92 | TMW 4.0433 | n.d. | - |
| | *F. culmorum* | DSM 62191 | TMW 4.0149 | n.d. | - |
| | *F. dimerum* | CBS 175.31 | TMW 4.0626 | n.d. | - |
| | *F. equiseti* | CBS 406.86 | TMW 4.0477 | n.d. | - |
| | *F. eumartii* | DSM 62809 | TMW 4.0303 | n.d. | - |
| | *F. heterosporum* | DSM 62231 | TMW 4.0224 | n.d. | - |
| | *F. longipes* | CBS 739.79 | TMW 4.0350 | n.d. | - |
| | *F. melanochlorum* | CBS 202.65 | TMW 4.0625 | n.d. | - |
| | *F. oxysporum* | DSM 62292 | TMW 4.0163 | - | - |
| | *F. oxysporum* f. sp. *dianthi* | CBS 310.87 | TMW 4.0113 | - | - |
| | | [h]LN 1.09 | TMW 4.0148 | - | - |
| | *F. scirpi* | CBS 448.84 | TMW 4.0410 | n.d. | - |
| | *F. solani* | DSM 62416 | TMW 4.0255 | n.d. | - |
| | *F. torulosum* | BBA 64465 | TMW 4.0437 | | - |
| *Geotrichum* spp. | *G. candidum* | TMW 4.0508 | TMW 4.0508 | n.d. | - |
| *Geomyces* spp. | *G. panorum* | BBA 66108 | TMW 4.0902 | n.d. | - |
| *Hypomyces* spp. | *H. rosellus* | CBS 521.81 | TMW 4.0400 | n.d. | - |
| *Memnoniella* spp. | *M. echinata* | CBS 627.61 | TMW 4.0711 | n.d. | - |
| *Microdochium* spp. | *M. majus* | TMW 4.0496 | TMW 4.0496 | n.d. | - |
| | *M. nivale* | TMW 4.0495 | TMW 4.0495 | n.d. | - |
| *Monascus* spp. | *M. ruber* | TMW 4.1426 | TMW 4.1426 | n.d. | - |
| *Mucor* spp. | *M. mucedo* | DSM 809 | TMW 4.0441 | n.d. | - |

| | | | | | |
|---|---|---|---|---|---|
| *Myrothecium* spp. | *M. roridum* | CBS 331.51 | TMW 4.0668 | n.d. | - |
| *Penicillium* spp. | *P. brevicompactum* | TMW 4.2279 | TMW 4.2279 | n.d. | - |
| | *P. camembertii* | DSM 1233 | TMW 4.0442 | n.d. | - |
| | *P. commune* | CBS 311.48 | TMW 4.1088 | n.d. | - |
| | *P. commune* | TMW 4.2270 | TMW 4.2270 | n.d. | - |
| | *P. crustosum* | CBS 499.73 | TMW 4.1080 | n.d. | - |
| | *P. expansum* | DSM 62841 | TMW 4.0466 | n.d. | - |
| | *P. glabrum* | TMW 4.2027 | TMW 4.2027 | - | - |
| | | [i]MUM 14.33 | TMW 4.2531 | - | - |
| | | TMW 4.2918 | TMW 4.2918 | - | - |
| | *P. janthinellum* | TMW 4.2318 | TMW 4.2318 | n.d. | - |
| | *P. jensenii* | TMW 4.2316 | TMW 4.2316 | n.d. | - |
| | *P. italicum* | DSM 62846 | TMW 4.1084 | n.d. | - |
| | *P. nalgiovense* | TMW 4.1371 | TMW 4.1371 | n.d. | - |
| | *P. nordicum* | [j]BFE 487 | TMW 4.2213 | n.d. | - |
| | *P. nordicum* | TMW 4.2271 | TMW 4.2271 | n.d. | - |
| | *P. olsonii* | TMW 4.1362 | TMW 4.1362 | n.d. | - |
| | *P. purpurescens* | CBS 223.28 | TMW 4.1082 | n.d. | - |
| | *P. purpurogenum* | CBS 286.36 | TMW 4.1079 | n.d. | - |
| | *P. roseopurpureum* | TMW 4.1770 | TMW 4.1770 | n.d. | - |
| | *P. rugulosum* | TMW 4.1902 | TMW 4.1902 | n.d. | - |
| | *P. stoloniferum* | TMW 4.2280 | TMW 4.2280 | n.d. | - |
| | *P. variabile* | CBS 385.48 | TMW 4.1081 | n.d. | - |
| | *P. verrucosum* | CBS 603.74 | TMW 4.1073 | n.d. | - |
| | *P. waksmanii* | TMW 4.2317 | TMW 4.2317 | n.d. | - |
| *Pseudogymnoascus* spp. | *P. destructans* | [k]OT-38-2010 | TMW 4.2509 | n.d. | - |
| *Stachybotrys* spp. | *S. chartarum* | [l]sp 2682 | TMW 4.0523 | n.d. | - |
| *Scopulariopsis* spp. | *S. acremonioides* | TMW 4.2366 | TMW 4.2366 | n.d. | - |

| | | | | | |
|---|---|---|---|---|---|
| *Trichoderma* spp. | *T. harzianum* | TMW 4.1502 | TMW 4.1502 | n.d. | - |
| | *T. virens* | CBS 344.47 | TMW 4.0710 | n.d. | - |
| *Trichothecium* spp. | *T. roseum* | CBS 5567.50 | TMW 4.0691 | n.d. | - |
| *Zygosaccharomyves* spp. | *Z. bailii* | DSM 70834 | TMW 3.058 | n.d. | - |
| | *Z. bisporus* | TMW 3.062 | TMW 3.062 | n.d. | - |
| | *Z. rouxii* | DSM 2531 | TMW 3.057 | n.d. | - |

*n.d. = not determined
[a]CBS = Westerdijik Fungal Biodiversity Institute Utrecht, NL
[b]TMW = Lehrstuhl für Technische Mikrobiologie Weihenstephan, Technical University of Munich, Freising, DE
[c]IBT = Institut for Bioteknologi strain collection at DTU Bioengineering in Denmark
[d]ITEM = Istituto Tossine e Micotossine da Parassiti Vegetali, CNR, Bari, IT
[e]NRRL = Northern Regional Research Laboratory, Peoria (Illinois), USA
[f]DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen, Darmstadt, DE
[g]BBA = Julius Kühn Institut, Bundesforschungsinstitut für Kulturpflanzen, Berlin, DE
[h]LN = Isolated from Prof. Dr. Ludwig Niessen, TMW
[i]MUM = Micoteca da Universidade de Minho - Portugal
[j]BFE = Max Rubner Institut, Bundeserforschungsanstalt für Ernährung, Karlsruhe, DE
[k]OT = Leibnitz Institute for Zoo and Wildlife Research (IZW), Berlin, DE
[l]sp = Lehrstuhl für Tierhygiene, Ludwigs-Maximilian-Universität, München, DE.

### 4.2.4 LAMP assay specificity and sensitivity using spore suspensions as template

In addition to testing purified gDNA, a serially diluted spore suspension (section 3.5.1.3) of *F. verticillioides* TMW 4.0850 ranging from $1\times10^7$ to $1\times10^1$ spores per reaction was tested by direct addition to the LAMP master mix prior to incubation at 65 °C. Results shown in Fig. 16B demonstrate that positive reactions were visible with a template concentration of $10^5$ spores per reaction. Further experiments showed that the sensitivity is increased to an LOD of 10 spores per reaction when approximately 200 mg of glass beads (1.25–1.65 mm) are used to support mechanical disruption by vortexing samples for 10 min prior to LAMP analysis (see Fig. 16C).

### 4.2.5 Fumonisin production potential by LC-MS/MS analysis

In order to check for a correlation between LAMP-assay results and the ability to produce fumonisins (Table 7, the literature was checked for reports of fumonisin production for each FSSC species (Table 1). Strains of species that were either unaccounted for fumonisin production in the literature (see Table 1 “na”) or which had a LAMP-result that did not match literature data (n = 48) were analyzed for $FB_1$ production using LC-MS/MS analysis. Forty-eight strains of *Fusarium* spp. were incubated at 25 °C with shaking at 180 rpm for four weeks in 100 mL Erlenmeyer flasks containing 50 mL of fumonisin-inducing liquid medium (section 3.2.4) inoculated with 2 agar disks (1 cm diameter each) from 7-day-old cultures grown on SNA solid medium (section 3.2.1) and following the sample preparation on section 3.6.1.

Results given in the right column in Table 7 show that six of the strains did not show a LAMP signal even though $FB_1$ was detected by LC-MS/MS analyses in concentrations above the LOD of 5.25 ng/mL growth medium (false negative results). Analysis of species for which no literature data on fumonisin production are currently available (Table 1) showed that *F. coicis*, *F. fracticaudum*, *F. mundagurra*, *F. newnesense*, *F. pininemorale*, *F. sororula, F. tjaetaba, F. udum* and *F. werrikimbe* displayed production of $FB_1$ in at least one of the tested isolates, respectively. No $FB_1$ production (< 5.25 ppb) was found in neither strain of *F. acutatum* (3/3), *F. bactridioides* (2/2), *F. fractiflexum* (2/2), *F. marasasianum* (1/1), *F. mexicanum* (2/2), *F. pseudocircinatum* (2/2), *F. thapsinum* (2/2) and *F. tupiense* (2/2).

Testing of species such as *F. agapanthi*, *F. bulbicola* and *F. sacchari* that have been described as non-producers in the literature showed that some of the tested strains were able to produce the toxin in small concentrations, which was in correspondence with LAMP results. The same was true for species such as *F. acutatum*, *F. anthophilum*, *F. dlaminii*, *F. napiforme*, *F. pseudocircinatum* and *F. thapsinum* that were described as fumonisin producers in the literature. In these species, non-producing strains tested negative in the LAMP-assay. All in all, comparison of LAMP results with fumonisin production in 47 FFSC species (100 strains) showed 8 % false positive results and 6 % false negatives.

### 4.2.6 LAMP assay using naturally contaminated samples as template

In order to demonstrate the usefulness of the developed LAMP assay for the analysis of naturally contaminated materials, 21 maize samples and maize based products were tested with the developed LAMP assay in triplicate analyses. Two samples, tortilla and corn flakes, were used as blank (negative controls). Surface washings of samples were prepared as

described in section 3.5.1.4. The debris was pelleted, washed and suspended in sterile distilled water. Five microliters of the suspension were added as template to the LAMP master mix and incubated at 65 °C. Table 9 displays the fumonisin levels (ppb) analyzed by high performance liquid chromatography (HPLC MS/MS) in 21 samples (section 3.6.2) collected in Argentina and Germany in comparison with the results of LAMP analysis. Fumonisin concentrations of samples ranged from 5260 to 40 ppb of total fumonisins content. Regardless of concentrations, all naturally contaminated maize samples were positive in the *fum1* LAMP assay demonstrating that the presence of fumonisin producers can be detected even at low levels of fumonisin contamination.

Table 9 Results of the LAMP assay with naturally contaminated maize and maize based products in comparison with fumonisin HPLC analysis.

| **Sample Number** | **Maize samples** | **Origin** | **$FB_1$ (ppb)** | **$FB_2$ (ppb)** | **$FB_3$ (ppb)** | **Total fumonisins (ppb)** | **LAMP assay** |
|---|---|---|---|---|---|---|---|
| 1 | Unground grains | Argentina | NA | NA | NA | 110 | + |
| 2 | Unground grains | Argentina | NA | NA | NA | 3730 | + |
| 3 | Unground grains | Argentina | NA | NA | NA | 550 | + |
| 4 | Unground grains | Argentina | NA | NA | NA | 2160 | + |
| 5 | Unground grains | Argentina | NA | NA | NA | 5260 | + |
| 6 | Unground grains | Argentina | NA | NA | NA | 1500 | + |
| 7 | Unground grains | Argentina | NA | NA | NA | 350 | + |
| 8 | Unground grains | Argentina | NA | NA | NA | 670 | + |
| 9 | Unground grains | Argentina | NA | NA | NA | 40* | + |
| 10 | Unground grains | Argentina | NA | NA | NA | 1470 | + |
| 11 | Ground Maize flour | BfR[1] | 240 | 40 | 10 | NA | + |
| 12 | Ground Popcorn | BfR | 1300 | 400 | NA | NA | + |
| 13 | Ground Corn Flakes | BfR | 260 | 40 | NA | NA | + |
| 14 | Ground Maize Chips | BfR | 160 | NA | NA | NA | + |
| 15 | Ground Honey Nuts | BfR | 180 | 15 | NA | NA | + |
| 16 | Ground Maize Flour | BfR | 400 | NA | NA | NA | + |
| 17 | Unground grains | BfR | 1100 | NA | NA | NA | + |
| 18 | Unground grains | BfR | 600 | NA | NA | NA | + |
| 19 | Unground grains | BfR | 4300 | NA | NA | NA | + |
| 20 | Ground Tortilla | BfR | Blank | Blank | Blank | Blank | - |
| 21 | Ground Corn Flakes | BfR | Blank | Blank | Blank | Blank | - |

[1]Federal Institute for Risk Assessment
*Detection limit of LAMP assay.

### 4.3 Detection of the *fum1* gene expression in *F. verticillioides* strains using RT-LAMP assay

#### 4.3.1 Design of RT-LAMP assay primers and optimization

LAMP primers were constructed in a way that allowed selective amplification of cDNA of the *fum1* gene in *F. verticillioides* (GenBank accession AF155773.5). Selective amplification was achieved by designing the F2 part of the forward inner primer (FIP-RT-*fum1*) to bind in a region of the cDNA that contained the 5′and 3′ flanks of the coding sequence after splicing of intron no. 4 (nt 27971-28033 in the AF15573 gDNA sequence) from the pre-messenger RNA. The remaining LAMP primers as well as the loop primers were designed by the Primer Explorer V5 software with primer F2 as fixes primer.

Table 10 List of RT-LAMP primers developed in the current study.

| Analysis | Primer name | Sequence 5' > 3' |
|---|---|---|
| RT-LAMP | F3 - *fum1* | TCA CAA TCG AAC CTC GAT G |
| | B3 - *fum1* | CGA GAT GGT TAT CTG CGT GG |
| | FIP - *fum1* | [a]GAACCCTCTAACCCGATGCCAA-CGCAGGATTGAATTTCCGGGA |
| | BIP - *fum1* | [a]CATCACAAAGGTCGGAGCAGGT-GCAGTTGTCGGCAAGGTAG |
| | LF - *fum1* | CAG TTG CTT CGA CGA CAC C |
| | LB - *fum1* | AAG TTC CAG CCT GGA GAC C |
| | F2 - *fum1* | CGC AGG ATT GAA TTT CCG GGA |
| | B2 - *fum1* | CTA CCT TGC CGA CAA CTG C |

[a]Hyphen indicates junction between F1c/B1c (left) and F2/B2 (right) parts of FIB/BIP primers.

Figure 17 shows the positions and orientations of the used RT-LAMP primers within the cDNA sequence of typical fumonisin producers. The strain *F. verticillioides* TMW 4.0709 was cultured in 500 mL Erlenmeyer flasks containing 110 mL of the following

fumonisin-inducing liquid medium (section 3.2.4). The culture was inoculated with 3 agar disks from 5 days-old SNA cultures (section 3.2.1) and incubated at 25 °C under static conditions for 7 days. After the cDNA sample preparation (3.5.1.2), the specificity of the designed primers was confirmed *in silico* by the nucleotides BLAST search tool on the NCBI database (Altschul et al., 1990). LAMP products resulting from purified cDNA of *F. verticillioides* TMW 4.0709 were separated on an agarose gel (section 3.5.4) for confirmation of amplification in positive LAMP reactions. To verify whether the amplified cDNA corresponds with the *fum1* gene sequence, the smallest amplified cDNA fragment was cut out from the gel and sequenced using primers F2- RT- *fum1* and B2- RT- *fum1* (see Table 10) in separate Sanger sequencing reactions. The LAMP product sequence was 100 % identic with the reference sequence for *F. verticillioides* TMW 4.0709 suggesting that the used primer set binds specially to the *fum1* gene of typical fumonisin producers.

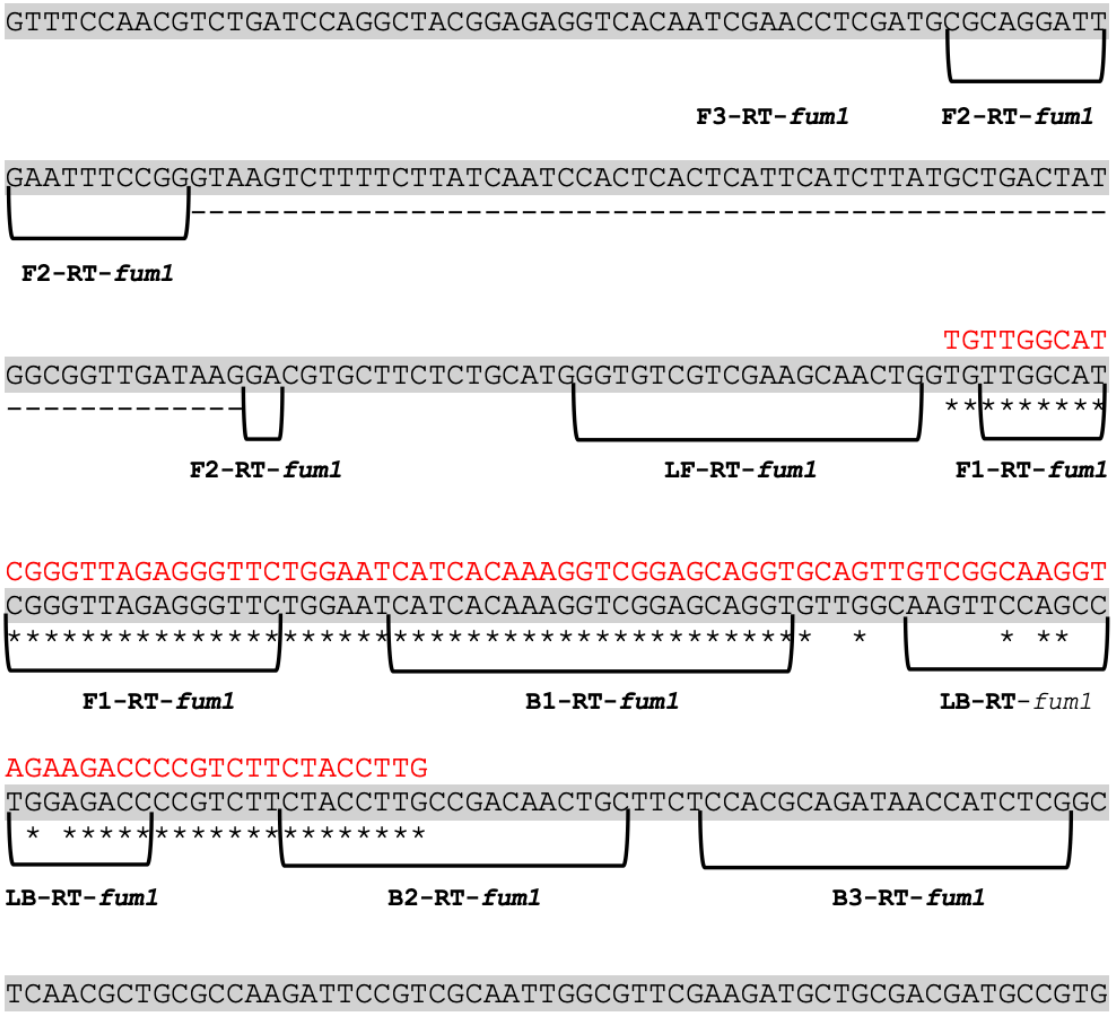


Figure 17 Illustration of binding sites of RT-LAMP primer set *fum1* within the whole genome partial sequence (NCBI reference sequence: GenBank AF155773.5) of the *F. verticillioides fum1* gene and sequence comparison of the *fum1* RT-LAMP-product consensus sequence (in red). The F2-RT-*fum1* is designed in order to bridge intronic sequences (***reference sequence match, --- intron sequence).

In order to optimize the incubation temperature of the RT-LAMP assay, the same concentrations of gDNA and cDNA as template were incubated at different constant temperatures for 40 minutes (Figure 18). The genomic DNA of *F. verticillioides* TMW 4.0709 was treated for 1 hour at 37 °C with RNase ONE™ Ribonuclease to remove residual RNA. The cDNA template was prepared as described previously. Figure 18 shows the results

of the incubation in a temperature gradient between 62 to 68.3 °C. No positive signal was observed for the RNA-free gDNA of TMW 4.0709. However, all temperatures of incubation showed positive signals after 40 minutes of incubation using cDNA as template. The first signal was detected after 20 minutes at 63, 64, 65 and 66 °C. To run the RT-LAMP assay under highly specific conditions, 65 °C was selected for isothermal incubation in all further experiments.

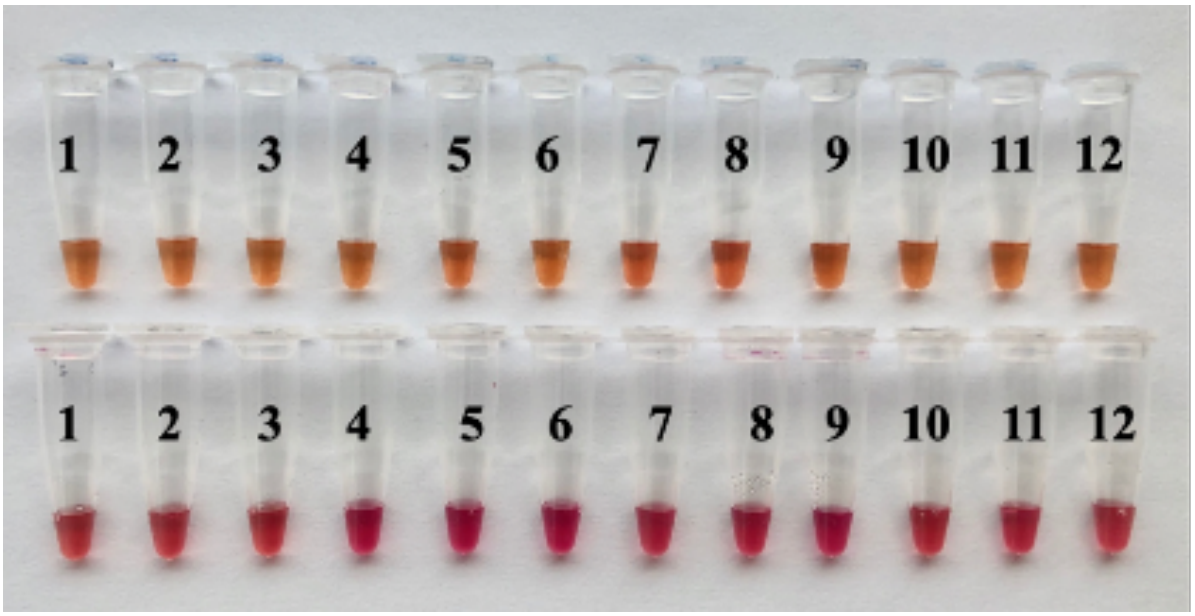


Figure 18 Temperature gradient of RT-LAMP assay from 62 °C to 68.3 °C with purified gDNA digested with RNAse One Ribonuclease (upper tubes) and cDNA (lower tubes) of *Fusarium verticillioides* TMW 4.0709 incubated during 40 minutes at 1= 62 °C, 2= 62.1 °C, 3= 62.5 °C, 4= 63 °C, 5= 63.7 °C, 6= 64.5 °C, 7= 65.3 °C, 8= 66.1 °C, 9= 66.9 °C, 10= 67.6 °C, 11 = 68 °C, 12 = 68.3 °C. Pink = positive reaction, orange = negative reaction.

### 4.3.2 Preliminary analyses using reverse transcription PCR

Before the sample was analyzed by RT-LAMP assay, RT-PCR analyses were performed using ß-tubulin gene and *fum1* gene specific primers (Table 2). Templates of gDNA and cDNA from *F. verticillioides* TMW 4.0709 was prepared as described in section

3.2.5 and incubated for 2, 3, 4 and 5 days. Results shown in Figure 19 demonstrated that mRNA was successfully transcribed into cDNA in all samples using ß-tubulin gene specific primers (see Figure 19A). Templates of gDNA and cDNA were clearly discernible by their different size. Also, the PCR products obtained with *fum1* gene specific primers had a clearly different length when gDNA or *fum1* specific cDNA were used as templates, respectively (see Figure 19B).

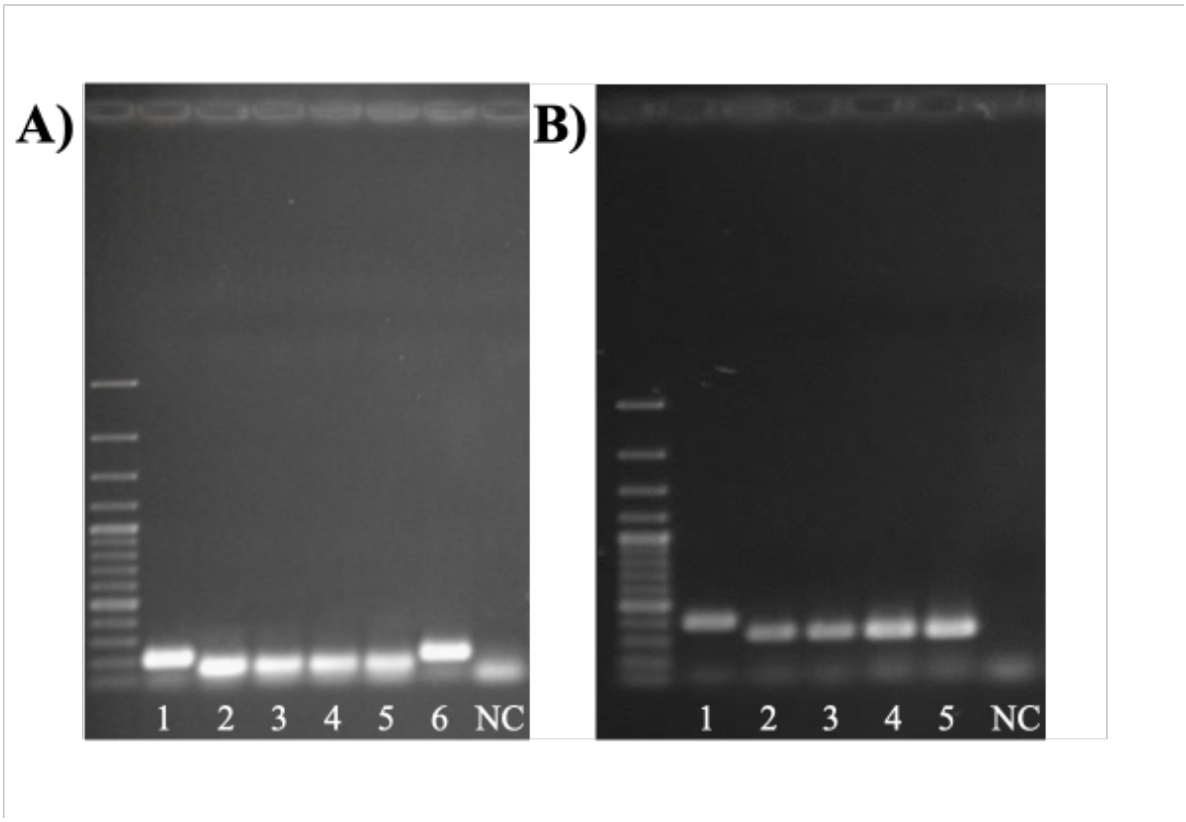


Figure 19 Analysis of RT-PCR ß-tubulin gene (19A) and RT-PCR *fum1* (19B) by gel electrophoresis of *Fusarium verticillioides* TMW 4.0709 incubated in Czapek's broth medium using fructose as carbon source. 1 and 6= gDNA of TMW 4.0709, 2= cDNA from 2 days of incubation, 3= cDNA from 3 days of incubation, 4= cDNA from 4 days of incubation, 5= cDNA from 5 days of incubation, NC= negative control using water as template. M= size marker (Gene Ruler 100 bp Plus DNA Ladder, Thermo Fisher Scientific Inc., Waltham, USA).

### 4.3.3 Biomass production using different liquid media

The production of mycelia was observed for each experiment in 100 mL of medium in biological duplicates. The fumonisin-inducing broth medium (FUMB, section 3.2.4), which contains fructose and other types of carbon sources such as malt extract, yeast extract and soya peptone, produces around 200 mg more mycelia than the Czapek's broth medium (section 3.2.5) containing only fructose during 5 days of incubation (data not shown). Incubation under static conditions or with shaking (200 rpm) had different impact on the biomass development. Static condition for FUMB medium yielded higher biomass than with shaking at 200 rpm. On the other hand, Czapek's broth medium resulted in higher mycelial biomass when shaken at 200 rpm. Using the best conditions for each medium, the amounts of mycelium during 3 to 5 d of incubation ranged from 286 mg to 900 mg and 219 mg to 676 mg in 100 ml of FUMB and Czapek's broth medium, respectively, both with fructose.

### 4.3.4 The *fum1* gene expression and $FB_1$ production using FUMB medium

In preliminary experiments, the TMW 4.0709 strain was incubated at 25 °C in FUMB medium (section 3.2.4) at static conditions during 3, 5 and 7 days in order to determine fumonisin concentrations by LC-MS/MS analysis (section 3.6.1) and to detect the *fum1* gene expression with the new RT-LAMP assay (section 3.5.2). According to LC-MS/MS analysis, 826 and 2890 ppb of fumonisin $B_1$ were determined at day 5 and 7 after inoculation, respectively. No fumonisin production was detected before day 5 of incubation. Nevertheless, RT-LAMP analysis resulted in positive signals for *fum1* gene expression in all samples tested, including day 3 of incubation in FUMB medium. For further analyses however,

Czapek's broth medium was selected in order to observe the effect of each carbon source separately on the *fum1* gene expression of *F. verticillioides* strains because the medium does not contain other types of carbon sources that would influence the experiment.

### 4.3.5 The impact of single carbon sources on the biomass production

In general, the amounts of fungal biomass in Czapek' broth medium (section 3.2.5) increased with time of incubation (2, 3, 4, 5 and 7 days) and variations were observed in relation to carbon source and *F. verticillioides* strains. Samples were incubated with 3 agar disks from 5-days-old SNA cultures (section 3.2.1) on a rotary shaker at 200 rpm in 500 mL Erlenmeyer flasks containing 110 mL of Czapek's broth medium (section 3.2.5). The production of biomass at 5 days of incubation for *F. verticillioides* TMW 4.0709 (maize isolate) was 676.25, 424.35, 346.25 and 530.25 mg for fructose, glucose, maltose and sucrose, respectively (Figure 20). After 7 days of incubation, the production of biomass was increased for glucose, maltose and sucrose. However, the fungal biomass was reduced by 200 mg compared to day 5 for fructose in strain TMW 4.0709 (Figure 20). For strain TMW 4.2444 (wheat isolate), the highest biomass yield was found in Czapek's broth medium with sucrose (1441.3 mg) and glucose (1081.8 mg) after 7 days of incubation, whereas lower biomass production was observed using fructose (1067.65 mg) and maltose (995.15 mg) as sole carbon source, respectively (Figure 20).

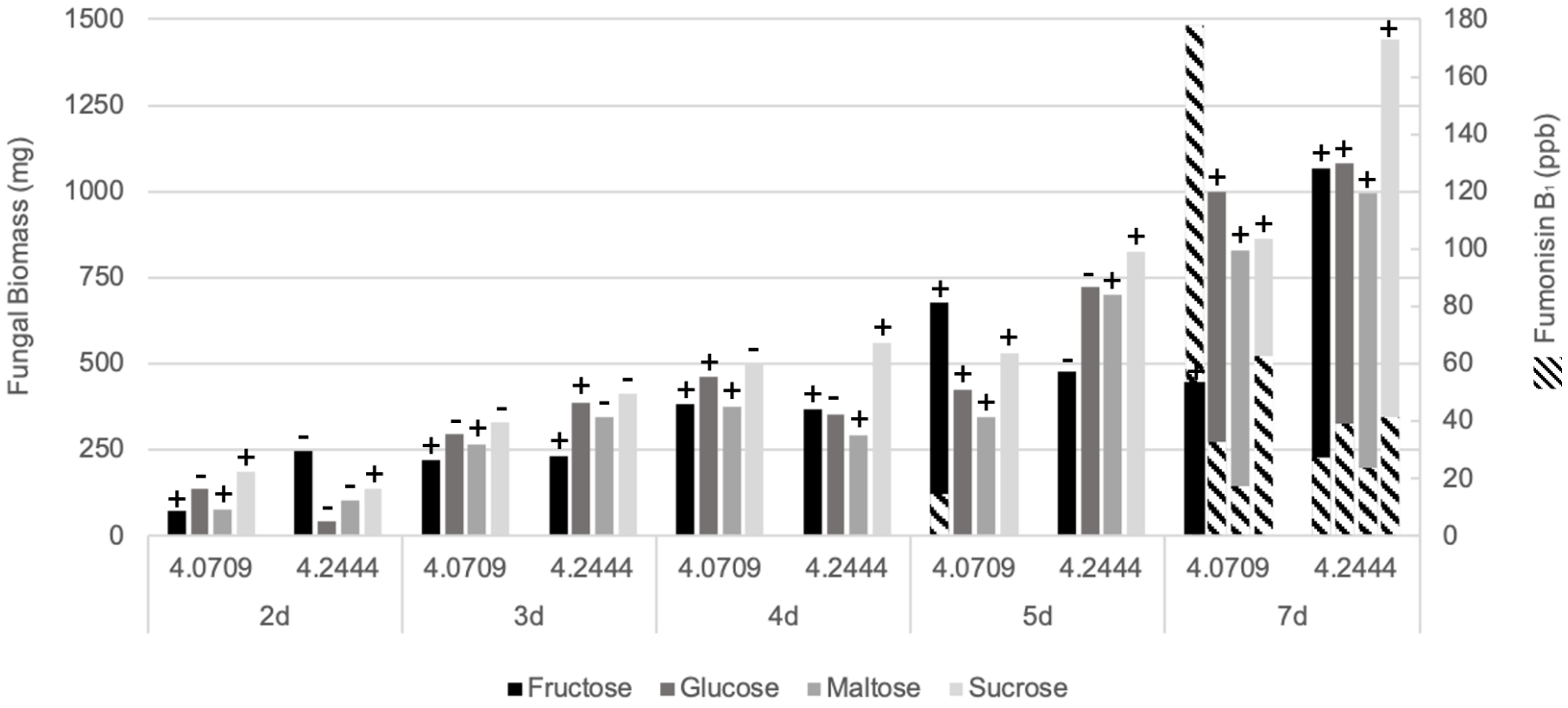


Figure 20 Illustration of the fungal biomass, *fum1* gene expression using RT-LAMP assay and $FB_1$ production by LC-MS/MS analysis for both *F. verticillioides* strains, TMW 4.0709 and TMW 4.2444, incubated in Czapek's broth medium using fructose, glucose, maltose and sucrose as sole carbon source at 25°C during incubation time.

### 4.3.6 The impact of single carbon sources on the *fum1* gene expression and $FB_1$ production

*F. verticillioides* strains, which originated from two different sources, maize and wheat, were selected and incubated in Czapek's broth medium using only one type of carbon sources per experiment in biological duplicates. Samples were incubated as described in section 4.3.5. The strains demonstrate different results according to RT-LAMP signal and fumonisin production (Figure 20). TMW 4.0709 resulted in RT-LAMP positive reactions with all samples collected using fructose and maltose during 2, 3, 4, 5 and 7 days of incubation. For the samples inoculated on glucose and sucrose, the first positive RT-LAMP signal with TMW 4.0709 was with 4 and 2 days of incubation, respectively. Although, the incubation of TMW 4.2444 on Czapek's medium showed the first RT-LAMP positive signals at 2 days for sucrose, 3 days for glucose and fructose and 4 days for maltose. For *F. culmorum* TMW 4.1040, the negative control, all samples showed negative signals for RT-LAMP analyses.

The LC-MS/MS analyses revealed production of fumonisin with all samples tested at 7 days of incubation, although TMW 4.0709 inoculated on fructose medium needed only 5 days (14 ppb of fumonisin $B_1$). Samples analyzed with TMW 4.0709 produced more fumonisin with fructose, sucrose, glucose and maltose, respectively (Figure 20). Moreover, the higher production for TMW 4.2444 was with sucrose, glucose, fructose and maltose as carbon sources (Figure 20). No production of fumonisin was observed when the negative control was analyzed by LC-MS/MS.

# 5 Discussion

Based on the initial hypotheses of this PhD thesis, the main results are summarized in the following theses:

(I) **MALDI-TOF mass spectrometry is a high-throughput technology enabling species level differentiation of the FFSC**

- MALDI-TOF MS database development and the implementation of adequate reference species to the database entries (MSPs) are of crucial importance for reliable outcomes, especially for identification of strains that are external to the supplementary database
- The developed MALDI-TOF MS supplementary database, containing sub-proteome mass spectra of 47 FFSC species, enables high identification and differentiation rates at species level
- Discriminant analysis of principal component based on MALDI mass spectra segregates the FFSC species in 3 groups
- A higher degree of taxonomic resolution for closely related species as *F. musae* and *F. verticillioides* is achieved using mature cell material for MALDI-TOF MS analysis.

(II) **The developed LAMP assay enables rapid and sensitive group-specific detection of fumonisin-producing *Fusarium* spp.**

- The designed LAMP assay primers reliably detect the presence of the *fum1* gene in FFSC species, which typically produce fumonisins

- The LAMP assay based on the presence of *fum1* gene in *Fusarium* spp. provides an alternative amplification technology, which includes many advantages comparing with traditional methods and offers high specificity and sensitivity in combination with high robustness and easy of use
- The LAMP assay permits the accurate detection of the *fum1* gene using gDNA of *Fusarium* spp. and conidial suspensions as well as a rapid diagnosis in maize and maize based products naturally contaminated with fumonisins.

(III) **The reverse transcription LAMP (RT-LAMP) enables *fum1* gene expression analysis upon fungal growth on different materials and thus prediction of fumonisin production upon growth on different plant materials**

- RT-LAMP primers designed detects exclusively cDNA of *fum1* gene, excluding gDNA of *F. verticillioides* strains
- The *fum1* gene expression starts between 2 to 4 days of incubation time, and positive RT-LAMP signals can be detected prior to the initial production of $FB_1$
- The amount of fungal biomass increased with time of incubation (2, 3, 4, 5 and 7 days) regardless the carbon source, while *fum1* expression differs
- The production of fumonisins by *F. verticillioides* differs upon growth on different carbon sources relating their adaptation to maize

The theses mentioned above are in the subsequent chapters of the discussion supported and explained in detail.

## 5.1 Differentiation of *Fusarium* spp. using MALDI-TOF MS technology

The identification of few FFSC species using MALDI-TOF technology has been published in communications of Becker et al. (2015), Cassagne et al. (2013), De Carolis et al. (2012), Dong et al. (2009), Marinach-Patrice et al. (2009), Ranque et al. (2014), Riat et al. (2015), Seyfarth et al. (2008) and Triest et al. (2015b). However, this is the first study that comprises a nearly full set of species belonging to the FFSC. Only three taxa (*F. parvisorum, F. temperatum* and *F. xylarioides*) were unavailable for this project. Other FFSC species as *F. brachiariae*, *F. caapi*, *F. desaboruense*, *F. ficicrescens*, *F. fredkrugeri*, *F. lumajangense*, *F. madaense*, *F. sudanense*, *F. terricola* and *F. volatile* have been newly described after the MALDI TOF MS experiments described here (Al-Hatmi et al., 2019; Costa et al., 2021; Ezekiel et al., 2020; Maryani et al., 2019; Moussa et al., 2017; Sandoval-Denis et al., 2018). Any prediction on their discernability by MALDI ToF MS from previously described species along sequence comparison of TEF1α may be misleading, as e.g., the respective sequences of *F. musae* and *F. verticillioides* a clearly different, while the standard MALDI differentiation was not effective using sample preparations based on young fungal material. Still, it should be easy to include forthcoming new species into the MALDI database and use the method, possibly in the modified form developed during this work. Most of the previous publications focused on aspects of clinical mycology (Al-Hatmi et al., 2015; Marinach-Patrice et al., 2009; Seyfarth et al., 2008; Triest et al., 2015b). Other authors (Becker et al., 2015) used MALDI-TOF MS to support the quality control performed on few strains of a culture collection after their conservation.

Marinach-Patrice et al. (2009) observed differences in the mass spectra obtained after inoculation of cultures on malt extract agar, PDA, and SCG medium. However, the differences did not interfere with the quality of identification, except for *F. verticillioides*,

which was misidentified when grown at higher temperatures. On the other hand, De Carolis et al. (2012) constructed a reference database with species such as *F. proliferatum*, *F. subglutinans*, and *F. verticillioides* based on direct MALDI-TOF MS analysis of intact fungal spores and/or hyphae (MALDI-TOF intact cell MS). Considering the strategy of constructing a supplementary database with spectra of both young and mature colonies of each of the three species, 96.8% of isolates were identified at the species level by the authors.

MALDI-TOF MS has recently been successfully applied for identification of other fungal genera. Abreu et al. (2014) reported that strains identified as *Clonostachys* sp. belonging to undescribed taxa according to phylogenetic analyses could be readily differentiated by MALDI-TOF MS fingerprinting, which suggests application of this new technique for the differentiation between known and possibly unknown species. Discriminating fingerprints were also acquired for *Stachybotrys chartarum* and *Stachybotrys chlorohalonata*, two closely related species that have darkly pigmented conidia, which are usually an obstacle for MALDI-TOF MS-based identification (Gruenwald et al., 2015). Authors reported that no differentiation of highly toxic isolates was possible from low toxic isolates using the method.

More recently, Luethy and Zelazny (2018) developed a protocol involving a one-step rapid protein extraction procedure from fungal material that utilizes a high-speed homogenizer. The protocol decreased the sample preparation procedure to 5 min. Other authors highlight the influence of different protocols for protein extraction, different matrices for MALDI-TOF MS analysis as well as different growth conditions on MALDI-TOF MS analysis. Lima and Santos (2017) and Rico-Munoz et al. (2018) recommended that researchers who use the new technology should have previous training in mycology and use only representative species/strains to construct a supplementary database for species identification.

### 5.1.1 Strategies for MALDI-TOF MS sample preparation

During the current study, a supplementary database was established including 47 of the currently described 60 species within the FFSC in order to differentiate them by MALDI-TOF mass spectrometry. The highly standardized protocol, both for sample preparation and database construction, is the most important prerequisite for sub proteome-based identification. Pre-analytical steps were assessed considering the relevant aspects for reproducible acquisition of spectra for MALDI-TOF MS-based analysis, i.e., pre-incubation time and media, incubation on solid or in liquid medium, and the number of biological and technical replicates. According to the results, a protocol with 2 days of pre-incubation on SNA followed by 2 days of incubation in liquid medium leads to cultures in which the cells are very uniform in their growth phase in order to minimize the variability of protein content and to provide high reproducibility of mass spectra.

The MALDI-TOF MS protocol established in the current study was primarily based on the biomass of young mycelial cells, which have delicate cell walls that are easily broken to release intracellular proteins and peptides for the acquisition of mass spectra. Furthermore, young hyphae do not accumulate pigmented compounds that might interfere with the reproducibility of proteomic mass spectra. It was therefore important to apply a two-step inoculation protocol. Pre-incubation on SNA for 2 days was chosen to synchronize the metabolic status of the mycelia before culturing in liquid medium for production of fresh mycelial biomass from both hypha and spores. In order to avoid low-intensity spectra with low peak numbers, an ethanol washing step was adopted.

The low – sugar synthetic medium, SNA, was chosen as the standard growth medium during the current study because it is frequently used for *Fusarium* cultivation (Nirenberg, 1981). In addition, SNA contains no proteins from foreign sources that could contaminate the

analysis. The liquid medium (SDB) does contain peptone and tryptone from casein and meat. Liquid culture mycelia must therefore be very extensively washed before the analysis. Panda et al. (2015) improved the yield of correct identification of molds by 30% by substituting SDA for SDB. This simple modification in the protocol allowed the reduction of effects of culture conditions and aided in the production of a uniform mycelium.

The growth medium and the incubation time used by Quéro et al. (2018) in sample preparation for MALDI-TOF MS have less influence on mass spectra than the kind of reference strains that are used to create the supplementary database. In the current study, this was reflected by the fact that MDS (Figure 5) showed a certain degree of separation between two strains of the same species. The second part of the sample preparation protocol consisted of cultivation in SDB. Riat et al. (2015), however, reported that this step was unnecessary because they found higher percentages of correct identifications (68.7%) without a subculture-based sample preparation protocol. Although it was not comparing the current protocol with results from a non-subculture-based protocol, results of the current study reached considerably higher percentage of correct identifications (96.41%, log score > 2.0) as compared to the former study, which speaks in favor of a two-step protocol rather than using only solid media for MALDI-TOF MS-based identification.

Also, Cassagne et al. (2013) tested liquid culture media for MALDI-TOF MS identification of clinical yeasts in comparison to direct identification from agar media. The authors reported that biological and technical reproducibility was significantly better with a protocol involving analysis from agar culture media. Nevertheless, only one species of *Fusarium* (*F. oxysporum*) was included in their analysis. These results suggest that peculiarities for each group of organisms need to be taken into consideration to obtain satisfactory spectrum quality. The present protocol that was chosen for the development of

the FFSC supplementary database by MALDI-TOF MS analysis yielded high quality spectra that enabled the separation of species, but also showed differences between strains (Figure 5).

### 5.1.2 Identification of FFSC strains external to the supplementary database

Species such as *F. ananatum*, *F. begoniae*, *F. mangiferae*, *F. fracticaudum*, *F. marasasianum*, *F. pininemorale*, *F. sterilihyphosum*, *F. sororula*, *F. succisae*, and *F. werrikimbe* were only represented by spectra of one strain in the database. Most of these species have only recently been described and are often represented in culture collections by only a few isolates or even none worldwide. In these cases, it was difficult to confirm the differentiation of these species with MALDI-TOF MS because no external isolate to the database was accessible. A minimum of two strains were at least available of all other studied species and for most of them there was at least one additional isolate present to challenge the database. Results of this challenging showed that the identification result was below 100% (see Table 3) for only 7 out of 49 species.

*Fusarium musae* was the only species that could not be identified as a separate species by the MALDI-TOF MS analysis using the method mentioned in section 3.4.1.1, even though sequencing data identified the isolates of the species as *F. musae*. Instead of *F. musae*, the best MALDI-TOF MS match for two out of three strains was *F. verticillioides*. Although both species are closely related phylogenetically, they show significant differences in their TEF1α barcoding sequences. However, their mass fingerprints seem to be too similar to allow for a clear distinction of these species. Similar observations were reported earlier by Triest et al. (2015b). The challenge of differentiating species by MALDI-TOF MS is higher both when the strains have very similar genetic background or a wide inter-species diversity

(Hou et al., 2019). Interestingly, no false identifications were observed with strains of the closely related *F. verticillioides* in which all seven isolates were correctly identify with top score values between 2.3 and 2.5 and no misidentification as *F. musae* occurred.

Considering the close phylogenetic relatedness between the FFSC species, a certain level of false identifications was to be expected using proteomic mass spectra. Nevertheless, only 9 strains (5.39%) out of a total of 167 strains analyzed by MALDI-TOF MS were incorrectly identified in regard to the established criteria and their sequence-based identification. A low percentage of erroneous matches within the FFSC is important, since many species of this group are mycotoxins producers, and their correct identification represents a major issue for health and food safety. Some species are known as typical producers of mycotoxins such as beauvericin, fumonisins, fusaproliferin, fusaric acid, fusarin C, and moniliformin (Gálvez et al., 2017; Moretti et al., 2007; Niehaus et al., 2014). Among these compounds, fumonisins are associated with esophageal and liver cancers, neural tube defects, and cardiovascular problems in regions where there is a high consumption of contaminated food (Marasas et al., 2004; Sun et al., 2007). Even though fumonisins have been found primarily in maize worldwide, there is also evidence for their occurrence in other crops such as sorghum, wheat, barley, maize, and rice (Bryla et al., 2016; Cendoya et al., 2018; Choi et al., 2018; Piacentini et al., 2015; Vismer et al., 2019).

It is therefore important to notice that no difficulties were observed during the current study with the identification of species known as the major fumonisin producers, i.e., *F. proliferatum*, *F. pseudonygamai*, and *F. verticillioides*, which was in contrast to studies conducted by Marinach-Patrice et al. (2009) and Triest et al. (2015b). In all three species, all tested strains were correctly identified with MALDI Biotyper log score values ranging from 2.2 to 2.5. Some studies have reported a higher percentage of correct identifications as compared to the present study (96.41%) using MALDI-TOF MS-based analysis (De Carolis

et al. 2012). Reducing the stringency of identification criteria is an option chosen by many authors that will increase the percentage of correct identifications. Also, strategies that consider only the one out of three replicated measurements with the best match score (Becker et al., 2014) as well as decreasing the cutoff score for species-level identification to 1.7 (Ranque et al., 2014) were used. However, stringent criteria in the measurements are important for increasing the reliability of the results.

In order to reach a high level of accuracy, the knowledge of possible limitations of the applied methodology is crucial for the detection of fungi (Rico-Munoz et al., 2018). Although the results of the current study show that MALDI-TOF MS can easily identify fungal pure cultures, identification of fungi from unprocessed samples will certainly limit its application. Despite publications reporting the use of MALDI-TOF MS directly with clinical specimens, the reliable species identification from samples with low microbial density in a complex matrix does not seem to be feasible. Recent reports have shown that the complexity of unprocessed samples can lead to fingerprints with uncertain identification results (Siller-Ruiz et al., 2017).

### 5.1.3 Arrangement of mass spectra from FFSC strains generated by MALDI-TOF MS

Even though MALDI-TOF MS-based analysis cannot provide any means for phylogenetic analysis of data, it was noteworthy to see that a correlation was observed between the phylogeographic concept described by O'Donnell et al. (1998a) and followed up by Kvas et al. (2009), Edwards et al. (2016) and Herron et al. (2015) and the discriminant analysis of principal components as shown in Fig. 7. Species grouping in group 1 was partially congruent with the Asian clade. All species grouping in group 2 were members of

the American clade and the majority of species grouping in group 3 of the DAPC analysis are nested in the African clade as shown in Herron et al. (2015). These observations were confirmed by results published earlier by Triest et al. (2015b) and also by De Carolis et al. (2012).

Considering that phylogeography represents the processes that influence the geographic distribution of genetic lineages through molecular data, MALDI-TOF MS is unable to give such information. Phylogeographic hypotheses consider that most species have some level of population structure with both spatial and temporal components (Summerell et al., 2010). O'Donnell et al (1998a) suggested a biogeographic hypothesis for the 3 groups of fungi formed based on phylogenetic analyses from sequencing a number of gene regions termed African, American and Asian clade. The hypothesis suggests that the biota area cladogram reflects vicariant events associated with the fragmentation of Gondwana in the upper Cretaceous through the Paleocene over the last 100 million years (O'Donnell et al., 1998a).

Hypothesis based on host origin presumes that all of the species in the respective clade have a close association and that the current host pathogen relationship has a long evolutionary history and can be used to inform the biogeography (Summerell et al., 2010). However, the placement of many species in the respective clade is not consistent with the geographic origin of its current major host, which leads many researchers to focus on species isolated from natural ecosystems because the species collected from agriculture ecosystems constantly mask the biogeographic information by the anthropogenic distribution patterns (Laurence et al., 2016). Although MALDI-TOF mass spectrometry is based on a sub-proteomic spectra database in the current study, surprisingly they showed a similar grouping as the phylogeographic groups suggested from DNA sequence data by Kvas et al. (2009). As is illustrated in table 1, most of the FFSC species are associated with plants, many of which

have a very strict geographical distribution. Other studies also proved the discriminatory power of MALDI-TOF MS, e.g., association of brewing yeast strains of *Saccharomyces cerevisiae* with different major beer types (Lauterbach et al., 2017).

Implementation of a reliable method to set up a supplementary database in MALDI-TOF mass spectrometry yielded a significant improvement in the process of filamentous fungi identification and permits identification even if a low number of strains are available for MSP construction. The availability of web-based publicly accessible spectra databases for fungi, such as the MSI platform (Normand et al., 2017) may improve the utility of MALDI-TOF MS as a tool for fungal species identification, since spectra can be compared with the database provided by the platform. Once the supplementary database has been implemented on the platform, users can submit MALDI-TOF mass spectra of their isolates as a query that will be returned by a species identification accompanied by a score value for evaluation of result.

### 5.1.4 Comparison of MALDI-TOF MS methods for reliable identification of closely related species

Banana is one of the most important fruits worldwide, both in trade and production volumes. In the last years, the trade of bananas was almost 20 % of the global fruit production (IISD, 2020) and nowadays compete with tomatoes for the world's most popular fruit (Mala, 2020). Latin America and the Caribbean are the leading export areas achieving an increase of 10.2 % of banana exportations in 2019 compared with 2018 (FAO, 2020). Adverse effects of extreme weather events and fungal diseases are usually the main

disruptors for the enhancement of banana supply observed through the years (Evans et al., 2020).

Among the post-harvest diseases affecting the balance in export/import of bananas, crown rot has gained importance due its negative impact on banana yield (Lassois et al., 2010). Recently, Molnár et al. (2015) and Kamel et al. (2016) reported the occurrence of *F. verticillioides* and *F. musae* on banana fruits from Dominican Republic, Ecuador, Ivory Coast, Columbia and Costa Rica. However, *F. musae* strains were demonstrated to have a greater ability to cause infection on banana fruits than *F. verticillioides* (Moretti et al., 2004). The description of *F. musae* by Van Hove et al. (2011) as a new species induced the re-identification of *Fusarium* spp. associated with keratitis cases, skin infection and sinusitis. As a result, *F. musae* has also been described as an opportunistic human pathogen (Triest et al., 2015a).

Although different authors (Moretti et al., 2004; Hirata et al., 2001) reported molecular phylogenetic differences between *F. verticillioides* strains when isolated from banana, only multilocus phylogenetic analyses allowed the separation of the species from each other (Van Hove et al., 2011). Furthermore, *F. musae* strains regularly produced the mycotoxin moniliformin in toxicity tests but none of the tested strains produced fumonisin $B_1$ or fumonisin $B_2$ (Moretti et al., 2004). Moreover, a molecular assay based on loop-mediated isothermal amplification (LAMP) indicated the absence of the $fum_1$ gene in *F. musae* strains as the polyketide synthase gene necessary for fumonisin production (table 7).

The application of new technologies such as MALDI-TOF MS analysis of subproteomic mass spectra has become attractive because successful identification and differentiation of species can be achieved with high precision (Chalupová et al., 2014). In the previous study, discussed here in section 5.1, MALDI-TOF was able to discriminate 46 species within the FFSC by MALDI-TOF MS using the sample preparation mentioned in

section 3.4.1.1. *F. musae* was the only species that showed miss identifications with *F. verticillioides* using the previous protocol (section 5.1). The background of the current study was therefore to determine whether liquid grown young mycelia (two days of growth, anaerobic) or older mycelia grown on solid medium (ten days of growth, aerobic) would result in distinguishable mass spectra to allow unambiguous separation of the very closely related species *F. musae* and *F. verticillioides*.

Species identification and separation by peptide mass fingerprinting is based on a high degree of diversity in the protein spectrum of organisms. However, there are cases, in which the diversity is too low between species to separate them properly under standard conditions. It is assumed that protein diversity can be improved by two different measures: i. using older and morphologically more differentiated mycelia in which structural and autolysis associated proteins as well as secondary pathway proteins are more diverse as compared to younger mycelia (Barros et al., 2010) and ii. using an extraction protocol that involves mechanical disruption of total cells as compared to chemical lysis of the cell membrane in the Bruker standard procedure for fungal organisms to improve extraction efficiency. In order to avoid spectral differences that may be based on variation of the sample preparation protocol, mycelia grown under the compared conditions received identical nutrients from medium and the same protein extraction protocols. The subsequently used method for MALDI-TOF MS was identical for protein extracts from both growth conditions to achieve highly reproducible and comparable peptide/protein profiles. Moreover, an additional layer of matrix was applied to improve the disintegration of proteins during laser desorption/ionization (Dong et al., 2009).

Comparing mass spectra from both protocols, it was found that using young fungal material, most of the identified peaks were distributed within a mass range of 3 - 8 kDa whereas they were distributed over a wider range of 2 to 13 kDa using mature fungal

material. Moreover, also the peaks detected in extracts of mature fungal material were higher as compared to young fungal material. Both effects lead to an increase of the number of peaks that are included in the differentiation of spectra from different species. MALDI-TOF mass spectrometry has been demonstrated to be a highly accurate identification tool, especially when identifications were compared with molecular analyses (Becker et al. 2014; Seyfarth et al., 2008). Experiments performed by De Carolis et al. (2011) concluded that neither culture medium, incubation temperature nor pretreatment of fungi involving different lysis steps had an influence on the accuracy of MALDI-TOF MS based identification. Only the length of incubation of the fungal cultures resulted in a significantly different number and intensity of mass peaks (De Carolis et al., 2011).

Among the closely related species of FFSC, Al-Hatmi et al. (2016a) recently proved the discrimination of *F. andiyazi* and *F. fiscicrescens*, two morphologically identical *Fusarium*-species using samples with 3 days of age by MALDI-TOF mass spectrometry. According to the results, an even higher level of morphological and physiological differentiation is required for the differentiation of *F. musae* and *F. verticillioides* (Figure 10). Although high percentage of correct identifications at species level were reported by Triest et al. (2015b), the use of fungal material at the initial stage of growth (3 days of incubation) was not able to discriminate *F. sacchari* from *F. proliferatum* and *F. musae* from *F. verticillioides*, in agreement with the initial results obtained for *F. musae* from young mature material (section 5.1).

## 5.2 LAMP assay for the rapid and sensitive group-specific detection of fumonisin producing *Fusarium* spp.

Since the introduction of fumonisins as a new class of mycotoxins by Gelderblom et al. (1988), their worldwide occurrence has been intensively investigated (Cendoya et al., 2018; Lee and Ryu, 2017). According to table 1, the group of fumonisin-producing *Fusarium* spp. comprises 22 species that belong to the FFSC. *F. oxysporum* and *F. polyphilalidicum* are the only non-FFSC species in the genus *Fusarium* which were identified as a fumonisin producers (Rheeder et al., 2002). According to the literature, only five of the FFSC-species that were newly described after 2009 were tested for their ability to produce fumonisins (*F. agapanthi*, *F. andiyazi*, *F. musae*, *F. secorum* and *F. sterilihyphosum*, see Table 1).

In order to facilitate the analysis of FFSC isolates for their potential to produce fumonisins and to enable the rapid detection of such fungi in contaminated materials, the aim of this part of study was to set up a rapid, sensitive and user-friendly DNA-based assay. Several attempts were made in the past to detect genes involved in fumonisin biosynthesis as a marker for the presence of potential fumonisin producing fungi using PCR-based assays (Bluhm et al., 2002; El-Yazeed et al., 2011; Gonzalez-Jaen et al., 2004; Proctor and Vaughan, 2017; Sreenivasa et al., 2006). However, compared to the high number of potential fumonisin $B_1$ producing *Fusarium* spp. currently known, (Choi et al., 2018; Moussa et al., 2017) these assays detect only a fraction of the total species with fumonisin producing potential.

Moreover, PCR-based detection assays require specific lab equipment to be properly performed. LAMP-based assays provide an analytical platform that may be used for the rapid, sensitive and user-friendly detection of microorganisms, including filamentous fungi (Niessen et al., 2013; Niessen, 2015). Such assays are especially interesting for the rapid detection of mycotoxinogenic fungi (Niessen et al., 2018). LAMP-based assays for the group

specific detection of trichothecene producers (Denschlag et al., 2014), aflatoxin producers (Niessen et al., 2018) and patulin producers (Frisch and Niessen, 2019) are available to date. Ferrara et al. (2020) published a LAMP-based assay for the detection of fumonisin $B_2$ producing isolates of *Aspergills niger* an *A. welwitschiae* in which they used primers that were designed to bind to the *fum10* gene of these species. No cross reactions with *Fusarium* species were observed with this assay. Very recently, Jiang et al. (2021) developed the detection of *F. fujikuroi* associated with rice bakanae disease on seeds as a powerful tool for the rapid and sensitive detection of the fungus and predicting the production of the gibberellin GA3.

### 5.2.1 Design of optimum LAMP primers

In the current study, the nucleotide sequence of the *fum1* gene was used as template for the design of LAMP primers, including a pair of loop primers that considerably decreased the time to signal. Besides the presence of the *fum1* gene, the gene expression of the encoded polyketide synthase (Alexander et al., 2009; Proctor et al., 2003) has also been studied and it was shown to be highly correlated with the production of fumonisins under various environmental conditions (Fanelli et al., 2012; Jurado et al., 2008; Marín et al., 2010; Medina et al., 2013). According to Samsudin et al. (2017), *fum1* gene expression is a highly significant marker for the potential of a strain to produce fumonisins. Hence, also the gene has been detected in the most important fumonisin producing species of the FFSC (Proctor et al., 2004).

Due to its fundamental role in the biosynthesis of fumonisins, it is assumed the gene to be a common trait also in species that have received little attention so far. Therefore, the

design of LAMP primers for the current study was based on a highly conserved region of the *fum1* gene as shown in Fig. 12. To confirm that the amplified LAMP product was not an artifact but rather a copy of the target gene, the smallest product from a LAMP reaction run with gDNA of *F. proliferatum* TMW and *F. verticillioides* TMW 4.0703 was sequenced. Based on NCBI database, the obtained sequence resulted in 93% of identity with the corresponding *fum1* sequence in *F. nygamai* and 100% for both *F. proliferatum* and *F. verticillioides*. To define the spectrum of detected species, 100 isolates representing 48 of the currently described in the FFSC were tested with the newly developed LAMP assay.

### 5.2.2 Comparison of LAMP results, available literature and fumonisin production

As shown in Table 7, the major fumonisin producers such as *F. fujikuroi*, *F. nygamai*, *F. proliferatum*, *F. ramigenum*, *F. subglutinans* or *F. verticillioides* were detected with low LODs. However, there were also species such as *F. acutatum*, *F. pseudocircinatum* and *F. thapsinum* which have been described in the literature as fumonisin producers but in which none of the analyzed strains was positive in the *fum1* LAMP assay. This result corresponded with the inability of those strains to produce $FB_1$ under the chosen conditions as determined by LC-MS/MS analysis. On the other hand, *F. agapanthi* that were described as non-producers of fumonisin in the literature showed positive LAMP reactions and also produced $FB_1$ according to LC-MS/MS analyses.

It was interesting to observe, that species such as *F. coicis*, *F. mundagurra*, *F. pininemorale, F. tjaetaba* or *F. udum,* which have never been tested for their potential to produce fumonisin, resulted in a positive LAMP signal that correlated with results of the LC-MS/MS analysis for at least 1 strain. From these results it can be deduced, that they may

harbor at least the *fum1* gene and could therefore be able to produce fumonisins. Those species have been isolated from soil, native plants, source of food and medicine for determined regions and also important crops as *Sorghum* sp. (Table 1). *F. mundagurra* was first isolated from soil of natural ecosystems in Australia (Laurence et al., 2016) and one year later was identified for the first time in a pediatric case of endobronchial fusariosis after an invasive pulmonary fungal disease in children (Al Yazidi et al., 2019), which contributes with the evidence that this species must receive more attention since high amount of fumonisin was produced by *F. mundagurra* in this study (Table 7). Other species such as *F. fractiflexum*, *F. mexicanum* and *F. tupiense* that have never been tested for fumonisin production did not result in a positive signal in the *fum1* LAMP assay and did not produce $FB_1$.

The fact that in many of the analyzed species only a percentage of the tested strains resulted in a LAMP signal and showed $FB_1$ production in the LC-MS/MS analysis was in accordance with observations according to which also fumonisins are regularly not produced by all analyzed strains of a species (Leslie et al., 1992; Proctor et al., 2006; Stępień et al., 2011). The reason for such inability to produce the toxin can be gene mutations either in the *fum1* gene or other genes involved in fumonisin biosynthesis which would result in a nonfunctional FUM proteins or problems with the intracellular regulatory system responsible for the biosynthesis of fumonisins. Comparison of LAMP signals and production of $FB_1$ above the LOD of 5.25 ng/mL of growth medium in 48 analyzed isolates showed that the LAMP assays resulted in 8 % and 6 % false positive and false negative results, respectively.

Testing of a great variety of fungal species other than FFSC revealed the high specificity of the *fum1* LAMP assay for potential fumonisin producing species (see Table 7 and 8). Evaluation of the sensitivity of the *fum1* LAMP assay was performed using serial dilutions of purified gDNA as template. This experiment was done with all isolates in Table 7

that showed a positive reaction in the assay. The lowest detection limits of the current assay were determined to be 5 pg/reaction of purified gDNA. This value represents similar sensitivity as compared to PCR-based detection of fumonisin producers (Bluhm et al., 2002). The sensitivity was also comparable to previously published gene specific LAMP assays (Denschlag et al., 2014; Frisch and Niessen, 2019; Niessen et al., 2018).

A number of different methods have been described in the literature so far which can be applied to monitor the LAMP reaction, both in real- time and endpoint applications (Shirato, 2019). However, reactions that provide a visually readable color change under day light conditions are especially interesting for the application of assays under field conditions because no post amplification processing steps are necessary. Two different indicators were used during the current study which follow different mechanisms for the induction of color change: i) manganese-loaded calcein which recovers its fluorescence upon out-complexing of the manganese ion by pyrophosphate as a by-product of enzymatic DNA synthesis (Tomita et al., 2008) and ii) neutral red as a pH indicator which changes its color from yellow to vivid pink upon pH drop induced by hydrogen ions as another by-product of DNA synthesis (Tanner et al., 2015; Zhang et al., 2019). Results showed no difference between the two indicators applied.

All positively tested FFSC species had a signal with both indicator dyes (see Table 7). However, the calcein system needs a UV-lamp for signal readout as opposed to the neutral red system which can be read visually under day light conditions. Neutral red enables a very clear differentiation between positive (pink) and negative (yellow) LAMP reactions that are readly visible with the naked eye (see Fig. 13). It is therefore the preferred choice for field applications. This indicator was also successfully applied in other LAMP applications (Alhassan et al., 2016; Poole et al., 2017; Vogt et al., 2017). Previously, laboratory colleagues also tested hydroxy naphthol blue (HNB) as an indicator that has a similar

mechanism as calcein with visual light detection (Almasi, 2019; Goto et al., 2009) but the color change was not convincing in our point of view. Recently, HNB was applied in a mixture together with manganese loaded calcein to result in a more pronounced visually detectable color change from blue to grey (Pang et al., 2019). However, this was not applied in this system to the LAMP assay described herein.

### 5.2.3 Application of LAMP assay for the detection of potential fumonisin producers in naturally contaminated samples

Usefulness of the new LAMP assay for its application in naturally contaminated samples was demonstrated in maize using a simplified protocol for sample preparation. It was observed that even for fumonisin concentrations as low as 40 ppb total fumonisins, the LAMP assay showed positive results in maize samples. Fungal material was washed off from the surface of grains and subjected directly to the LAMP assay as amplification template. Direct LAMP-based analysis of sample materials was previously demonstrated with a variety of different sample matrices such as food (Salim et al., 2018), feed (Takabatake et al., 2018) or water (Stedtfeld et al., 2016).

Since the goal of this part of study was to provide a rapid method for testing, washing of unground grains instead of analyzing ground sample material has the advantage of taking only a fraction of time as compared to extracting DNA from ground samples. Direct addition of fungal conidia had a LOD of $1 \times 10^5$ conidia per reaction. Given a genome size of 41.79 Mbp and a single copy gene and an LOD of 5 ng, the minimum detectable number of gene copies is $1.11 \times 10^5$ per reaction. This value corresponds well with the detection limit found for the direct analysis of *F. verticillioides* conidia as shown in Fig. 16B and demonstrates that

cells undergo a rather complete lysis under the conditions prevailing during incubation of the LAMP reaction. Moreover, Fig. 16C shows that application of an additional mechanical cell disruption treatment results in a positive signal when only $10^3$ conidia are added to the reaction. These results suggest that the application of a very simple protocol for sample preparation was sufficient to detect fumonisin producers in sample materials with a total time to result of only 60 min.

### 5.3 The influence of carbon sources on the *fum1* gene expression using reverse transcription LAMP (RT-LAMP)

The production of mycotoxins is part of the evolution process of some fungi to colonize a wide range of ecosystems. There is still significant discussion about the origin, purpose and importance of products of secondary metabolism. Evidence suggests that mycotoxins produced by fungi confer a competitive advantage to the producer and under stress conditions. Despite the role of mycotoxins probably may operate only within natural ecosystems (Magan and Aldred, 2007), the investigation of many possibilities of production in laboratory conditions give an insight about the favorable conditions involved on the field of food mycotoxicology.

Significant research effort has been focused on the prevention of *F. verticillioides* contamination since this species is regularly associated with high fumonisin contamination of agricultural commodities (Marín et al., 2004; Picot et al., 2010). The study of the influence of biotic and abiotic factors on fumonisin production can improve strategies that are aimed at the prediction of colonization and mycotoxin formation in agricultural substrates. These different plants may harbor different sets of carbohydrates, which can be determinative

nutrients for infesting fungi. However, only little information is available about the influence of different carbon sources on the network of genes, which are involved in fumonisin biosynthesis.

### 5.3.1 Influence of carbon sources on mycotoxins production

Studies on the influence of carbon sources on mycotoxin-producing fungi other than *F. verticillioides* showed that the carbon sources analyzed for most of the species has a strong influence on the kind and concentration of mycotoxin produced. Brzonkalik et al. (2011) noted that *Alternaria alternata* produced more mycotoxins such as alternarional, alternariol monomethyl ether and tenuazonic acid when sodium acetate, rhamnose and maltose were added as carbon sources, respectively. The highest ochratoxin production of *A. ochraceus* strains isolated from grapes (Aso 2) was found with sucrose as carbon source (Medina et al., 2008), whereas *F. graminearum* isolates from wheat ears that displayed the classical *Fusarium* head blight symptoms showed enhanced trichothecene production with sucrose as carbon source (Jiao et al. 2008).

Literature research shows that most of the relevant publications are related to growth assessment and/or quantification of $FB_1$ production in *F. verticillioides* rather than gene expression. Only recently, Achimón et al. (2019) demonstrated that sugar sources caused differences in the hyphal morphology and that polysaccharides such as starch, amylose and amylopectin resulted in a significantly lower production of $FB_1$ compared to monosaccharides such as maltose and glucose. On the contrary, Bluhm and Woloshuk (2005) found large amounts of $FB_1$ production when *F. verticillioides* was grown on amylopectin or dextrin, a product of amylopectin hydrolysis.

According to the current study, the lowest concentrations of fumonisin $B_1$ (LC-MS/MS) were observed at day 7 post inoculation (pi) in both *F. verticillioides* strains, from maize and wheat (17.1 and 23.45 ppb, respectively) when Czapek's broth medium with maltose as sole carbon source was used. Nevertheless, Jiménez et al. (2003) observed that the addition of malt extract, yeast extract and mycological peptone to the basal medium with maltose increased strongly the fumonisin production in strains of *F. verticillioides*. Higher $FB_1$ concentrations and higher fungal biomass production were observed in the current study with fructose and sucrose, respectively, both for TMW 4.0709 (from maize) and TMW 4.2444 (from wheat). Although similar production of $FB_1$ was observed for both strains with glucose (approximately 35 ppb) as sole carbon source, the strain from maize (TMW 4.0709) produced the highest amount of $FB_1$ (178 ppb) with fructose as sole carbon source.

Maize has a generally higher content in free glucose, fructose, sucrose and maltose as compared to wheat (Zilic et al., 2017). Especially the mean concentrations of free fructose and sucrose are 50 % and 270 % higher, respectively as compared to wheat. The observed higher concentrations of $FB_1$ found for the two sugars in the maize associated strain TMW 4.0709 may therefore be the result of its adaptation to growth on that commodity. Also, the closely related maize pathogen *F. proliferatum* showed a significant increase in the production of fumonisins when grown on sucrose as sole carbon source (Jian et al., 2019).

A significant decrease of the $FB_1$ concentrations was observed after days 9 and 11 pi for most of the carbon sources in the maize associated strain TMW 4.0709, but also for the fructose grown culture of strain TMW 4.2444. Such behavior has been described previously in the literature. López-Errasquín et al. (2007) noticed the reduction of fumonisin concentrations in *F. verticillioides* strains between 14- and 28-days pi. This delay probably occurred because the basal medium used was richer in nutritional sources providing an extension of the lag phase. The reduction of oxygen during incubation or simply the

degradation of $FB_1$ and subsequent *de novo* production in a cyclic manner were discussed by Le Bars et al. (1994). Also, the conversion of fumonisins to masked compounds, e.g., fumonisin glycosides, or the enzymatic cleavage of the toxin have been suggested by Alberts et al. (1990) to explain this phenomenon that is typical for fumonisins.

### 5.3.2 Influence of environmental conditions on the fumonisin production

Various studies have been conducted to analyze the influence of environmental factors on the expression of genes that are involved in the biosynthesis of fumonisins in *F. verticillioides*, including *fum1*. Although the detection of *fum1* gene expression on different carbon sources experiments have only a few findings, other environmental factors involving *F. verticillioides* have been enhancing the progress of fumonisin biosynthesis subjects. Examinations of water activity (aw) and temperature have been demonstrated to assert a profound effect on growth, *fum1* gene expression and fumonisin production. However, the correlation of gene expression and production of the toxin seems to be variable, because usually the expression of only a few genes is directly correlated with the toxin biosynthesis (Medina et al., 2013).

Although Jurado et al. (2008) noticed some level of independence of growth rate and *fum1* expression under solute and matric potential stresses, *fum1* gene expression decreased progressively or was maintained through mild water stress levels (-0.7 and -2.8 MPa) and higher growth rates. Different lights and their wavelengths have been demonstrated to stimulate the growth of *F. verticillioides* and increase the $FB_1$ production as well as the expression level of *fum1*, *fum21* and *FvVE1* (Fanelli et al., 2012). Among the fungicides already tested, most of the treatments using benomyl resulted in a strong reduction in *fum1*

expression (Cruz et al., 2014). Recently, Samsudin et al. (2017) compared the effect of 2 biological control agents under different ripening stages of maize cobs. In general, *Clonostachys rosea* 016 significantly reduced $FB_1$ contamination more efficiently than the gram-negative bacterium. However, this study showed many fluctuations in temporal $FB_1$ production, regardless of *fum1* gene expression, which highlight the variation of factors associated with fumonisin production.

### 5.3.3 Influence of carbon sources on the *fum1* gene expression using reverse transcription LAMP

So far, most of the published studies used reverse transcription real-time PCR or microarrays to detect and quantify (relatively or absolutely) the gene expression. However, reverse transcription real-time PCR has a high demand for sophisticated lab equipment and well-trained staff. Therefore, results take relatively long to be produced. Similar to LAMP-based assays, also reverse transcription LAMP is a less demanding and time-consuming alternative to PCR-based methods. The current study was therefore aimed at the development and application of a reverse transcription LAMP (RT-LAMP) assay for the rapid detection of *fum1* gene expression. Feasibility of the new method was demonstrated by analysis of two strains of *F. verticillioides* that were isolated from two different commodities.

The use of cDNA as template allowed synthesis of large amounts of DNA in a short period of time (less than 1 hour) at constant temperature (Notomi et al., 2000). The primers for the current RT-LAMP assay were designed to exclude amplification of genomic DNA by bridging the most downstream of the four introns present in the *fum1* gene. Results showed that a LAMP reaction with the designed primers only occurred, when cDNA of *F.*

*verticillioides* was added as template and not with genomic DNA isolated from the same strain even when the incubation temperature was low. Under such conditions, unspecific amplification would have occurred if primer binding was unspecific. Therefore, the incubation temperature of the assay was chosen at 65 °C, which is the activity optimum of the *Bst* polymerase.

Reverse transcription LAMP is widely used for the LAMP-based detection of viruses with a RNA genome, including SARS-CoV-2 (Baek et al., 2020). The usefulness of reverse transcription LAMP for the expression analysis of fungal genes was first demonstrated by Minenko et al. (2014) who managed to detect the expression of housekeeping genes coding for EF1α and β-tubulin in *Fusarium graminearum* in a one-step assay that combined mRNA transcription and cDNA amplification in one single reaction. In the current study, the technology was applied for the first time to monitor the expression of a gene that is involved in the production of a secondary metabolite in a fungus, *F. verticillioides*.

The results of the current study showed *fum1* gene expression already in a very early growth stage in most of the cultures, long before production of $FB_1$ was observed from day 5 pi onward. However, *fum1* gene expression in all tested cultures was observed from day 7 pi, in a stage in which $FB_1$ was found in all cultures. It was interesting to observe that the *fum1* expression was rather stable over time in the maize associated strain TMW 4.0709 but showed some discontinuity in the wheat related strain TMW 4.2444. It is suggested that this effect is triggered by the fact that *fum1* as a gene that codes for a polyketide synthase early during fumonisin biosynthesis is up- or downregulated in accordance with the concentrations of later compounds in subsequent biosynthesis steps. With the newly developed reverse transcription LAMP assay, a tool is now available that can be used to further elucidate *fum1* expression in a simple yet highly specific and sensitive manner.

# 6 Summary

Members of the *Fusarium fujikuroi* species complex (FFSC) are commonly involved in devastating diseases of many economically important plants. They invade developing seeds and other plant tissues in the field causing significant annual losses in crop plants. In addition, fungal spoilage can also affect human and animal health because some species in this group, especially *F. proliferatum* and *F. verticillioides*, are mycotoxin producers occurring in food/feed worldwide. Since morphology-based species identification is of limited value in the FFSC, Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI–TOF MS) analysis of subproteomes has been applied as a promising tool for the discrimination of closely related species. A standardized protocol for the MALDI-TOF MS based identification of FFSC species was developed and validated. Forty-seven of the currently described 60 species were identified by DNA sequencing analysis and their mass spectra were included as reference in a supplementary MALDI-TOF MS database. The discriminative potential of the database was evaluated with more than 80 non-reference FFSC isolates and resulted in 96.41 % of correct identifications at the species level. It was demonstrated that MALDI-TOF MS is a suitable and accurate technology for the identification and differentiation of species within the FFSC as well as an innovative, time efficient alternative to the Multi Locus Sequencing Technology (MLST).

In order to improve the differentiation of very closely related species within the FFSC, e.g., *F. verticillioides* and *F. musae*, spectra from 2-day old liquid cultures and 10-day old agar grown cultures of *F. musae* and *F. verticillioides* were compared with each other. Although the application of the protein extraction method obtained high quality spectra with both young and mature fungal material, discriminant analysis of principal component (DAPC) indicated differentiation of *F. verticillioides* and *F. musae* only when spectra from

the agar grown older fungal material was analyzed. After the construction of a supplementary database, 100 % of isolates of the respective species were correctly identified resulting in top log scores between 2.1 – 2.6 for *F. verticillioides* and 2.4 – 2.5 for *F. musae*. The results underline the potential of MALDI-TOF MS-based analysis even for the discrimination of closely related taxa within the FFSC since the use of mature cell material enables a higher degree of taxonomic resolution in more complex mass spectra.

Fumonisins are mycotoxins that contaminate maize and maize-based food products, and feed. They have been associated with nerve system disorders in horses, pulmonary edema in swine as well as neural tube defects and esophageal cancer in humans. The *fum1* gene codes for a polyketide synthase involved in the biosynthesis of fumonisins. It is present in the genomes of all fumonisin producing *Fusarium* spp. Reliable detection of *fum1* can provide an estimate of the toxicological potential of cultures and food sources. A *fum1* specific LAMP assay was developed and tested with purified DNA of 47 different species from the FFSC. The *fum1* gene was detected in 28 species among which *F. fujikuroi, F. globosum, F. nygamai, F. proliferatum, F. subglutinans* and *F. verticillioides* are the most important fumonisin producers. None out of 92 tested non-*Fusarium* species showed cross reactions with the new assay. The limit of detection (LOD) of the new assay as 5 pg of genomic DNA per reaction for *F. fujikuroi, F. nygamai* and *F. verticillioides*. Higher LODs were found for other LAMP positive species. Apart from pure genomic DNA, the LAMP assay detected fumonisin-producers when a crude extract from $10^3$ conidia/reaction were used as template after mechanical lysis. LAMP-results were well correlated with fumonisin $B_1$ ($FB_1$) production. The current study reports for first on $FB_1$ production in strains of *F. coicis, F. mundagurra, F. pininemorale, F. sororula, F. tjataeba, F. udum* and *F. werrikimbe*. Usefulness of the LAMP assay was demonstrated by analyzing $FB_1$ contaminated maize grains. The new LAMP assay was shown to rapid, sensitive and reliable for the diagnosis of

typical fumonisin producers and that it can be used as a versatile tool in HACCP concepts that target the reduction of fumonisins in the food and feed chain.

*Fusarium verticillioides* is the most important producer of $FB_1$ and the production of the toxin as well as the development of the fungus is influenced by environmental conditions, especially carbon sources. In order to determine the expression of *fum1* as a key gene in the biosynthesis of $FB_1$, a reliable and fast reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay was developed and optimized. The new assay was applied to the detection of *fum1* expression in two *F. verticillioides* strains that were isolated from maize and wheat, respectively. Both strains were cultivated in Czapek's medium containing four different sugars as sole carbon sources. Analysis of *fum1* expression was accompanied by $FB_1$ determination using LC-MS/MS analysis. High growth rates and production of $FB_1$ were observed in fructose-containing medium for the strain that originated from maize. Less production of $FB_1$ occurred using maltose as sole carbon source for both strains. The *fum1* gene expression started between 2 to 4 days of incubation, and positive signals were detected prior to the initial production of $FB_1$. The RT-LAMP assay was effective in the detection of *fum1* gene expression at very early stages of *F. verticillioides* growth and can therefore be a useful tool in the early prediction of $FB_1$ formation.

# 7 Zusammenfassung

Pilzarten, die zum *Fusarium fujikuroi*-Spezieskomplex (FFSC) gerechnet werden, sind an verheerenden Krankheiten vieler wirtschaftlich wichtiger Pflanzen beteiligt. Sie dringen in die Samenanlagen und andere Pflanzengewebe während der Infektion auf dem Feld ein und verursachen erhebliche jährliche Verluste bei Kulturpflanzen. Darüber hinaus können diese Pilze auch die Gesundheit von Mensch und Tier beeinträchtigen, da einige Arten aus dieser Gruppe, insbesondere *F. proliferatum* und *F. verticillioides*, Mykotoxinproduzenten sind, die in Lebensmitteln / Futtermitteln auf der ganzen Welt vorkommen. Da die morphologiebasierte Spezies-Identifikation im FFSC nur von begrenztem Wert ist, wurde in der vorliegenden Arbeit die *matrix-assisted laser desorption/ionization time of flight* Massenspektrometrie (MALDI-TOF MS) von Subproteomen als vielversprechendes Instrument zur Unterscheidung nahe verwandter Spezies eingesetzt. Ein standardisiertes Protokoll zur MALDI-TOF MS-basierten Identifizierung von FFSC-Arten wurde entwickelt und validiert. Jeweils mehrere Stämme von siebennundvierzig der derzeit beschriebenen 60 Arten wurden durch DNA-Sequenzierungsanalyse identifiziert und ihre Massenspektren wurden als Referenz in eine MALDI-TOF MS-Datenbank aufgenommen. Das Differenzierungspotential der Datenbank wurde mit mehr als 80 FFSC-Isolaten der betreffenden Arten bewertet, die jedoch nicht Teil der Referenzdatenbank waren. Dabei ergab sich ein Anteil von 96,41% an korrekten Identifizierungen auf der Artenebene. MALDI-TOF MS hat sich als geeignete und akkurate Technologie zur Identifizierung und Differenzierung von Arten innerhalb des FFSC sowie als innovative, zeiteffiziente Alternative zur Multilocus-Sequenzierungs Technologie (MLST) erwiesen. Um die Differenzierung sehr eng verwandter Arten innerhalb des FFSC zu verbessern, z. B. von *F. verticillioides* und *F. musae*, wurden von 2-Tage alten

Flüssigkulturen und von 10-Tage alten Agarkulturen von *F. musae* und *F. verticillioides* miteinander verglichen. Obwohl die Verwendung der Proteinextraktionsmethode sowohl mit jungem als auch mit reifem Pilzmaterial hochqualitative Spektren ergab, zeigte die *discrimant analysis of principal components* (DAPC) nur bei der Analyse von Spektren aus dem älteren Pilzmaterial eine ausreichend genaue Differenzierung zwischen *F. verticillioides* und *F. musae*. Nach dem Aufbau einer ergänzenden Datenbank wurden 100% der Isolate der jeweiligen Spezies korrekt identifiziert, was zu *top-log-scores* zwischen 2,1 und 2,6 für *F. verticillioides* und 2,4 bis 2,5 für *F. musae* führte. Die Ergebnisse unterstreichen das Potenzial der MALDI-TOF MS-basierten Analyse auch für die Differenzierung eng verwandter Taxa innerhalb des FFSC, da die Verwendung von reifem Zellmaterial einen höheren Grad an taxonomischer Auflösung in komplexeren Massenspektren ermöglicht.

Fumonisine sind Mykotoxine, die Mais und Lebensmittelprodukte auf Maisbasis sowie Tierfutter kontaminieren. Sie wurden mit Störungen des Zentralnervensystems bei Pferden, Lungenödemen bei Schweinen sowie Neuralrohrdefekten und Speiseröhrenkrebs beim Menschen in Verbindung gebracht. Das *fum1*-Gen kodiert für eine Polyketidsynthase, die an der Biosynthese von Fumonisinen beteiligt ist. Es kommt im Genom aller Fumonisin-produzierenden *Fusarium* spp. vor. Ein zuverlässiger Nachweis von *fum1* kann eine Abschätzung des toxikologischen Potenzials von Pilzkulturen aber auch von Nahrungsmitteln liefern. Ein *fum1*-spezifischer LAMP-Assay wurde entwickelt und mit gereinigter DNA von 47 verschiedenen Spezies aus dem FFSC getestet. Das *fum1*-Gen wurde in 28 Arten nachgewiesen, unter denen *F. fujikuroi*, *F. globosum*, *F. nygamai*, *F. proliferatum*, *F. subglutinans* und *F. verticillioides* die wichtigsten in der Literatur beschriebenen Fumonisinproduzenten sind. Keine der 92 getesteten Pilzarten, die nicht der Gattung *Fusarium* gehören, zeigte Kreuzreaktionen mit dem neuen Assay. Die Nachweisgrenze (LOD) des neuen Assays beträgt 5 pg genomische DNA pro Reaktion für *F. fujikuroi*, *F.*

*nygamai* und *F. verticillioides*. Für andere LAMP-positive Spezies wurden höhere LODs gefunden. Abgesehen von reiner genomischer DNA konnten Fumonisinproduzenten auch nach Zugabe mechanisch lysierter Pilzkonidien mit einer LOD von $10^3$ Konidien / Reaktion als Matrize nachgeweisen werden. Die LAMP-Ergebnisse korrelierten gut mit der Produktion von Fumonisin $B_1$ ($FB_1$). Die aktuelle Studie berichtet erstmalig über die $FB_1$-Produktion in Stämmen von *F. coicis*, *F. mundagurra*, *F. pininemorale*, *F. sororula*, *F. tjataeba*, *F. udum* und *F. werrikimbe*. Die praktische Anwendbarkeit des LAMP-Assays wurde durch Analyse von $FB_1$-kontaminierten Maiskörnern demonstriert. Es wurde gezeigt, dass der neue LAMP-Assay zur Diagnose typischer Fumonisinproduzenten schnell, empfindlich und zuverlässig ist und als vielseitiges Instrument in HACCP-Konzepten zur Reduzierung von Fumonisinen in der Lebensmittel- und Futtermittelkette eingesetzt werden könnte.

*Fusarium verticillioides* ist der wichtigste Produzent von $FB_1$.Die Produktion des Toxins sowie die Entwicklung des Pilzes wird durch Umweltbedingungen, insbesondere Kohlenstoffquellen, beeinflusst. Um die Expression von *fum1* als Schlüsselgen in der Biosynthese von $FB_1$ zu detektieren, wurde ein zuverlässiger und schneller RT-LAMP-Assay (*Reverse Transcription Loop-mediated isothermal amplification*) entwickelt und optimiert. Der neue RT-LAMP Assay wurde zum Nachweis der *fum1*-Expression in zwei *F. verticillioides*-Stämmen angewendet, die aus Mais bzw. Weizen isoliert wurden. Beide Stämme wurden in Czapeks Medium kultiviert, das vier verschiedene Zucker als einzige Kohlenstoffquelle enthielt. Die Analyse der *fum1*-Expression wurde von einer $FB_1$-Bestimmung unter Verwendung einer LC-MS/MS-Analyse begleitet. Hohe Wachstumsraten und die Produktion von $FB_1$ wurden in Fructose-haltigem Medium für den Stamm beobachtet, der aus Mais stammte. Bei Verwendung von Maltose als einziger Kohlenstoffquelle für beide Stämme trat eine geringere Produktion von $FB_1$ auf. Die *fum1*-Genexpression begann zwischen 2 und 4 Tagen nach der Inokulation, und positive Signale

wurden vor dem Auftreten messbarer Mengen von $FB_1$ nachgewiesen. Der RT-LAMP-Assay war beim Nachweis der *fum1*-Genexpression in sehr frühen Stadien des Wachstums von *F. verticillioides* wirksam und kann daher ein nützliches Werkzeug für die frühe Vorhersage der $FB_1$-Bildung sein.

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# 9 Publications

The thesis was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – Brazil) with the project grant number 201391/2015-5. The financial assistance included for the author of this thesis did neither influence the design of the study nor the interpretation of its results.

**First authorship publications in peer-reviewed journals derived from this thesis (Tatsch, É.F. and Wigmann, É. F. are the same person):**

- **Évelin F. Tatsch**, Rudi F. Vogel, Ludwig Niessen. 2021. Identification of the banana fruit pathogen *Fusarium musae* under two different culture conditions using MALDI-TOF mass spectrometry. (under revision)

  Parts of this publication are constituents of the results and discussion chapters 4.1. and 5.1. and revisited in the introduction and methods parts.
- **Évelin F. Tatsch**, Karsten Meyer, Rudi F. Vogel, Ludwig Niessen. 2021. Characterization of the influence of carbon sources on *fum1* gene expression in the fumonisin producer *Fusarium verticillioides* using RT-LAMP assay. International Journal of Food Microbiology 354, 109323

  Parts of this publication are constituents of chapters 4.3. and 5.3., and revisited in the introduction and methods parts.
- **Évelin F. Wigmann**, Karsten Meyer, Eugenia Cendoya, Ronald Maul, Rudi F. Vogel, Ludwig Niessen. 2020. A loop-mediated isothermal amplification (LAMP) based assay for the rapid and sensitive group-specific detection of fumonisin producing *Fusarium* spp. International Journal of Food Microbiology 325, 108627

Parts of this publication are constituents of chapters 4.2. and 5.2., and revisited in the introduction and methods parts.

- **Évelin F. Wigmann**, Jürgen Behr, Rudi F. Vogel, Ludwig Niessen. 2019. MALDI-TOF MS fingerprinting for identification and differentiation of species within the *Fusarium fujikuroi* species complex. Applied Microbiology and Biotechnology 103, 5323 – 5337.

  Parts of this publication are constituents of chapters 4.1. and 5.1., and revisited in the introduction and methods parts.

**First authorship of presentations at international conferences derived from this thesis:**

- **Oral presentation** at International Commission on Food Mycology, Freising – Germany. 2019
- **Poster presentation** at European *Fusarium* Seminar EFS14, Tulln – Austria. 2018
- **Poster presentation** at 40$^{th}$ Mycotoxin Workshop, Munich – Germany. 2018
- **Poster presentation** at IV International Conference on Microbial Diversity, Bari – Italy. 2017

**Collaborations:**

- LC-MS/MS analyses of pure culture samples were conducted in collaboration with Dr. Karsten Meyer and René Mamet (Chair of Animal Hygiene, Technical University of Munich, Weihenstephaner Berg 3, 85354 Freising, Germany)

- LC-MS/MS analyses of naturally contaminated samples were conducted in collaboration with Dr. Eugenia Cendoya (Research Institute on Mycology and Mycotoxicology (IMICO), CONICET – Universidad Nacional de Río Cuarto, Ruta 36 Km 601, 5800 Río Cuarto, Cordoba, Argentina) and Dr. Ronald Maul (Unit Plant Toxins and Mycotoxins, Dept. Safety in the Food Chain, German Federal Institute for Risk Assessment, Max-Dohrn-Str. 8-10, 10589 Berlin, Germany.

## 10 Statutory declaration

I hereby declare that I wrote the present Ph.D. thesis with the topic:

"**Taxonomy, diagnosis and mycotoxicology of species belonging to the *Fusarium fujikuroi* species complex (FFSC)"**

independently and used no other aids than those cited. In each individual case, I have clearly identified the source of the passages that are taken word for word or paraphrased from other works. Other contributions to this work in terms of collaboration are clearly indicated and acknowledged in the "publications" section.

Freising,

Évelin Francine Tatsch.