Ecological interactions of *Fusarium* species and the meal beetle *Tenebrio molitor*

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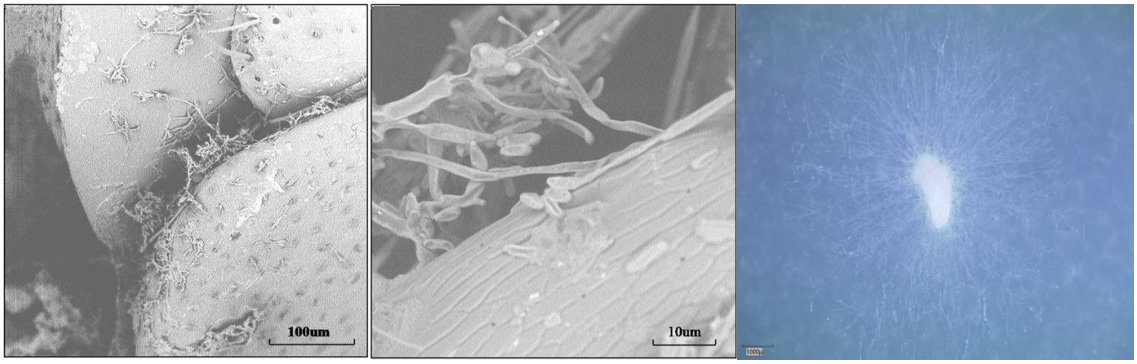

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Dedicated to my parents, my husband,

and the youth time faded away

Abstract

Ecological interactions of *Fusarium* species and the meal beetle *Tenebrio molitor*

Contamination of stored grains by moulds and their mycotoxins results in quality and nutritional reduction. Ingestion of contaminated products and feed poses a significant hazard to human and animal health. The moulds deterioration and storage insects are of major concern in poor post-harvest management conditions. The interactions between the storage moulds (*Fusarium* spp.) and insects (*T. molitor*) are bilateral.

Fusarium species colonized wheat kernels affected the feeding behaviors, weight gain and survival rate of *T. molitor* mature larvae. Wheat kernels colonized by *F. proliferatum* and *F. poae* attracted *T. molitor* larvae significantly more than untreated (control) kernels; whereas kernels colonized with *F. avenaceum* or *Beauveria bassiana* were avoided by the larvae. However, larvae fed on *F. culmorum*, *F. avenaceum* or *B. bassiana* colonized kernels had enhanced larvae mortality. Our results indicated that *T. molitor* larvae have the ability to sense potential survival threats of kernels colonized with *F. avenaceum* or *B. bassiana*.

T. molitor acted as vehicle for *Fusarium* spp. dissemination within grains. After feeding on *Fusarium* mycelia and conidia for 24 h, live conidia were traceable in beetles' excreta. Beetles were capable of contaminating a high proportion of wheat kernels up to 20 days. Kernels contamination and fungal colony forming unit density of *F. proliferatum* were higher than other tested *Fusarium* species. *T. molitor* beetles disseminated tested *Fusarium* fungal conidia internally and externally. Fungal contamination by beetle copulation to eggs was first described in our present research.

F. proliferatum drew our research attention for its attracting meal beetles property. Pathogenicity of different *F. proliferatum* strains on *T. molitor* was assessed according to mortality on 4th instar larvae. Radiate growth rate, sporulation, and DNA biomass in larvae tissue were evaluated as parameters to determine the contribution to pathogenicity. For pathogenicity on wheat plants, we demonstrated that *F. proliferatum* strains can systemically colonize the wheat plant (cv. Taifun) from soak-inoculation of mature wheat seeds to stem, leaf and then to wheat kernels. The pathogenicity on *T. molitor* larvae and wheat plants was not consistent, which indicated different pathogenicity mechanisms.

Kurzfassung

Ökologische Interaktion zwischen *Fusarium* Spezies und dem Mehlkäfer *T. molitor*

Die Kontamination eingelagerten Getreides mit Vorratspilzen und deren Mykotoxinen sowie der Befall mit Vorratsschädlingen führen zur Minderung von Qualität und Nährwert. Durch die Aufnahme kontaminierter Nahrungs- und Futtermittel entstehen erhebliche Gefahren für die menschliche und tierische Gesundheit. Die Interaktionen zwischen Vorratspilzen (*Fusarium* spp.) und Insekten (*T. molitor*) sind bilateral. Von *Fusarium* besiedelte Weizenkörner beeinflussten Nahrungsaufnahme, Gewichtszunahme und Überlebensrate von *T. molitor* Larven. Von *F. proliferatum* und *F. poae* befallene Körner lockten signifikant mehr *T. molitor* Laven an als unbehandelte Körner, während Körner mit *F. avenaceum* oder *Beauveria bassiana* von den Larven gemieden wurden. Larven, die mit *F. culmorum*, *F. avenaceum* oder *B. bassiana* befallen Körner gefressen hatten, wiesen eine höhere Sterblichkeit auf. Unsere Ergebnisse deuten darauf hin, dass *T. molitor* Larven die Fähigkeit haben, potentielle Schadwirkungen von Körnern mit *F. avenaceum* oder *B. bassiana* zu erkennen. *T. molitor* fungierte als Vehikel für *Fusarium* spp. Nachdem die Käfer 24 h mit *Fusarium* Myzel und Konidien gefüttert worden waren, waren lebende Konidien in ihren Exkrementen nachweisbar. Die Käfer waren 20 Tage lang in der Lage, große Anteile exponierter Weizenkörner zu kontaminieren. Die Menge der durch die Käfer kontaminierten Weizenkörner und die Dichte der koloniebildenden Einheiten waren bei *F. proliferatum* höher als bei den anderen untersuchten *Fusarium* Spezies. *T. molitor* verbreitete die Konidien von *Fusarium* intern und extern. Die Kontamination von Eiern durch die Kopulation wird erstmals in unserer vorliegenden Arbeit beschrieben. Die Pathogenität verschiedener *F. proliferatum* Stämme wurde anhand der Sterblichkeitsrate des vierten Larvenstadiums von *T. molitor* bewertet. Als Parameter der Pathogenität wurden radiale Wachstumsrate, Sporulation und Pilz-DNA in Larvengewebe untersucht. Es konnte gezeigt werden, dass *F. proliferatum* Stämme in der Lage sind, ausgehend von einer Tauchinokulation von Saatgut die daraus entstehenden Pflanzen systemisch über Stängel und Blätter bis zu den Körnern zu besiedeln. Die Pathogenität für Weizenpflanzen korrelierte nicht mit der für *T. molitor* Larven, was auf unterschiedliche Mechanismen der Pathogenität hinweist.

List of abbreviations

ANOVA	:	Analysis of variance
° C	:	Celsius
CI	:	Confidence Interval
CLA	:	Carnation Leaf-piece Agar
cm	:	Centimeter
CTAB	:	Cetyltrimethyl Ammonium Bromide
cv	:	Cultivar
CYP	:	Cytochrome P450
CZID-Agar	:	Czapek-Dox-Iprodione-Dichloran Agar
d.f.	:	Degree of freedom
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxynucleotide triphosphate
EDTA	:	Ethylenediaminetetraacetic acid
FB	:	Fumonisins B type
FHB	:	*Fusarium* Head Blight
Fig.	:	Figure
FUM	:	Fumonisins synthesis gene
GFP	:	Green Fluorescent Protein
GLM	:	Generalized linear model
h	:	Hour
HPLC	:	High-performance liquid chromatography
HT-2	:	HT-2 toxin
LOD	:	Limits of detection
LOQ	:	Limits of quantification
mg	:	Milligram
ml	:	Milliliter
min	:	Minutes
mm	:	Millimeter

mM	:	Millimole
N	:	Number of replications
ng	:	Nanogram
ns	:	No significant differences
PCR	:	Polymerase Chain Reaction
PDA	:	Potato Dextrose Agar
pg	:	Picogram
qPCR	:	quantitative real-time Polymerase-Chain-Reaction
r	:	Regression
RH	:	Relative Humidity
rpm	:	Rotation per minute
S.D.	:	Standard deviation
SEM	:	Scanning electron microscopy
Sig.	:	Significance
spp.	:	species
$S=\pi r^2$	:	Square, Radius
T-2	:	T-2 toxin
TE	:	Tris-ethylendiamin-tetra acetat
TEF	:	Translation Elongation Factor
tri	:	Trichothecenes synthesis gene
µg	:	Microgram
µl	:	Microliter

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Chapter 1 General introduction

1. Important of *Fusarium* species

The genus *Fusarium* (Ascomycota, Nectriaceae) was introduced by Link in 1809 (Link, 1809) as species with fusiform conidia borne on a stroma (Cai et al., 2011). *Fusarium* species are the most diverse and ubiquitously distributed soil-borne plant-pathogenic fungi (Summerell et al., 2010). They cause serious diseases of maize (Logrieco et al., 2002b), small-grain cereals like wheat, barley, rice, sorghum, oat (Parry et al., 1995), vegetables such as tomato, eggplant (Katan et al., 1976), cucumber (Liu et al., 1995) and asparagus (Seefelder et al., 2002), and even trees, for example chestnuts (Chen & Zhu, 2011) and others (Dochinger & Seliskar, 1962). Decreased yields as well as diminished quality of plant products due to *Fusarium* infection cause significant economic losses worldwide (Placinta et al., 1999, Glenn, 2007) Beside plants, the members of this genus incite directly diseases in humans (Nir-Paz et al., 2004), and domesticated animals (Goyarts & Dänicke, 2006, Kallela & Ettala, 1983, Marasas et al., 1988)

Fusarium head blight (FHB) of wheat and other small grain cereals is a destructive disease with huge economic impact in many wheat and barley growing area around the world, especially in the humid and semi-humid wheat-growing regions (Bai & Shaner, 1994). The infection of heads of small grain cereals and maize plants with *Fusarium* species impairs both grain yields and quality (Parry et al., 1995) in various ways, including adversely affected grain size, weight, germination rate, protein content, baking quality of the flour, fungal colonization of the ear and cutting off the supply of nutrients to the upper spikelet, seedling blight as a result of seed infections and mycotoxins in the grain products (Brandfaß, 2006, Snijders, 1990). FHB is caused by several fungal species. The predominant *Fusarium* species in European are *F. graminearum*, *F. culmorum*, *F. avenaceum* and *F. poae* (Bottalico & Perrone, 2002).

Cereals contaminated by *Fusarium* usually take place in the field before or during harvest, but infection may develop in the field or in storage (Agrios, 2005).

1.1 *Fusarium avenaceum* description

Fusarium avenaceum (teleomorph *Gibberella avenacea* R. Cook) is the main pathogen that causes head blight of wheat (Kang et al., 2005). *F. avenaceum* is predominantly soil-borne and causes damaged grain kernels. The infected kernels are smaller, shriveled, and discolored (white to pale pink). Additionally, the quality and yield are reduced and accompanied with accumulation of toxic secondary metabolites in the infected kernels and chaff (Abramson et al., 1987). *F. avenaceum* also causes damping-off, root rot, stalk rot, or fruits rot under conditions adverse to vegetable host plants like broccoli and fruits rot (Gerlach & Nirenberg, 1982, Mercier et al., 1991).
F. avenaceum can synthesize significant quantities of secondary toxic metabolites in vitro, such as beauvericin, enniatins (Logrieco et al., 2002c), fusarin C (Farber & Sanders, 1986), moniliformin (Marasas et al., 1984b). Beauvericin and enniatins have similar cyclic hexadepsipeptides structure with a specific cholesterol acyltransferase inhibitor activity (Tomoda et al., 1992). Beauvericins have toxicity to several human cell lines (Logrieco et al., 2002a) and induce cell apoptosis and DNA fragmentation (Logrieco et al., 2002a, Ojcius et al., 1991). Furthermore, beauvericin is suggested as a potential risk of cardiotoxicity in contaminated grains and foods. Enniatins cyclic peptides, primarily enniatins A, B and B_1, are synthesized by some strains of *F. avenaceum* under laboratory (Logrieco et al., 2002c) and field conditions (Jestoi et al., 2004). Enniatins are reported that to a role in plant pathogenicity (Herrmann et al., 1996). *F. avenaceum* is not a trichothecenes producer because of the absence of carrying tri5 gene, which is essential for the production of trichothecenes (Tan & Niessen, 2003).

F. avenaceum toxicoses to any animals or human beings haven't been reported, but ground crop products are toxic to chicks and mice, and are dermotoxic to rabbits

(Marasas et al., 1984a). *F. avenaceum* possesses resistance to most clinical antifungals, while amphotericin B is reported as the most effective (Pujol et al., 1997).

1.2 *Fusarium culmorum* description

Fusarium culmorum is one of predominant *Fusarium* species associated with *Fusarium* head blight, particularly in cool parts of Europe like north, central and west Europe (Parry et al., 1995). Beside head blights, it also causes cereal foot rots (Leslie et al., 2006). Plants ears with head blights have shrunken and chalk kernels, but these infected kernels are not responsible for disease dispersal in the field conditions (Gilbert et al., 2003). *F. culmorum*, closely related with *F. graminearum*, are two important fungi becoming the more commonly observed cause of head blight disease (Waalwijk et al., 2003). Well-known as a soil fungus, *F. culmorum* can be splash dispersed to the heads of some small grain cereals, such as: wheat and barley (Jenkinson & Parry, 1994), where head blight symptoms are shown and grains are extensively colonized (Jackowiak et al., 2005). Arabidopsis thaliana is susceptible to the cereal ear blight fungal pathogen *F. culmorum*, which may assist the research of host pathogen interactions of this fungus (Urban et al., 2002).

F. culmorum synthesizes mycotoxins such as moniliformin (Scott et al., 1987), deoxynivalenol and related trichothecenes (Quarta et al., 2005, Marasas et al., 1984b), fusarin C (Farber & Sanders, 1986), zearalenone (Marasas et al., 1984b) and steroids (Burmeister & Vesonder, 1990). Reports of the production of T-2 toxin and neosolaniol by *F. culmorum* have not been substantiated. *F. culmorum* is a trichothecenes producer because it carries tri5 and tri6 genes (Covarelli et al., 2004), They are essential for the production of trichothecenes. Trichothecene production is an important factor in *Fusarium* head blight of small grains (Snijders & Perkowski, 1990, Miedaner & Reinbrecht, 2001).

1.3 *Fusarium poae* description

Parryet al. (Parry et al., 1995) reported that up to 17 *Fusarium* species have been associated with *Fusarium* head blight. *Fusarium poae* is one of most common of *Fusarium* complex species responsible for ear (head) blight of small grain cereals according to the survey in Great Britain, Argentina, Canada, Germany, and Italy, and other countries (Parry & Nicholson, 1996). *F. poae* was associated with the species most commonly isolated from glumes affected by ear blight symptoms according to the ear diseases survey of winter cereals. It is a relatively weak pathogen with a high frequency, however, Abramson et al. (Abramson et al., 1993) found that *F. poae* alongside with *Gibberella zeae* and *F. sporotrichioides* synthesized the greatest amount and the greatest number of trichothecene mycotoxins.

Trichothecenes mycotoxins can cause human and animal toxigenicity through consumption of colonized cereal grains by *F. poae* (Leslie et al., 2006). Nivalenol (NIV), a type B trichothecene, was cited as one of the most common mycotoxins among all mycotoxins synthesized by *F. poae*. (Stenglein et al., 2014, Jestoi et al., 2008, Vogelgsang et al., 2008). *F. poae* also can produce trichothecenes of type A such as T-2 and HT-2, among others (Thrane et al., 2004). Moreover, mycotoxins syntheses depend on the host plants. For example, fusarenone-X and nivalenol (Foremska et al., 1999) are produced by *F. poae* strains inoculated maize. Nivalenol is produced when *F. poae* inoculated onto barley (Salas et al., 1999). Isolates of *F. poae* can produce beauvericin (Logrieco et al., 1998) and fusarin C (Farber & Sanders, 1986).

1.4 *Fusarium proliferatum* description

Fusarium proliferatum (sexual stage: *Gibberella intermedia*) is an important pathogen infecting numerous crop plants worldwide and causes serious economic problems on agricultural cultivation. In various climatic zones, *F. proliferatum* colonizes an extra -ordinarily broad range of host plants including maize (Marín et al., 1998), wheat (Desjardins et al., 2007), barley (Jurado et al., 2010), rice (Park et al., 2005), sorghum

(Leslie et al., 1990), asparagus (Seefelder et al., 2002), banana (Jimenez et al., 1993), date palm (Abdalla et al., 2000), garlic, onion (Stankovic et al., 2007) and so on. Infection of plants with *F. proliferatum* leads to products quality decline, and a decrease in yield to unprofitable level.

The toxicity of *F. proliferatum* to ducklings was determined by feeding on contaminated feed, the results indicatedthat the toxicity was corresponding with the amount of moniliformin produced instead of the amount of fumonisins produced (Vesonder & Wu, 1998). After experimental rats ingested in contaminated grain, also causes death, they appeared hemorrhage and diarrhea symptoms (Abbas et al., 1988). *F. proliferatum* is the main producer of fumonisins mycotoxins with high levels in food and feed products (Leslie et al., 2004). Moreover, the FUM gene cluster required for fumonisin biosynthesis has been sequenced and characterized in some detail (Waalwijk et al., 2004).

Besides, *F. proliferatum* is capable of synthesizing other mycotoxins, including beauvericin (Leslie et al., 2004), enniation (Meca et al., 2010) , fusaric acid (Bacon et al., 1996), fusarin (Miller et al., 1995, Bacon et al., 1996), fusaproliferin (Leslie et al., 2004, Reynoso et al., 2004, Ritieni et al., 1995) and moniliform (Logrieco et al., 1995, Miller et al., 1995).

2. Mycotoxins: trichothecenes, zearalenones and fumonisins

Microorganisms produce primary metabolites during active cell growth: such as amino acids, carbohydrates, acetone, ethanol, vitamins, organic acids. Micro organisms also can synthesize secondary metabolites such antibiotics, alkaloids and toxins, which are not essential for their growth, but play an important role for protecting themselves to survive from the enemy.

Mycotoxins are worldwide discovered in many different agricultural commodities and foods because pathogens (molds) can be dispersed by soil, air, water, insects and

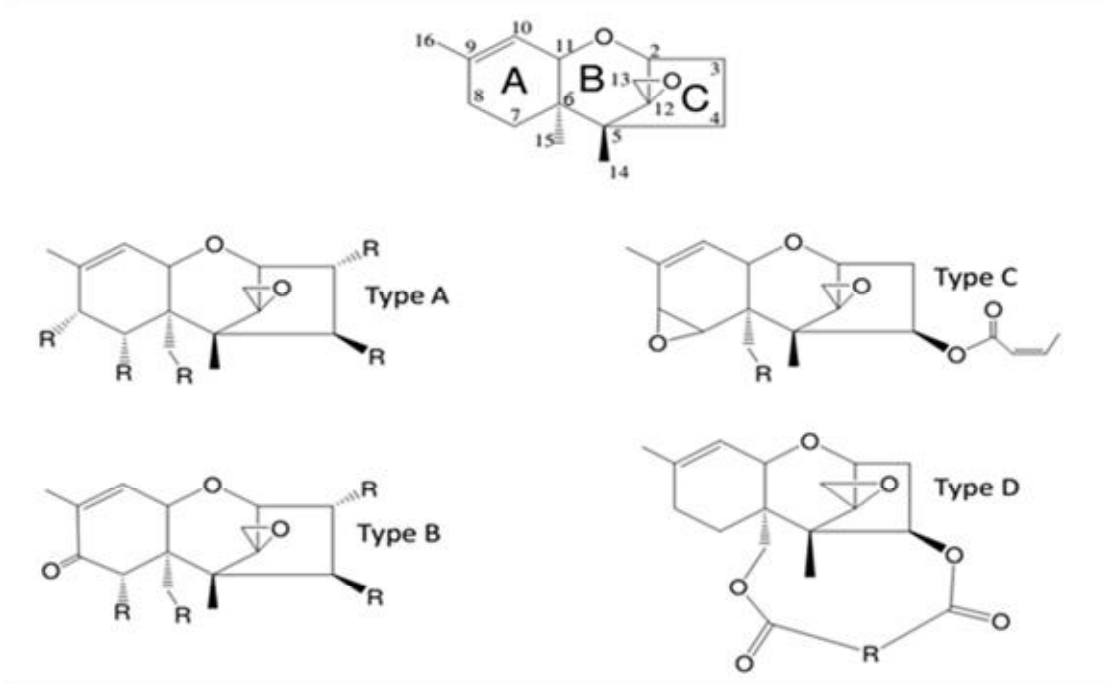
vegetation (Smith, 2005). Mycotoxins can be produced during the growing seasons, the post-harvest period or in some cases, during all storage, transport and food processing stages (Smith, 2005). The six main genera synthesizing mycotoxins are *Aspergillus*, *Claviceps*, *Fusarium*, *Penicillum*, *Neotyphodium* associated with plant tissue and *Stachybotrys* related with construction materials (Smith, 2005). *Fusarium* mycotoxins are produced due to complex interaction of biological, chemical, and physical factors and have been found in India, Japan, several African and European countries, and North America (Fratamico et al., 2005). Most *Fusarium* mycotoxins are produced in the field and remain in the storage grain.

Chelkowski (Chelkowski, 1998) reported that although about 20 *Fusarium* species can infect small grains worldwide, only six species cause more concern because of the mycotoxins they produce: *F. culmorum* and *F. graminearum* produce deoxynivalenol or vomitoxin, nivalenol, and zearalenone; *F. poae* produces nivalenol; and *F. avenaceum* produces moniliform and *F. verticilliodes* and *F. proliferatum* produce fumonisins in maize (Smith, 2005).

Three major classes of mycotoxins produced by *Fusarium* that have been proven to cause animal disease outbreaks: trichothecenes, zearalenones and fumonisins (Desjardins, 2006).

2.1 Trichothecenes

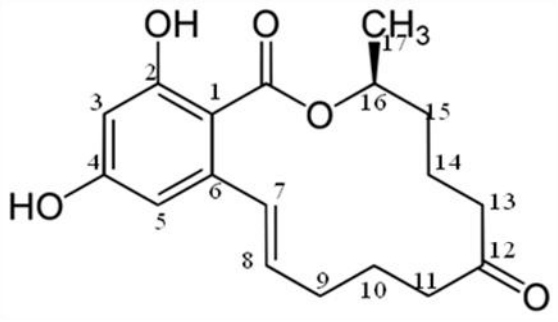
Trichothecenes are agriculturally important mycotoxins of relevance to chronic and fatal toxicosis of human and animals (Desjardins, 2006). All trichothecenes contain an epoxide at the $C_{12,13}$ position, which is responsible for their toxicological function. Different classifications of trichothecenes (Type A, B, C, D) depend on chemical structures on C position (Sudakin, 2003).



Main structure of trichothecenes (McCormick et al., 2011)

2.2 Zearalenones

Zearalenones are not acutely toxic and have not been associated with any fatal mycotoxicoses in humans or animals. Zearalenones are non-steroidal estrogenic mycotoxins and have been associated with estrogenic syndromes in swine and experimental animals (Desjardins, 2006).



Main structure of zearalenones (Desjardins, 2006)

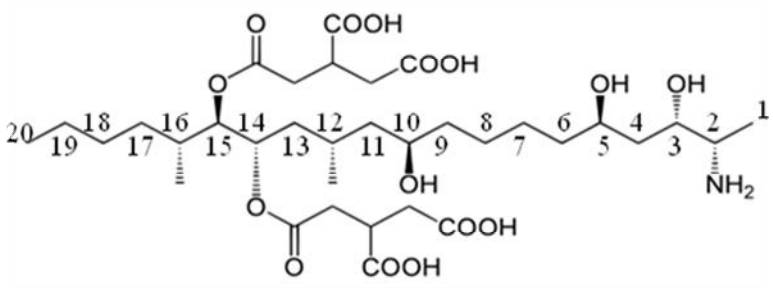
Zearalenone	Molecular Weight	Carbon position[a] C-4	C-5	C-7—C-8	C-11	C-12	C-14
Zearalenone	318	OH	H	C=C	H	1=O	H
α-and β-Zearalenol	320	OH	H	C=C	H	OH	H
α-and β-Zearalanol	322	OH	H	C-C	H	OH	H
11-Hydroxyzearalenone	334	OH	H	C=C	OH	1=O	H
14-Hydroxyzearalenone	334	OH	H	C=C	H	1=O	OH
4-Acetylzearalenone	360	OAc	H	C=C	H	1=O	H
5-Formylzearalenone	346	OH	CHO	C=C	H	1=O	H

a Numbering system has been simplified for clarity (Desjardins, 2006)

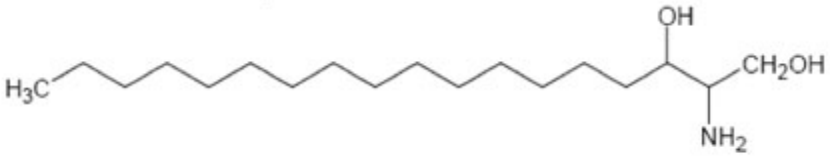
2.3 Fumonisins

Fumonisins are a family of toxic and carcinogenic mycotoxins produced by *F. verticillioides* (formerly *Fusarium moniliforme*) and *F. proliferatum*. The chemical structures of six different molecules of the fumonisin group (FA_1, FA_2, FB_1, FB_2, FB_3 and FB_4) have been described. Only 'B type' fumonisins have been shown to be toxic. FB_1 is the most abundant and most toxic of fumonisins found in contaminated maize (Visconti & Doko, 1994). FB_1 is hepatotoxic and hepatocarcinogenic for rats (Gelderblom & Sny136 man, 1991; Gelderblom et al., 1991). It causes weight loss in chickens and injuries to several organs (kidney, liver and heart) (Javed et al., 1992a, b). In vitro assays on animal cell cultures show that FB_1 inhibits cellular multiplication and causes cytotoxicity in some cell strains (Shier et al., 1991; Norred et al., 1992; Yooet al., 1992; Abbas et al., 1993). Recently, Wang et al., (1991) demonstrated that FB_1 inhibits the biosynthesis of sphingolipids, which play critical roles in a number of cellular functions, such as cell-cell communication, differentiation and cell transformation. FB_1 is the first naturally-occurring inhibitor of sphinganine and sphingosine-N-acyltransferase (Abado‐Becognee et al., 1998, Wang et al., 1991, Yoo et al., 1992, Riley et al., 1993).

The striking structural similarity of fumonisins to sphinganine and other long-chain sphingoid based the possibility that fumonisins might disturb sphingolipid metabolisim (Desjardins, 2006).

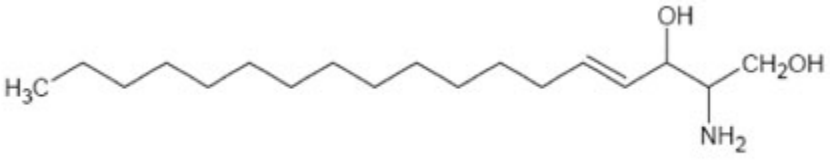


Main structure of fumonisins (Desjardins, 2006)



Sphinganine

(Griessler & Encarnação, 2009)



Sphingosine

(Griessler & Encarnação, 2009)

Fumonisins	Molecular Weight	Carbon Position C-2	C-5	C-10
Fumonisin B_1	721	NH_2	OH	OH
Fumonisin B_2	705	NH_2	OH	H
Fumonisin B_3	705	NH_2	H	OH
Fumonisin B_4	689	NH_2	H	H
Fumonisin A_1	763	N-acetyl	OH	OH
Fumonisin A_2	747	N-acetyl	OH	H
Fumonisin A_3	747	N-acetyl	H	OH
Fumonisin P_1	800	N-hydroxypyridinium	OH	OH
Fumonisin P_2	784	N-hydroxypyridinium	OH	H
Fumonisin P_3	784	N-hydroxypyridinium	H	OH

(Desjardins, 2006)

Mechanism of fumonisins: the toxicity of fumonisin is based on the structural similarity to the sphingoid bases: sphingosine and sphinganine. They are inhibitors of sphinganine (sphingosine) N-acyltransferase (ceramide synthase), a key enzyme in the lipid metabolism, resulting in a disruption of this pathway. This enzyme catalyzes the

acylation of sphinganine in the biosynthesis of complex sphingolipids and also the deacylation of dietary sphingosine and the sphingosine that is released by the degradation of complex sphingolipids (ceramid, sphingomyelin and glycosphingolipide) (Wang et al. 1991). Sphingolipids are basically important for the membrane and lipoprotein structure and also for cell regulations and communications (second messenger for growth factors) (Berg et al. 2003). The other consequence of inhibition ceramide synthase is rapid increase of sphingoid bases (sphinganine and sphingosine). Free sphingoid bases are toxic to most cells by affecting cell proliferation and induce apoptosis or necrotic cell death (Riley et al. 1996; Stevens et al. 1990 (Griessler & Encarnação, 2009).

3. Post-harvest infection of *Fusarium*

Knowledge of a wide variety of abiotic and biotic factors influence grain quality after harvest has been studied in the stored grain ecosystem. Important factors include respiration of crop kernels and contaminant mould, storage insects and mites, and the key mini-environmental factors of water availability and temperature. Associations between these factors influence the dominance of fungi, particularly mycotoxigenic species.

A post-harvest survey was conducted in cereals like wheat, barley and oats to determine *Fusarium* species occurrence and geographic distribution in Norwegian (Kosiak et al., 2003). According to the investigation results, *F. avenaceum*, *F. poae*, *F. tricinctum* and *F. culmorum* were most frequently isolated *Fusarium* species (Kosiak et al., 2003). Poor post-harvest management of cereals can cause fast deterioration in kernels nutritional quality. Microbial (bacteria, yeasts and filamentous fungi) activities can cause undesirable effects in grains including discoloration, contribute to increasing the temperature and relative humidity in the microenvironment and losses in dry matter like starch through the utilization of carbohydrates as energy sources, degrade lipids and proteins or alter their digestibility, produce volatile metabolites

giving off-odors, cause loss of germination rate of seeds, reduce baking and malting quality; affect utilization as animal feed or as seed (Magan et al., 2004). Studies have shown that growth, mycotoxin production, competitiveness and niche occupation by mycotoxigenic species are influenced by the presence of other contaminant moulds and environmental factors. This has been demonstrated for both *Fusarium culmorum* and deoxynivalenol production, *Aspergillus ochraceus*/*Penicillium verruscosum* and ochratoxin production and *Fusarium* section Liseola and fumonisin production. Certain species of both fungi and insects in grain storage are primary causes of deterioration and often these are also intimately associated (Dunkel, 1988). Therefore, a more holistic ecological view is needed when considering long-term-safe storage management approaches to of cereal grains after harvest (Magan et al., 2003).

4. Storage insects *Tenebrio molitor*

Tenebrio molitor (Coleoptera: *Tenebrionidae*) is an important and globally distributed insect of stored grain ecosystem and its capability of selecting optimal ratios of dietary components (Morales-Ramos et al., 2011). It reacts to protein quality changes of the nutritional feed substrate (Davis & Sosulski, 1974) and is sensitive to small quantitative variations of dietary composition (Davis, 1969). *T. molitor* mature larvae perform preference to *F. proliferatum* 21.1 colonized wheat kernels and avoidance to *F. avenaceum* colonized wheat kernels causing high mortality (Guo et al., 2014), which indicate that *T. molitor* larvae are capable to screen adverse factors, such as mycotoxins synthesize by different *Fusarium* species.

5. Fungi transmission by storage insects

The close relationship between fungi and storage insects in the stored grain ecosystem has been well studied (Dunkel, 1988). Insects damage grains by ingestion, oviposition and also the body friction. All of these actions break the seed envelop which allows entry of fungi (Dunkel, 1988). Storage insects play an important role in the distribution of spores. Some insects externally disseminate the fungal spores attached

on the cuticle of the insects (antenna, mouthpart, wings and legs) and internally disseminate the fungal spores survived from the gut passage by excretion. Windels also supposed that picnic beetles *Glischrochilus quadrisignatus* are attracted to variety of habitats could acquire various *Fusarium* species. *Fusarium* species were isolated from both external and internal of larvae, pupae and newly emerged beetles (Windels et al., 1976). Some insects are known to use spoilage moulds as a food source. Some fungi can provide B vitamins not available in their stored product diet (Dunkel, 1988). With their metabolic activity water and heat production, storage insects can increase the water activity and temperature of grain to levels suitable for fungal growth. Fungi affect the behavior, growth and reproduction of insects. In addition, fungi are important olfactory signals for insects in the storage system (Dunkel, 1988).

6. Management of postharvest grain contamination

Post-harvest grains contamination by fungi has not drawn so much attention. The management of postharvest deterioration and spoilage by of grains, and commercial feeds depend on certain precautions and storage conditions that must be fulfilled before and during harvest, and during transport and storage to minimize mycotoxins. The crop products should be healthy and high quality when they are harvested. Taken the following strategies into consideration, its subsequent infection and spoilage in storage will be diminished. First, the moisture content should be kept below the minimum levels which required for the growth of the common storage fungi. For example, when moisture content reaches to 13.0 to 13.2%, *Aspergillus* species will grow and cause starchy cereal seeds spoilage. Accurate and regular moisture measurements to ensure safe thresholds are not breached. Second, low temperature decelerates the respiration of grain and microorganisms. Most storage fungi grow very slowly at the temperature range 12 to 15 °C, and their growth almost ceases at 5 to 8 °C. Therefore, the temperature of stored grains should be kept as low as possible. Meanwhile, an increase of moisture in grain should be concerned. Third, infestation

of stored products by storage insects (*Tenebrio moliter*, *Tribolium castaneum*) and mites should be kept to a minimum through the use of fumigants. This helps keep the storage fungi from getting dispersed and growing rapidly. At last, the stored grain should avoid impurity, and be free of mechanical damage and broken seeds. Such grains decrease the risk infected by storage fungi compared to weakened or cracked grain. In addition, quick airflow drying through storage bins to remove excess moisture and heat is also an efficient approach. Strategies mentioned above can be combined for the prevention of spoiled grains and mycotoxins entering the human and animal food chains (Agrios, 2005).

7. Research objectives

Fusarium species are among the most diverse and widely dispersed plant-pathogenic fungi. *Fusarium* species can infect the plants in the field, even they contaminate the crops products after harvest, during the transport, or storage period. The general objective of this research is to investigate ecological interactions of the meal beetle *Tenebrio molitor* with diverse *Fusarium* species on wheat kernels. On one hand, whether *Fusarium* species or *Fusarium* colonized kernels affect the *T. molitor* larvae feeding behavior and mortality after ingestion on *Fusarium* mycelia or *Fusarium* colonized kernels. Different *F. proliferatum* strains pathogenicity on *T. molitor* larvae and wheat was assessed. Or the *T. molitor* larvae can benefit from feeding on the *Fusarium* mycelia or *Fusarium* colonized kernels. On the other hand, storage insects also disperse fungal spores in storage barn and cause more deterioration. In addition, insects can increase the water activity and temperature of grain to levels suitable for fungal growth.

The specific objectives of the research are as follows:

1) Investigate effects of fungal colonization of wheat grains with *Fusarium* spp. on food choice, weight gain and mortality of meal beetle larvae (*Tenebrio molitor*) to convince whether the larvae can screen adverse situations

2) *Fusarium* species dissemination within grain by the meal beetle *T. molitor* and the fungi dissemination routine and duration by beetle *T. molitor* to assess the deterioration and to improve post-harvest management approaches

3) Illustrate *F. proliferatum* strains pathogenicity on 4th instar *T. molitor* larvae and whether *F. proliferatum* mycelia penetrate and propagate in the larvae tissue from cuticle or gut

4) Quantification of fungal DNA biomass and mycotoxins amount in wheat kernels and stem, flag leaves to determine if *F. proliferatum* can systemically infect wheat plants

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Chapter 2 Effects of fungal colonization of wheat grains with *Fusarium* spp. on food choice, weight gain and mortality of meal beetle larvae (*Tenebrio molitor*)

Abstract

Species of *Fusarium* have significant agro-economical and human health-related impact by infecting diverse crop plants and synthesizing diverse mycotoxins. Here, we investigated interactions of grain-feeding *Tenebrio molitor* larvae with four grain-colonizing *Fusarium* species on wheat kernels. Since numerous metabolites produced by *Fusarium* spp. are toxic to insects, we tested the hypothesis that the insect senses and avoids *Fusarium*-colonized grains. We found that only kernels colonized with *F. avenaceum* or *Beauveria bassiana* (an insect-pathogenic fungal control) were avoided by the larvae as expected. Kernels colonized with *F. proliferatum, F. poae* or *F. culmorum* attracted *T. molitor* larvae significantly more than control kernels. The avoidance/preference correlated with larval feeding behaviors and weight gain. Interestingly, larvae that had consumed *F. proliferatum*- or *F. poae*-colonized kernels had similar survival rates as control. Larvae fed on *F. culmorum*-, *F. avenaceum*- or *B. bassiana*-colonized kernels had elevated mortality rates.

HPLC analyses confirmed the following mycotoxins produced by the fungal strains on the kernels: fumonisins, enniatins and beauvericin by *F. proliferatum*, enniatins and beauvericin by *F. poae,* enniatins by *F. avenaceum,* deoxynivalenol and zearalenone by *F. culmorum*. Our results indicated that *T. molitor* larvae have the ability to sense potential survival threats of kernels colonized with *F. avenaceum* or *B. bassiana*, but not with *F. culmorum*. Volatiles potentially along with gustatory cues produced by these fungi may represent survival threat signals for the larvae resulting in their avoidance. Although *F. proliferatum* or *F. poae* produced fumonisins, enniatins and beauvericin during kernel colonization, the larvae were able to use those kernels as diet without exhibiting increased mortality. Consumption of *F. avenaceum*-colonized kernels, however, increased larval mortality; these kernels had higher enniatin levels than *F. proliferatum* or *F. poae*-colonized ones suggesting that *T. molitor* can tolerate or metabolize those toxins.

1. Introduction

Fusarium species (Ascomycota, Nectriaceae) are among the most diverse and widespread plant-infecting fungi (Summerell et al., 2010). They cause important diseases of maize, small-grain cereals, vegetables and even trees (Parry et al., 1995). Decreased yield as well as diminished quality of plant products due to *Fusarium* infection cause significant economic losses worldwide (Placinta et al., 1999, Glenn, 2007). Moreover, *Fusarium* species are prominent producers of medically relevant mycotoxins (D'mello et al., 1999, Desjardins, 2006). Toxicologically most important mycotoxins produced by *Fusarium* species comprise sesquiterpenoids trichothecenes such as T-2 toxin and deoxynivalenol (McCormick et al., 2011), polyketides such as fumonisins (Nelson et al., 1993), and depsipeptides such as beauvericin or enniatins (Moretti et al., 2007, Wang & Xu, 2012). Mycotoxin exposure resulting from the ingestion of contaminated products poses a hazard to human and animal health (Pestka, 2007, Placinta et al., 1999, Voss et al., 2007). Moreover, immunocompromized patients occasionally develop invasive fusariosis caused most often by *F. solani*, *F. oxysporum,* or *F. verticillioides* (Nucci & Anaissie, 2007, Cambuim et al., 2007) and some mycotoxins were found to suppress humoral as well as cell-mediated immunity in mammals (Voss et al., 2007, Pestka, 2007).

Beauvericin a cyclic hexadepsipeptide was isolated from an entomopathogenic fungus *Beauveria bassiana* and was demonstrated to be toxic to invertebrates (Hamill et al., 1969) before it was identified in extracts of two *Fusarium* species that were toxic to Colorado potato beetle (Gupta et al., 1991). Toxicity of beauvericin to insects is now well established (Grove & Pople, 1980, Wang & Xu, 2012). Enniatins, chemically closely related to beauvericins, were purified from extracts of *Fusarium* species because of their antibiotic activity (Gäumann et al., 1947). Insecticidal properties of enniatins were discovered only after enniatins were purified from cultures of entomopathogenic (and plant pathogenic) species *Fusarium lateritium* (Grove & Pople, 1980). The demonstration of the toxicity of enniatins was later extended to further insect species (Strongman et al., 1988). Although beauvericin and enniatins are most prominent insecticidal mycotoxins of *Fusarium* species, toxic effects of other *Fusarium* mycotoxins on insect individuals as well as tissue cultures were reported (Dowd et al., 1989, Fornelli et al., 2004, Teetor-Barsch & Roberts, 1983). Studies performed with purified mycotoxins are inherently limited because toxic effects in nature result from exposures to mixtures of compounds with additive or synergistic effects, involving known mycotoxins as well as numerous less- or unknown metabolites. Moreover, while the

biological function of trichothecenes as virulence factors in plant infection (Proctor et al., 1995) and zearalenone as agent of interference competition and protection against mycoparasitic fungi (Utermark & Karlovsky, 2007) have been demonstrated, little is known about the biological functions and ecological roles of numerous other *Fusarium*-mycotoxins.

With few exceptions, *Fusarium* species are not known as entomopathogens (Teetor-Barsch & Roberts, 1983) and our understanding of ecological interactions of grain-feeding insects and grain-colonizing fungi is still scarce. Here, we used *T. molitor* to investigate interactions of meal beetles with diverse *Fusarium* species on wheat kernels. *B. bassiana* is a potent pathogen of tenebroid beetles (Knorr et al., 2009) and was used as a positive control and uninfected kernels were used as negative control. *T. molitor* is an important and globally distributed pest of stored products and its capability of selecting optimal ratios of dietary components (Morales-Ramos et al., 2011) indicates that beetles may also sense and avoid toxic fungi-colonized diet. Our working hypothesis was that *T. molitor* can distinguish among kernels colonized with diverse *Fusarium* species or *B. bassiana* and that the insect's repulsion/attraction of respective kernels correlates with their impact on larvae's survival.

2. Materials and methods

Isolation and identification of the strains was described in a previous study (Görtz, 2009). In brief, *F. avenaceum* 1.27 was isolated from colonized wheat kernels in the year 2008 at Poppelsdorf, Bonn, Germany, and taxonomically characterized as described in this study; *F. culmorum* 3.37 was isolated from colonized wheat in the year 2004 at Klein-Altendorf, Bonn, Germany; *F. poae* DSM 62376 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany); *F. proliferatum* 21.1 was isolated from colonized maize in the year 2007 at Hainichen, Germany; and *B. bassiana* Bea2 was isolated from infected *Otiorhynchus sulcatus* (the black vine weevil) in the year 1989 at Stuttgart, Germany. The strains were grown on potato dextrose agar (PDA) plates in darkness at 23 °C.

The meal beetle *T. molitor* is a common storage pest and destructive insect species. *T. molitor* larvae were reared on whole wheat flour with 5% yeast extract in a climate chamber in darkness at 27 ± 2 °C and a relative humidity of 65 ± 5%. Last instar larvae were starved for 72 h and were randomly selected prior use in experiments.

2.1 Inoculation of wheat kernels

For preparing diet contaminated with fungi, spring wheat kernels (cultivar Taifun) were soaked in distilled water for 18 h at room temperature and placed into 1 litre plastic bags separately, subsequently autoclaved for 30 min. Autoclaved wheat kernels were inoculated with fungal mycelia on PDA agar and incubated at room temperature for 4 weeks to ensure complete colonization of the kernels. Control diet was handled in a same way with un-inoculated PDA agar.

2.2 Larvae's preference/avoidance experiments

To determine preference or avoidance reactions of the larvae, feeding experiments were performed on Petri dishes with a diameter of 142 mm. The dishes were marked to generate four equal sectors in the form of identical pie slices (1, 2, 3 and 4). Into opposing sectors each 6 g uninfected kernels were placed and in the remaining two sectors each 6 g of kernels of interest (fungi-colonized or non-colonized). Then, 10 individuals of *T. molitor* were placed randomly in the centre of the Petri dishes. After 20 min in darkness and without any disturbance, the number of larvae in each sector was determined. In total, 20 repetitions with using each 10 naive, inexperienced larvae per sample were performed within one replicate and in total three independent replicates were performed.

2.3 Larval weight gain determination

To monitor feeding of larvae, we determined the cumulative weight gain of 10 larvae within 24 h when fed on fungi-colonized or control kernels. Integral weights of 10 individuals were measured before and 24 h after placing on respective kernels. Per treatment 10 independent determinations were performed and in total three independent replicates were performed.

2.4 Survival rate analysis of larvae on fungal mycelium, colonized kernels or upon stabbing or injection-based infections

Per treatment 30 individuals of *T. molitor* were reared on mycelia of diverse *Fusarium* strains grown on PDA plates and survival rates were monitored daily for 15 days. In total five independent replicates were performed. For survival analysis on fungi-colonized kernels, 30 individuals of *T. molitor* per treatment were reared on respective kernels and survival rates were monitored for 15 days. In total three independent replicates were performed. For stabbing-based infection experiments, *F. avenaceum*, *F. culmorum*, *F. poae*, *F. proliferatum* or *B. bassiana* were inoculated on PDA plates and incubated for 2 weeks. Sterilized insect

minutin pins were used to scratch a mixture of mycelia and conidia from the plate and to wound larvae dorso-laterally leaving mycelia and spores as small plug at the wounding site. In three independent replicates survival of larvae was monitored for the period of one week. Additionally, infection with spores using a syringe-based injection of approx. 10^4 conidia into each larva in 5 µl of water with 0.01% Tween 20 was performed. Survival of larvae was monitored after 5 days incubation in three independent replicates, with 120 larvae per group and per replicate. Living larvae were harvested at 0 day, 5 days, 10 days and 15 days time points then freeze-dried at -50 °C for 48 h. The freeze-dried larvae were ground for subsequent DNA extraction and quantitative real time PCR analysis.

2.5 Mycotoxin analysis[1]

Wheat kernels and larvae were freeze-dried, ground and extracted as described (Nutz et al., 2011). Samples analyzed for beauvericin and enniatin content were not defatted to avoid losses of the mycotoxins in the organic phase. HPLC separation was performed on a RP column at 40 °C and trichothecenes A, B and zearalenone were detected by tandem mass spectrometry using triple quadrupol 1200 L (Varian, Darmstadt, Germany) based on published methods (Adejumo et al., 2007a, Adejumo et al., 2007b). Two mass transitions were used for each toxin. Beauvericin, fumonisin B1 and enniatins were separated on the same HPLC system but detected using ion trap 500MS (Varian, Darmstadt, Germany) as described (Nutz et al., 2011). For each mycotoxin detected on the ion trap, three mass transitions were used. Calibration curves were constructed using analytical standards dissolved in methanol/water (1:1) with a correction for recovery and matrix effects. The limits of quantification for deoxynivalenol, nivalenol, fusarenon X, T2-toxin, diacetoxyscirpenol and neosolaniol, beauvericin, enniatin A, B, A_1, B_1 and fumonisin B_1 ranged between 9 and 170 µg/kg in kernels and 9 and 130 µg/kg in larvae.

2.6 Molecular analyses of *Fusarium* species

qPCR was conducted to monitor if any of tested *Fusarium* strains had the ability to proliferate in *Tenebrio* body cavity or tissue. Total DNA from *T. molitor* larvae was extracted from 30-50 mg of freeze-dried material using a CTAB method (Brandfass & Karlovsky, 2008), purified by phenol extraction, precipitated with isopropanol and dissolved in 50 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). DNA was diluted fifty fold prior to PCR analysis.

[1] The mycotoxins analyses experiments were done by Dr. Katharina Pfohl

Thermocycler (CFX384^{TM}, BioRad, USA) was used for real-time PCR analysis (qPCR) in a total volume of 4 µl. Primers MGBF/R (Waalwijk et al., 2004), OPT 18F/R (Schilling et al., 1996), Fp 82F/R (Parry & Nicholson, 1996), Fp3F/4R (Jurado et al., 2006) and P1/P3 (Hegedus & Khachatourians, 1996) were used for *F. avenaceum*, *F. culmorum*, *F. poae, F. proliferatum* and *B. bassiana*, respectively. The reaction mixture consisted of buffer (16 mM $(NH4)_2SO_4$; 67 mM Tris-HCl; 0.01% (v/v) Tween-20, pH 8.8 at 25 °C, Bioline, Lükenwalde, Germany), 0.15 mM of each dNTP (Bioline, Lükenwalde, Germany), 2.5 mM $MgCl_2$, 0.1 U of *Taq* DNA polymerase (BIOTaq, Bioline, Lükenwalde, Germany), 0.3 µM of each primer, 0.1 x SYBR Green I (Invitrogen, Karlsruhe, Germany) and 1 mg/ml bovine serum albumin. The lowest standards set as limits of quantification (LOQ) were 14-40 ng/g for all four *Fusarium* spp. and 1.0 µg/g for *B. bassiana*.

To amplify translation elongation factor 1-alpha (TEF) gene region, primers ef1/ ef2 (O'Donnell et al., 1998) were used. PCR was performed in reaction mixture described above with hot-start DNA polymerase (Immolase DNA Pol, Lükenwalde, Germany) in a total volume of 25 µl The following cycling conditions were used: 1 cycle of 10 min at 95 °C, 30 cycles of 60 s at 94 °C, 45 s at 58.5°C, and 60 s at 72 °C, followed by a final extension cycle at 72 °C for 5 min. Amplified DNA products were sequenced (LGC Genomics, Berlin, Germany) by Sanger method in both directions. The sequence of the TEF-1a gene of *F. avenaceum* strain 1.27 was deposited at EMBL Nucleotide Sequence Database with the accession number HG794242. The taxonomical identity of the strain was determined with the help of morphological characters (Leslie & Summerell, 2006), its mycotoxin profile (Desjardins, 2006) and TEF-1a sequence (Geiser et al., 2004).

2.7 Statistical analyses

Statistical analyses were performed with R 2.15.3 Weight gain values were log transformed before ANOVA. Survival rates over time were analyzed with a Cox regression model (coxph function) and survival proportion values were fitted to a generalized linear model (GLM) with quasibinominal error structure and logit link function.

3. Results

3.1 Survival rates of *Tenebrio molitor* larvae fed on mycelium of *Fusarium* species

In a first step we determined the capability of *Fusarium* species to induce mortality of larvae. We monitored the survival rates of *T. molitor* larvae reared on PDA plates covered with mycelium of four *Fusarium* species during a period of 15 days. Under these conditions fungal mycelium on PDA was the sole diet available for the larvae. We fitted a Cox's proportional hazards model with censoring on the data set and found evidence for significant differences between the survival curves (log-rank-test, $\chi^2 = 35.53$; d.f. = 8; $P < 0.001$) with a significant effect of *Fusarium* strains ($\chi^2 = 27.17$; d.f. = 4; $P < 0.001$) when compared to controls (Fig. 1). We also found a significant effect of the second variable replicate ($\chi^2 = 10.3$; d.f. = 4; $P = 0.036$) with one out of five replicates showing some difference ($z = 2.01$; $P = 0.045$) when compared to the other replicates. Our analysis revealed that the daily hazard of larvae survival increased by 7.85 times (confidence interval at 95% level (CI) = 1.89 to 32.65 times) when feeding on *F. culmorum* mycelium, by 3.76 times (95% CI = 0.89 to 15.81 times) on *F. avenaceum* mycelium, by 4.96 times (95% CI = 1.19 to 20.76 times) on *F. poae* mycelium and by 5.34 times (95% CI = 1.28 to 22.29 times) on *F. proliferatum* mycelium, respectively, when compared to controls.

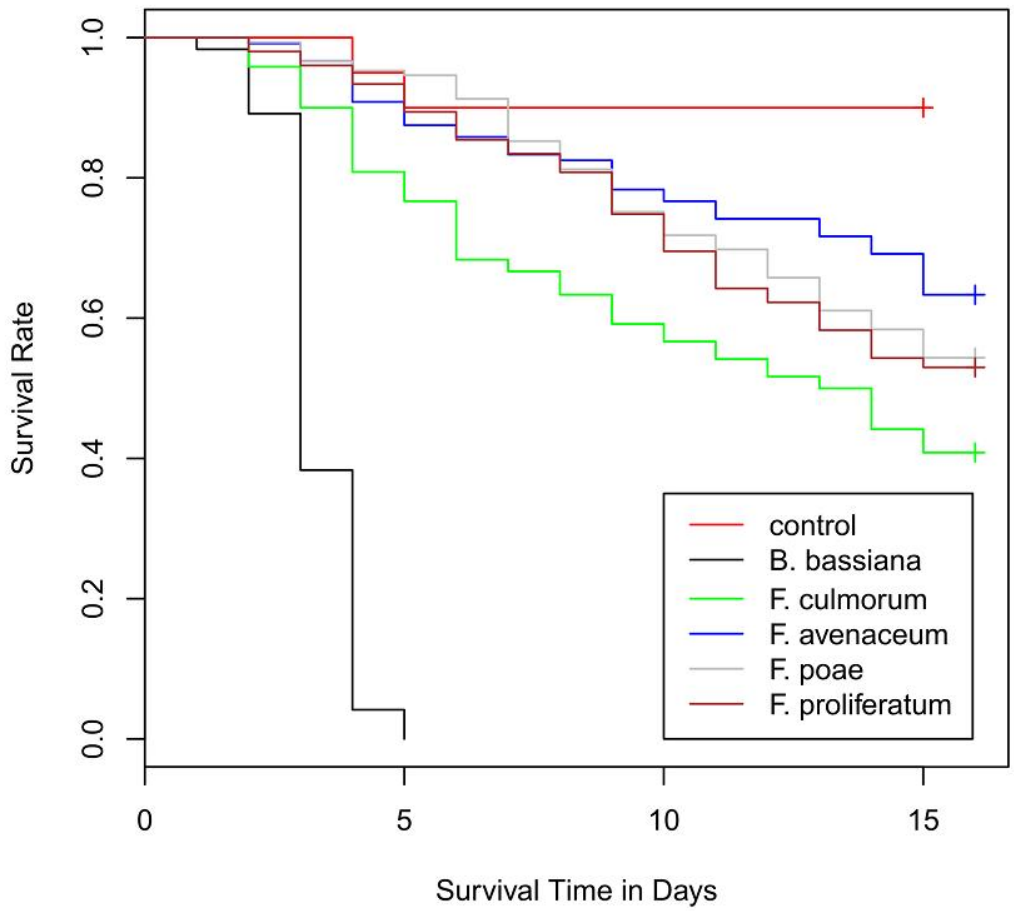

Fig. 1: Survival of larvae feeding on mycelium of diverse *Fusarium* species grown on PDA. Survival curves of larvae reared on PDA plates covered with mycelium of diverse *Fusarium* species were significantly reduced when compared to controls (Cox regression model, $\chi^2 = 35.53$; d.f. = 8; $P < 0.001$; N=560). In addition, survival curve on mycelium of the entomopathogen *B. bassiana* is shown as positive control.

3.2 Larval preference or avoidance to fungi-colonized wheat kernels

In a next step we investigated potential selective feeding behaviors of the larvae as we expected avoidance reactions towards kernels that were colonized with different *Fusarium* species. *T. molitor* larvae showed significant preference or avoidance reactions to colonized kernels depending on the fungal strain (Fig. 2A). Inspection of diagnostic plots as well as use of the Fligner-Killeen test for equality of variances (Conover et al., 1981) revealed that values were normally distributed and that there was homogeneity of variance for the examined groups. Therefore, an ANOVA test was performed on the data set. Unexpectedly, we found that larvae significantly preferred wheat kernels colonized with *F. proliferatum* (mean ± S.D. = 77 ± 4 %; CI = +21 to +33%), *F. poae* (70 ± 5 %; CI = +14 to +26 %) or *F. culmorum* (60 ± 2 %; CI = +4 to +16%) while avoided kernels colonized by *F. avenaceum* (43 ± 2 %; CI = -7 to -13%) or *B. bassiana* (18 ± 5 %; CI = -26 to -38%) when compared to control kernels (50 ±

1 %; CI = 46 to 54 %) (ANOVA, F = 113.3; d.f. = 5 and 12; P < 0.001).

The preference or avoidance behaviors correlated with significant changes in larval weight gain within 24 h on kernels (Fig. 2B). Inspection of diagnostic plots as well as use of the Fligner-Killeen test for equality of variances revealed that logarithmically transformed values were normally distributed and that there was homogeneity of variance for the examined groups. Therefore, an ANOVA test was performed on the data set with the log-transformed response variable weight gain. When compared to larvae reared on control kernels (cumulative weight gain of 10 larvae within 24 h: 138.1 ± 22.1 mg; CI = 132.2 to 153.4 mg) larvae significantly gained more weight on wheat kernels colonized by *F. proliferatum* (173 ± 35.2 mg), *F. poae* (168.4 ± 32 mg) or *F. culmorum* (167.8 ± 29.1 mg), while gained less weight on kernels colonized by *F. avenaceum* (108.5 ± 20.6 mg) or *B. bassiana* (79.1 ± 13.6 mg) (ANOVA, F = 66.79, d.f. = 7 and 172, P < 0.001). Of note, we found a significant effect of the explanatory variable replicate (F = 3.31; d.f. = 2; P = 0.039) with one out of three replicates showing significant difference (t value = -2.57; P = 0.011) when compared to the other two replicates.

3.3 Survival rates on wheat kernels colonized with mycotoxin-producing fungi

We determined the survival rate of larvae ingesting fungi-colonized kernels for a period of 15 days. Fitting the data to a generalized linear model with quasibinominal error structure and logit link function revealed that the survival rate of the *T. molitor* larvae feeding on *F. proliferatum* (80 ± 12 %; odds ratio (OR) = 0.77 and CI = 0.53 to 1.13) or *F. poae* (79 ± 13 %; OR = 0.72 and CI = 0.49 to 1.05) colonized kernels were not significantly different when compared to survival rates on control kernels (84 ± 11 %), even when there was a slight reduction (Fig. 2C). However, survival rates of larvae feeding on *F. avenaceum* (73 ± 14 %; OR = 0.53 and CI = 0.37 to 0.77; P < 0.001), *F. culmorum* (73 ± 13 %; OR = 0.51 and CI = 0.35 to 0.73; P < 0.001) or *B. bassiana* (41 ± 36 %; OR = 0.13 and CI = 0.09 to 0.19; P < 0.001) colonized kernels were significantly reduced when compared to larvae feeding on control kernels (Fig. 2C) (GLM, F = 40.635; d.f. = 5 and 354; P < 0.001).

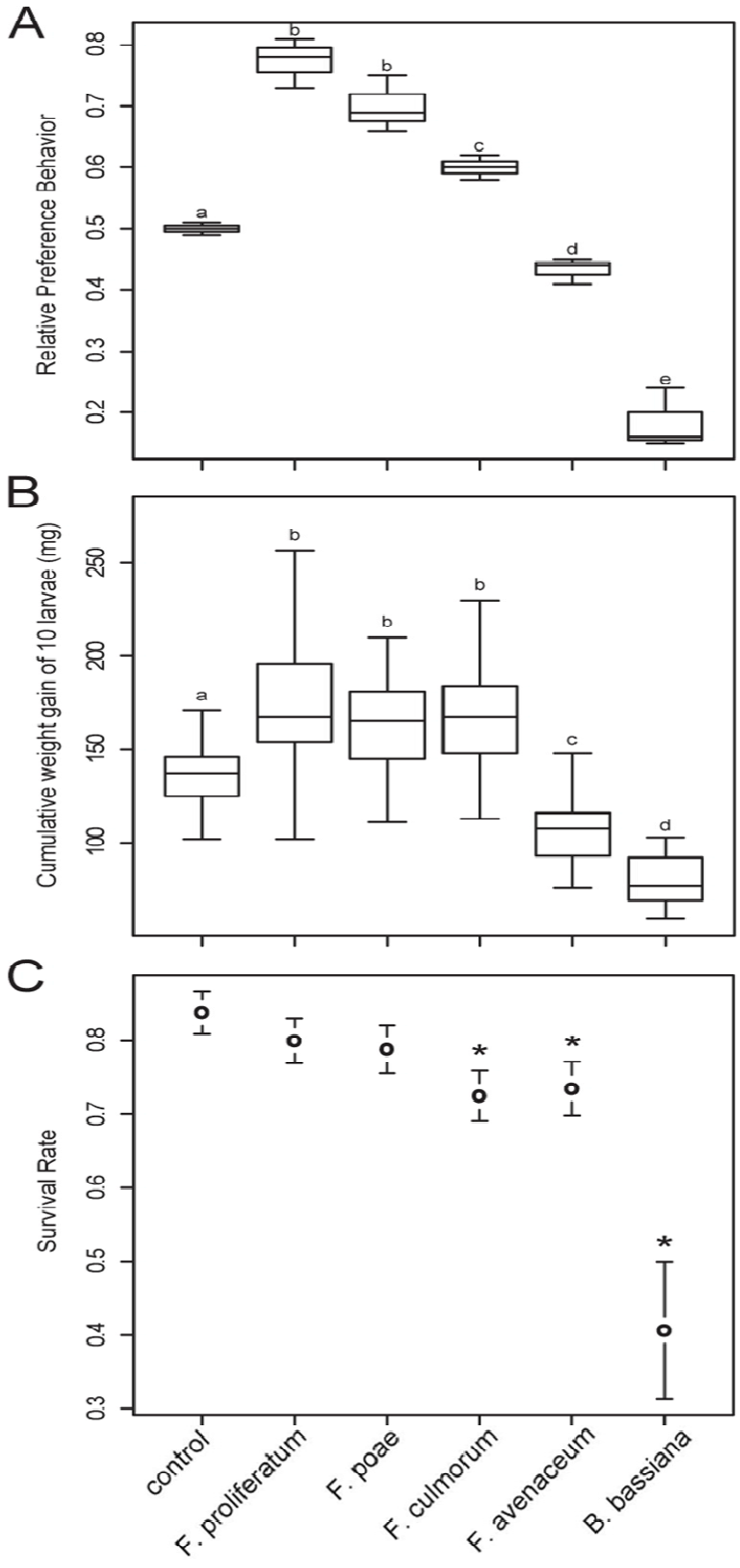

Fig. 2: Reactions of larvae to fungi-colonized wheat kernels. (A) Boxplot of relative preference or avoidance reactions of larvae within 20 min towards fungi-colonized wheat kernels. N=360. (B) Boxplot of cumulative weight gain of each 10 larvae per data point within 24 h on colonized wheat kernels with in total N=1,800. (C) Relative survival rate of larvae on colonized kernels was determined within 15 days. Results are shown as mean values ± CI at 95% levels with N=360. Significant differences are indicated by letters or by asterisks.

To test our initial hypothesis that larvae's avoidance levels against fungi-colonized kernels correlate with the capability of respective kernels to induce higher mortality rates in larvae, we performed a Kendall's tau statistical analysis. This analysis estimates a rank-based measure of association of not normally distributed values. The results of this test indicated that avoidance levels of larvae were negatively correlated with their survival rates in our examined cases.

Table 1: Correlations between percentage of larvae avoidance the colonized kernels and survival rate

			Avoidance (%)	Survival rate
Kendall's tau_b	The percentage of the larvae avoiding colonized kernels [%]	Correlation Coefficient	1.000	-.579**
		Sig. (2-tailed)		.003
		N	360	360
	Survival rate	Correlation Coefficient	-.579**	1.000
		Sig. (2-tailed)	.003	
		N	360	360

**. Correlation is significant at the 0.01 level (2-tailed).

3.4 Mycotoxin analysis

To examine whether mycotoxins accumulated in colonized kernels were responsible for the reduction of survival rates and avoidance behavior towards colonized kernels, we estimated the content of mycotoxins in wheat kernels and in larvae that died within 15 days. The analysis of kernels confirmed the production of fumonisin B_1, enniatins and beauvericin by *F. proliferatum*, of enniatins and beauvericin by *F. poae,* enniatins by *F. avenaceum,* beauvericin by *B. bassiana*, and deoxynivalenol and zearalenone by *F. culmorum* (Table 1). Of note, low amounts of enniatins were found in kernels colonized with *B. bassiana* that likely originated from naturally contaminated kernels. In general, mycotoxins found in kernels were also found in larvae fed on the respective kernels, with the only exception of deoxynivalenol.

Table 1: Mycotoxin content in kernels or *T. molitor* larvae

	B. bassiana		***F. avenaceum***		***F. culmorum***		***F. poae***		***F. proliferatum***	
	Kernels	**Larvae**	**Kernels**	**Larvae**	**Kernels**	**Larvae**	**Kernels**	**Larvae**	**Kernels**	**Larvae**
							(µg mycotoxin/g meal)			
Beauvericin	0.03	<LOQ	<LOQ	<LOQ	-	-	30.10	1.19±0.32	36.36	0.24±0.34
Enniatin A	<LOQ	<LOQ	14.26	0.04±0.03	-	-	<LOQ	<LOQ	0.03	0.01±0.01
Enniatin A_1	<LOQ	<LOQ	60.51	0.33±0.25	-	-	<LOQ	<LOQ	<LOQ	<LOQ
Enniatin B	0.21	0.19±0.04	>90	13.00±5.96	-	-	0.32	1.84±2.59	27.09	0.09±0.06
Enniatin B_1	<LOQ	<LOQ	>90	0.99±0.35	-	-	0.03	0.21±0.31	2.89	0.01±0.01
Fumonisin B_1	<LOQ	<LOQ	<LOQ	<LOQ	-	-	<LOQ	<LOQ	39.74	1.86±0.64
Diacetoxyscirpenol	-	-	<LOQ	<LOQ	-	-	<LOQ	<LOQ	-	-
Neosolaniol	-	-	<LOQ	<LOQ	-	-	<LOQ	<LOQ	-	-
T-2 toxin	-	-	<LOQ	<LOQ	-	-	<LOQ	<LOQ	-	-
Deoxynivalenol	-	-	<LOQ	<LOQ	10.24	<LOQ	<LOQ	<LOQ	-	-
Nivalenol	-	-	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	-	-
Zearalenone	-	-	<LOQ	<LOQ	>210	0.02±0.01	<LOQ	<LOQ	-	-
Fusarenon X	-	-	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	-	-

The values indicate mean values and respective standard deviation. Hyphens indicate that the mycotoxin was not analyzed.

3.5 Altered feeding behavior of larvae on colonized kernels

Interestingly, we observed that *T. molitor* larvae showed varying preference in feeding outer or inner parts of colonized kernels, probably as consequence of concentration variations of more water soluble or insoluble fungal metabolites within the kernels (Fig. 3). To quantify these preferences of larvae, we first compared values of respective percentages of kernels with visible feeding traces for all groups. We fitted a generalized linear model with quasibinominal error structure and logit link function to the data and observed that there were significantly more wheat kernels with feeding sign when colonized with *F. proliferatum* (62.93 ± 3.28 %; OR = 1.8; CI = 1.56 to 2.06), *F. poae* (61.55 ± 2.69 %; OR = 1.69; CI = 1.47 to 1.94) or *F. culmorum* (59.18 ± 6.49 %; OR = 1.53; CI = 1.33 to 1.76) and less kernels with sign of feeding colonized with *F. avenaceum* (31.67 ± 3.32 %; OR = 0.49; CI = 0.43 to 0.56) or *B. bassiana* (20.29 ± 2.57 %; OR = 0.27; CI = 0.23 to 0.31) when compared to controls (48.6 ± 2.22 %) (GLM, $F = 216.32$; d.f. = 5 and 54; $P < 0.001$). Next, we compared kernels with feeding signs divided by the number of kernels with additional signs of caving. Since uninfected kernels as well as kernels colonized with *F. avenaceum* or *B. bassiana* showed no signs of caving at all, these groups were excluded from the analysis. Using a generalized linear model with quasibinominal error structure and logit link function we found significant differences between the groups (GLM, $F = 946.31$; d.f. = 2 and 27; $P < 0.001$); kernels colonized with *F. proliferatum* with no signs of caving were 92.1 ± 1.99 % (OR = 19.05; CI = 15.85 to 23.04), with *F. poae* 90.43 ± 1.83 % (OR = 15.46; CI = 13.01 to 18.47) and with *F. culmorum* were 37.93 ± 3.71 % (OR = 0.61; CI = 0.56 to 0.67), respectively.

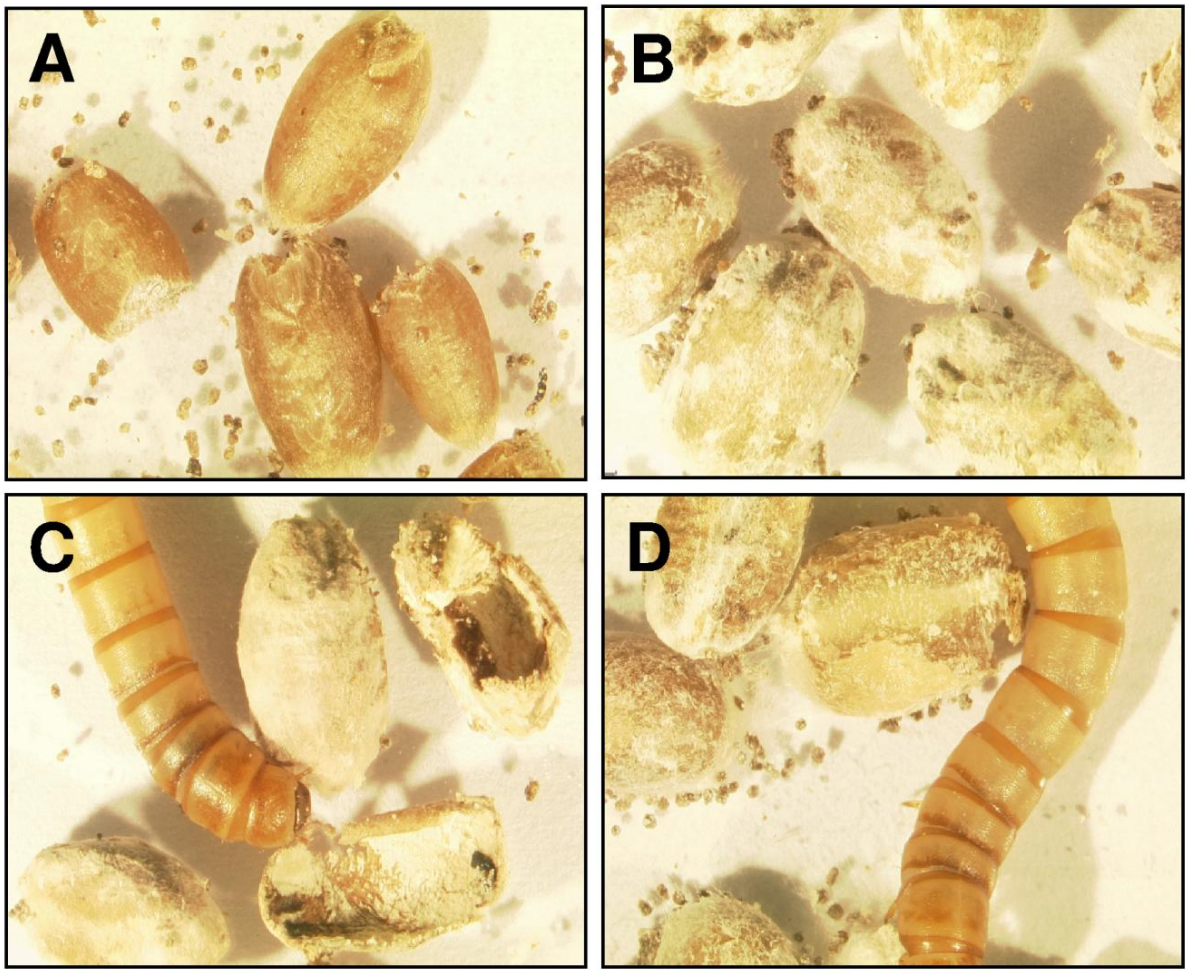

Fig. 3: Selective feeding behavior of larvae on fungi-colonized wheat kernels. (A)Un-inoculated wheat kernels as negative control; (B) *F. avenaceum*, (C) *F. culmorum* and (D) *F. proliferatum* colonized kernels are shown. Larvae preferred feeding on the inner parts of the kernels infested with *F. culmorum* and on the outer parts of the kernels infested with *F. proliferatum* whereas larvae avoided feeding on *F. avenaceum* colonized wheat kernels

3.6 Survival rates of larvae infected by inoculation of conidia into the hemocoel

To address the question whether *Fusarium* species are capable of replicating in the insect's hemocoel and thereby contributing to the reduction of larvae's survival rate we injected fungal strains into larvae. As a first experiment, we performed a stabbing-based infection route using fungi-contaminated needles. Using this approach, we found no significant effect on larval survival rates by injected *Fusarium* strains; however, as expected, a significantly reduced survival rate by entomo-pathogenic *B. bassiana* (GLM, $F = 17.66$; d.f. = 6; $P < 0.001$) (Figure S1).

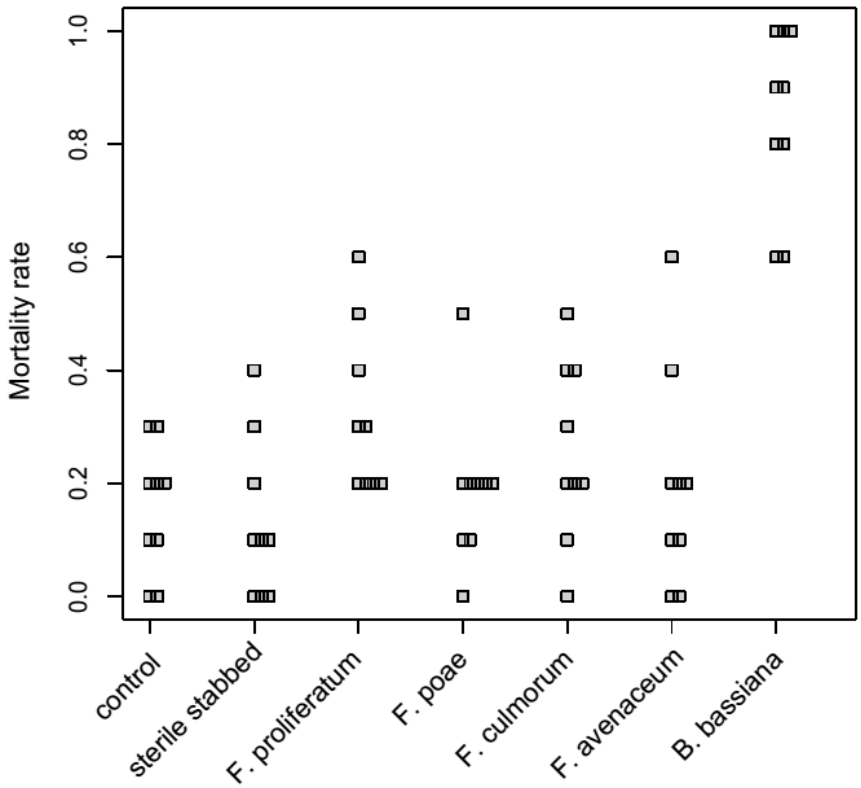

Figure S1: Mortality rates of larvae stabbed with fungi-contaminated minutin pins within 7 days. The figure showed the accumulative mortality rate of the larvae. Short vertical lines in the small box indicated the amount of the dead larvae. N=630.

In a second experiment, we used a syringe-based injection method resulting in an inoculation with approx. 10^4 fungal conidia per larva. Using this second approach with a relatively high infection dose, we determined a significant reduction in larvae's survival rate post injection with each fungus. Fitting the data to a generalized linear model with quasibinominal error structure and logit link function revealed that larvae's survival rate was reduced by *F. proliferatum* (65.19 ± 1.7 %; OR = 0.53; CI = 0.42 to 0.67), *F. poae* (66.3 ± 2.31 %; OR = 0.56; CI = 0.45 to 0.71), *F. culmorum* (29.63 ± 3.57 %; OR = 0.12; CI = 0.09 to 0.15) or *F. avenaceum* (32.6 ± 2.31 %; OR = 0.14; CI = 0.11 to 0.17) when compared to wounded controls (77.78 ± 5.09 %) or sterile puffer injected animals (74.81 ± 1.69 %) (GLM, F = 300.32; d.f. = 6 and 14; $P < 0.001$). Values from *B. bassiana* challenged larvae were excluded from the analysis, since all treated animals died resulting in a variance of 0 thereby disturbing statistical analysis.

The DNA content of used *Fusarium* species in inoculated larvae was estimated by qPCR after a period of 5, 10, and 15 days to determine whether the fungi can germinate and proliferate in the larvae. *Fusarium* species DNA biomass in the living larvae was under limits of

quantification; only DNA of *B. bassiana* used as a positive control was measurable.

4. Discussion

The present study provides insights into ecological interactions of the meal beetle *T. molitor* with selected *Fusarium* species and *B. bassiana* on wheat kernels. When fungal mycelium was provided as the only food source, survival rates of the larvae were reduced with all tested *Fusarium* species. However, in a more ecologically relevant situation, when fungus-colonized wheat kernels were used as diet, we observed that the survival rates of *T. molitor* larvae fed on *F. proliferatum*- or *F. poae*-colonized kernels were similar to controls and larvae fed on *F. avenaceum*- , *F. culmorum*- or *B. bassiana*-colonized kernels were significantly reduced when compared to survival rates of larvae fed on non-colonized kernels. *T. molitor* larvae preferred feeding on kernels colonized with *F. proliferatum*, *F. poae,* or *F. culmorum* over control kernels and avoided feeding on kernels colonized with *F. avenaceum* or *B. bassiana*. These behavioral reactions correlated with the capability of fungal species to reduce larvae survival on colonized wheat kernels, except for *F. culmorum*-colonized kernels.

None of the tested *Fusarium* species multiplied in living larvae when injected into the insect's hemocoel, in contrast to the entomopathogen *B. bassiana*. The differences within some independent replicates in our experiments provide some evidence for the hypothesis that *Fusarium* mycotoxins are responsible for increased insect mortality rates, since mycotoxin levels tend to vary between replicates even if environmental factors are strictly controlled (Waskiewicz et al., 2010).

Larve fed on grain colonized with *F. culmorum* exhibited the highest mortality rate. Major mycotoxins produced by *F. culmorum* are deoxynivalenol and zearalenone. High levels of both mycotoxins were found in kernels colonized with *F. culmorum* but only low or undetectable concentrations were found in larvae fed on *F. culmorum*-colonized kernels. This may be a result of insects avoiding kernel parts with high toxin content and/or that these mycotoxins were efficiently metabolized. However, the survival of larvae fed on these kernels was low indicating that either transformation products of deoxynivalenol or zearalenone were still toxic or other toxic products of *F. culmorum* were present. In *F. avenaceum*-colonized kernels high levels of enniatins may have contributed to the elevated mortality rates of larvae fed on colonized kernels; insect toxicity of enniatins is well documented (Grove & Pople, 1980, Strongman et al., 1988). Interestingly, we detected only low amounts of beauvericin in

wheat kernels colonized with *B. bassiana,* which probably killed insects by utilizing other mechanisms including multiplication within the host. Another evidence that beauvericin may not be responsible for larval mortality in this study is the high survival rates of larvae fed on *F. proliferatum* and *F. poae*-colonized kernels, which contained high levels of beauvericin.

Natural selection is likely to act on multiple levels in both insects and fungi. Insects may use fungi as diet or may avoid potentially pathogenic fungi or fungal toxins. For example, the beetle *Coccinella septempunctata* a predator of many insects was shown to avoid *B. bassiana*-colonized insect preys or contaminated leaf surfaces (Ormond et al., 2011). A similar avoidance behavior was found in the bug *Anthocoris nemorum* (Meyling & Pell, 2006). Fungal volatiles represent key repellence signals for these insects and probably also for *T. molitor* in our study. A recent study with the termite *Macrotermes michealseni* provided evidence that a mixture of the volatiles 4, 5-dihydro-5-pentyl-2-(3H)furanone, borneol, 4-nonanone, 2-nonanone, butyrolactone, and camphor contributed largley to the repellency of *B. bassiana* to this termite species (Mburu et al., 2013). On the other side, insects may increase their tolerance or resistance against potentially pathogenic fungi or their toxins. Supporting this view, larvae of *T. molitor* have recently been reported to be much more tolerant or resistant to tested *Fusarium* mycotoxins than other insect larvae such as armyworms; Moore and Davis reported that *T. molitor* larvae were about 100 times more tolerant to dietary T-2 toxin than the armyworm *Mamestra configurata* (Moore & Davis, 1983). Genomic data of a related tenebrionid beetle, the red flour beetle *Tribolium castaneum,* further supports this view by identifying specific genetic adaptations including gene duplications of the CYP450 subfamilies CYP6 and CYP9, which are known to be involved in toxin resistances (Richards et al., 2008), or of immune-inducible anti-fungal thaumatins (Altincicek et al., 2013). Taken together, this indicates that tenebrionid beetles may have adapted to counteract to toxic fungi in their environments and determination of the genome sequence of *T. molitor* may further help to address this hypothesis. Fungi, on the other side, may produce chemical compounds to react to competition or may increase tolerance against grazing. Moreover, producing diverse volatile components fungi could easily gain fitness benefits by attracting, repelling or remaining invisible to potential insect hosts, competitors or vectors depending on selective pressures. Indeed, recent studies revealed the role of fungal toxins in interactions of fungi with fungivorous arthropods (Rohlfs & Obmann, 2009, Staaden et al., 2010, Trienens & Rohlfs, 2012, Rohlfs & Churchill, 2011). Enhanced production of fungal toxins as a defense response of *Aspergillus nidulans* against fungivorous collembolans

was recently established (Döll et al., 2013). In the present study we have not investigated induced reactions of *Fusarium* fungi to the presence or feeding of beetles; this will be the subject of subsequent studies.

In conclusion, our study shed light on ecological interactions of *T. molitor* larvae with four *Fusarium* species. We found that larvae have evolved to sense threats derived from *F. avenaceum*- or *B. bassiana*-colonized kernels, but not the threats of *F. culmorum*-colonized kernels. Kernels infested with *F. poae* or *F. proliferatum* showed no significant impact on larvae's survival rates under tested conditions. To identify the nature of the threat signals for the larvae will be part of our future studies. Knowledge on these signals as well as their correlations with the presence of specific mycotoxins may help better understand mutual ecological adaptations of meal beetle and *Fusarium* species.

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Chapter 3 *Fusarium* spp. dissemination within grains by *Tenebrio molitor*

Abstract

Fusarium species have negative impact on food security by infecting a wide array of crop plants and producing numerous human-health threatening toxins. Recently, we found that the common pest of stored products, the meal beetle *Tenebrio melitor*, was preferably feeding on wheat grains colonized with *F. proliferatum*. This was surprising since *F. proliferatum* was capable of increasing beetle's mortality after *T. molitor* larvae grazing *F. proliferatum* mycelia and conidia or *F. proliferatum* colonized wheat kernels. We draw the hypothesis that *F. proliferatum* attracts meal beetles to gain fitness benefits by getting dispersed using the beetles as vector.

After feeding on *Fusarium* mycelium and conidia, live conidia were traceable in beetles' excreta and beetles were capable of contaminating a high proportion of wheat kernels. The duration of fungal dissemination by beetles and fungal colony forming unit density in beetles excreta were recorded for up to 20 days. Fungal DNA in live beetle was quantified by qPCR at different time points after feeding on *Fusarium* spp. mycelia and conidia to monitor whether *Fusarium* species survive or proliferate in live beetles. Conidia were traceable for at least up to 20 days in beetles' excreta and also *F. proliferatum* DNA were positively detectable in living beetles collected at 1 day, 5 days, 10 days and 15 days time points. HPLC-MS was performed to detect beauvericin, fumonisins, enniatins in live beetles. Beauvericin was found in beetles feeding on *F. poae*, *F. proliferatum*, or *Beauveria bassiana* mycelium.

Our study indicated that 1) Conidia of *Fusarium* species survived through beetles' gut passage. Fungal DNA of *Fusarium* and mycotoxins including beauvericin, enniatins were detectable in beetles and 2) Kernels contamination and fungal colony forming unit density of *F. proliferatum* were higher than other tested *Fusarium* species, although *F. culmorum* spores were also detectable in beetles' feces for up to 20 days. We believe that our results provide new insights for the hypothesis that *F. proliferatum* attracts meal beetles to gain fitness benefits by getting dispersed. A deeper understanding of the impact of insects on toxin-producing fungi dissemination within storage grains is essential for food security and may help in the improvement of post-harvest management approaches.

1. Introduction

Colonization of kernels by *Fusarium* species causes serious problems in food security by diminishes the quality of the crop products. *F. proliferatum* has a wide host plant range and has frequently been isolated from cereal plants, including maize, wheat, barley, rice, sorghum, oats as well as other plants such as banana (Jimenez et al., 1993), citrus fruits (Hyun et al., 2000), asparagus (Elmer, 1995), orchids , date palm (Abdalla et al., 2000) and pine seedlings (Ocamb et al., 2002). *F. proliferatum* (Gibberella intermedia, *Gibberella fujikuro* imating population D) is together with *F. verticillioides* (Gibberella moniliformis, *G. fujikuroimating* population A) the main source of fumonisin toxin production in food and feed products (Jurado et al., 2010, Desjardins et al., 2007). The primary health concerns resulting by high dose fumonisin ingestion are acute toxic effects as well as potential carcinogenic effects by lower dose intake over time. Consumption of fumonisin-contaminated maize was reported to be associated with higher rates of esophageal cancer and neural tube birth defects (Meeting & Organization, 2001). Besides fuminisins, *F. proliferatum* is capable of synthesizing other mycotoxins including beauvericin , fusaproliferin , fusarins (Miller et al., 1995), and moniliformin (Logrieco et al., 1995, Miller et al., 1995).

Insects represent an important route in the transmission of numerous pathogens between plants (Feldman et al., 2008). Fermaud and Menn reported that the grape berry moth *Lobesia botrana* transmitted *Botrytis cinerea* from infected to healthy grape berries in 1992 (Fermaud & Le Menn, 1992). Paine et al. discussed that the fungal species *Ophiostoma* may benefit from the association with the bark beetles by transport to new host trees. Beetle species (*Dendroctonus*) may benefit from the association with fungi by feeding on the fungi, or by the fungi contributing to the death of the host trees through mycelia penetration of host tissue, toxin release, interactions with preformed and induced conifer defenses (Paine et al., 1997). Prom et al.demonstrated that contaminated adult corn earworm feeding on the honeydew secreted by *Claviceps africana* could transmit the sorghum ergot from diseased to healthy panicles when environmental conditions are favorable for infection (Prom et al., 2003). Rust fungus *Puccinia monoica* inhibits flowering in its host plants (*Arabis* species) and radically transformed infected leaves that mimic true flowers of unrelated species in shape, size, color and nectar production. These fungal pseudoflowers are highly successful in attracting pollinating insects which fertilize the rust (Roy, 1993). Moreover, some pathogens were found to synthesize volatile organic compounds and sugars to attract insects for dissemination (Feldman et al., 2008). Friedli and Bacher presented a

by-product-purloined mutualism between stem-boring weevil and rust fungus, weevil purloined benefits from feeding on rust-infected tissue and rust fungus receiving by-product benefits from the weevil transmission spores (Friedli & Bacher, 2001).

Recently, we found that colonization of wheat grains with *Fusarium* spp. had a significant impact on food choice, weight gain and mortality of the meal beetle *Tenebrio molitor* an important cosmopolitan pest of stored products (Guo et al., 2014). We found that *F. proliferatum* colonized wheat kernels were more attractive as food source to *T. molitor* compared negative control, which led us to the hypothesis that *F. proliferatum* attracts these insects for their dissemination. Here, we investigated potential dissemination routes of *F. proliferatum* on wheat kernels by *T. molitor*.

2. Materials and Methods

2.1 Study organisms and general medium

T. molitor larvae were reared on whole wheat flour with 5% yeast extract in a climate chamber in darkness at 27 ± 2 °C and a relative humidity of 65 ± 5%. *T. molitor* beetles were used in the experiment because of the most dispersive stage (Dunkel, 1988). The beetles were starved for 24 h and were randomly selected prior use in experiments. *T. molitor* beetles were starved for 24 h for cleaning up the remaining food also to make sure the adults will feed on the mycelia (Davis et al., 1975). For beetles surface sterilization, the beetles were carefully washed with autoclaved distilled water to wash away the attachments on the cuticle, then with 1.2~1.3% sodium hypochlorite for 3 min followed by 3 times washing with autoclaved distilled water for 2 min. The beetles were dried on autoclaved paper with covering sterile plastic cover over at room temperature under laminar flow cabinet (super-clean bench).

Single-spore fungal strains *F. avenaceum* 1.27, *F. culmorum* 3.37, *F. poae* DSM 62376, *F. proliferatum* 21.1, and *Beauveria bassiana* Bea2 were grown on potato dextrose agar (PDA) plates and incubated in darkness at 23 °C. Isolation and identification of the strains was described in a previous study (Goertz et al., 2010). The identification of all species was confirmed by real time PCR with species-specific primers besides morphological identification. *F. avenaceum* 1.27 was isolated from infected wheat kernels in the year 2008 at Poppelsdorf, Bonn, Germany, and taxonomically characterized as described (Guo et al., 2014); *F. culmorum* 3.37 was isolated from infected wheat in the year 2004 at Klein-Altendorf, Bonn, Germany; *F. poae* DSM 62376 was purchased from Deutsche Sammlung von

Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany); *F. proliferatum* 21.1 was isolated from infected maize in the year 2007 at Hainichen, Germany *B. bassiana Bea2* was isolated from infected *Otiorhynchus sulcatus* (the black vine weevil) in the year 1989 at Stuttgart, Germany.

CLA (Carnation Leaf-piece Agar): aseptically placing sterile 2 pieces of carnation leaves into 2% water agar plate (20 g agar in 1 L of water).The carnation leaf pieces are prepared from fresh carnation leaves free from fungicide or insecticide residue. Immediately after collection, the leaves are dried in oven with the temperature 70 °C for 3-4 hours until brittle. The dried leaves are sterilized by 1.5 % sodium hypochlorite and dry again under clean bench and then 2 pieces of leaves are placed on water agar plate (Leslie & Summerell, 2006).

CZID-Agar: Czapek-Dox-Iprodione-Dichloran Agar (Abildgren et al., 1987, Thrane, 1996), when the CZID medium cools down to around 55 °C after autoclave, add 1 ml Penicillin, 1 ml Streptomycin, 1 ml Ampicillin with the concentration 1 g/10 ml distilled water and 10 ml Chlortetracyclin with the concentrction 1 g/100 ml distilled water. And 1 ml Rovral (BASF, Germany) solution with the concentration 60 mg/10 ml autoclaved distilled water was added.

2.2 Fungi conidia and mycelia attachment on beetles

T. molitor beetles were starved for 24 h and 10 beetles were subsequently placed on one PDA (potato dextrose agar) plate fully covered with *F. proliferatum* 21.1 mycelia (2 weeks growth) for 24 h feeding (Jayasinghe & Parkinson, 2009). Live individual beetles were frozen to death at -20 °C prior analysing using scanning electronic microscopy (desktop Phenom™ G2 Pro by Phenom-World, Netherland). Figures of mycelia and conidia attached on the antennae, mouthparts, wings, abdomen and legs were taken for observing fungal mycelia and conidia attachment to body parts of the beetles.

2.3 Fungi colonized the dead beetles

To determine fungi colonization and *Fusarium* conidia germination on dead beetles, beetles which died within 15 days after feeding on *F. proliferatum* mycelia and conidia were collected. Dead beetles were surface sterilized with 1% sodium hypochlorite for 3 min. Subsequently, beetles were rinsed 3 times with sterile distilled water and placed on CZID (selective medium priority for *Fusarium* growth) plates. Images of the specimens observed under light microscope were recorded digitally with a camera incorporated to the Leitz DMRB Leica

light microscope using software Diskus 4.2 (Hilgers, Königswinter, Germany).

2.4 Time duration of fungi dissemination by feeding behaviour and cuticle friction

Autoclaved wheat kernels were investigated for contamination by beetles' cuticle friction and mouthparts ingestion. After feeding on *F. avenaceum*, *F. culmorum*, *F. poae*, *F. proliferatum*, or *B. bassiana* mycelia and conidia for 24 h, beetles were individually placed in the sterilized small Petri dishes with autoclaved wheat kernels. Autoclaved kernels were changed each day. Kernels at the time points 1, 5, 10, 15, and 20 days grazing were harvested for assessment time duration of contamination, percentage of the contaminated wheat kernels out of all the tested wheat kernels was considered as parameter to value tested *Fusarium* species. 15 wheat kernels were collected and placed on CZID medium for the contamination test. 5 repetitions were conducted. The whole experiments were repeated twice.

2.5 Fungal conidia detection in beetle's excreta

To investigate fungal dissemination by survived conidia in excreta from gut passage, *T. molitor* excreta were collected 1, 5, 10, 15 and 20 days time points after beetles feeding on *F. avenaceum*, *F. culmorum*, *F. poae*, *F. proliferatum*, or *B. bassiana* mycelia and conidia for 24 h. Excreta were mashed and suspended in the sterilized water, then the suspension was spread on CZID plates to clarify the time spores existing in adults' excreta. After 3-4 days incubation, fungal colony forming unit density was recorded and treated as parameter to value the difference among species (colony forming unit/mg excreta). 5 repetitions were done. The whole experiments were repeated twice.

2.6 Fungi DNA quantification and mycotoxin analyses in beetles[2]

qPCR was performed to monitor if any of tested *Fusarium* species as follows: *F. avenaceum*, *F. culmorum*, *F. poae*, *F. proliferatum*, or *B. bassiana* had the ability to proliferate in living beetles. After beetles grazed 24 h mycelia and conidia of different *Fusarium* spp. above on PDA plates, beetles feed on autoclaved wheat kernels as unique food source and were harvested at 1, 5, 10, and 15 days. Beetles were stored in 96% ethanol and then evaporated in the speed vacuum at 40 ℃ overnight to evaporate. Then dry beetles were grinded into fine powder.

[2] The mycotoxins analyses experiments were done by Dr. Katharina Pfohl.

Fine beetles powder of 15~17 mg was weighted and transferred into 2 ml new eppis. 1 ml acetonitrile: water (84:16) was added. After thoroughly stirring the samples, they were shaken overnight at 180 rpm. Next day the samples were centrifuged at 14,500 rpm for10 min. 950 ul supernatant was transferred into 2 ml eppis and stored in -20 ℃ for mycotoxin analyses.

The samples analyzed for trichothecene B and zearalenone were additionally defatted with cyclohexane. Beauvericin, fumonisin B_1 and enniatins were separated on a RP column at 40 °C followed by electrospray ionization in positive mode connected to an ion trap 500 MS (Varian, Darmstadt, Germany) as described (Nutz et al., 2011). For each mycotoxin detected, three mass transitions were used. Nivalenol, deoxynivalenol and zearalenone were separated on the same HPLC system but detected by tandem mass spectrometry using triple quadrupol 1200 L (Varian, Darmstadt, Germany) based on published methods (Adejumo et al., 2007a, Adejumo et al., 2007b). Two mass transitions were used for each toxin. Calibration curves were constructed using analytical standards dissolved in methanol/water (1:1) with a correction for recovery and matrix effects. The limits of quantification for beauvericin, enniatin A, B, A_1, B_1 and fumonisin B_1 were 150 and 390 ng/g in beetles respectively.

After mycotoxin extraction, the beetle meal was dried with the vacuum concentractor at 40 ℃for 4 h. Total DNA of *T. molitor* beetle was extracted from dried beetles fine powder using a CTAB method (Brandfass & Karlovsky, 2008). The pellet was dissolved in 50 ul TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). DNA was diluted fifty fold prior to PCR analysis. Inhibition assay was done to test if any matrix effects on the amplification of fungi DNA.

Thermocycler (CFX384TM, BioRad, USA) was used for real-time PCR analysis (quantitative PCR) in a total volume of 4 ul. Primers MGB F/R (Waalwijk et al., 2004), OPT18 F/R (Schilling et al., 1996), Fp 82F/R (Parry & Nicholson, 1996), Fp 3F/4R (Jurado et al., 2006) were used for the species-specific detection of *F. avenaceum*, *F. culmorum*, *F. poae* and *F. proliferatum* respectively. The reaction mixture consisted of buffer (16 mM $(NH_4)_2SO_4$; 67 mM Tris-HCl; 0.01% (v/v) Tween-20, pH 8.8 at 25 ℃, Bioline GmbH, Luckenwalde, Germany), 0.15 mM of each dNTP (Bioline GmbH, Luckenwalde, Germany), 2.5 mM $MgCl_2$, 0.1 U of Taq DNA polymerase (BIOTaq, Bioline GmbH, Luckenwalde, Germany), 0.3 mM of each primer, 0.1 x SYBR Green I (Invitrogen, Karlsruhe, Germany) and 1 mg/ml bovine serum albumin. The lowest standards set as limits of quantification were 0.169 pg/ul for *F. avenaceum*, *F. culmorum*, *F. poae* and 2.09 fg/ul for *F. proliferatum*.

2.7 Fungal dissemination by copulation between beetles

Male *T. molitor* beetle reared on *F. proliferatum* 21.1 mycelia and conidia for 48 h, subsequently, the male beetle was individually placed in the plastic Petri dishes with surface sterilized female beetle. Eggs were picked up one by one with the sterilized forceps 5-7 days after copulation from dissected female beetle cavity and then placed on CZID plates. Mycelia grew out from the eggs were recorded.

2.8 Fungi re-isolation and confirmation

Finally, to confirm Koch's postulates, *F. proliferatum* 21.1 as a representative species was re-isolated from gut of the adult beetle, contaminated wheat kernels by the beetles feeding, excreta of the adult beetles feeding on fungal mycelia and conidia, and eggs from female copulated with male that ingested on *F. proliferatum* mycelia and conidia for 24 h. Hypha tip was scratched and transferred on CZID plate, three replicates were done. Later, single mycelium tip was scratched and transferred again on PDA and CLA to confirm the pathogen through the pigment and colony, microconidia and macroconidia characteristics (Leslie & Summerell, 2006). Three discs of 50 mm diameter PDA filled with mycelia were cut and transferred into 50 ml Erlenmeyer flask with PDB medium under 150 rpm and 25 ℃ dark incubator condition for 4 days. Using four-layer cheese cloth for filtering the mycelia, mycelia were freeze-dried at -50 °C for 48 h. Mycelia were ground into fine powder with micro-disintegrator. 18~20 mg fine powder was used for DNA extraction.

DNA was extracted according to the manufacturer's protocol of DNeasy Plant Mini kit (QIAGEN, Germany) and the samples were eluted in 50 µl of elution buffer and stored at -20 °C. The DNA of samples was estimated by gel electrophoresis in 1.0% agarose gels (Agarose NEEO Ultra-Quality, Carl Roth GmbH, Karlsruhe, Germany) prepared in 1 x TAE buffer (diluted from 50 x TAE buffer, Applichem GmbH, Darmstadt, Germany). The electrophoresis was carried out at 100 V/6 cm for 30 min. The agarose gel was stained with 10,000 x dilution Gel Red (5 ul Gel Red diluted in 50ml 1x TAE buffer, Biotium, Darmstadt, Germany) and documented with a digital imaging system (Gel Doc^{TM}, Bio-Rad, USA). PCR also be done with *F. proliferatum* species-specific primers. PRO1: 5'-CTTTCCGCCAAGTTT CTTC-3' and PRO2: 5'-TGTCAGTAACTCGACGTTGTTGTT-3' (Mulè et al., 2004) and the fragment is 585 bp.

3. Results

3.1 *Fusarium* conidia and mycelia attached on different body parts of *T. molitor* beetles

To determine the attachment of mycelia and conidia on different body parts the beetles as transmission route, we utilized scanning electronic microscopy. Mycelia (Fig. 1A) and conidia (Fig. 1B) of *F. proliferatum* 21.1 were detected on the beetles' cuticle. In detail we found conidia on antennae (Supplementary Information Fig. 1: B, A as control), mouthparts (Supplementary Information Fig. 1: D, C as control), wings (Supplementary Information Fig. 1: F, E as control) and legs (Supplementary Information Fig. 1: H, G as control) of *T. molitor* beetles. By attachment on beetle cuticle, conidia and mycelia could easily be disseminated by friction or contact to new kernels of cereals. Moreover, *Fusarium* mycelia grew on beetles' carcass (Fig. 2: A and B), which could represent a further route of dissemination.

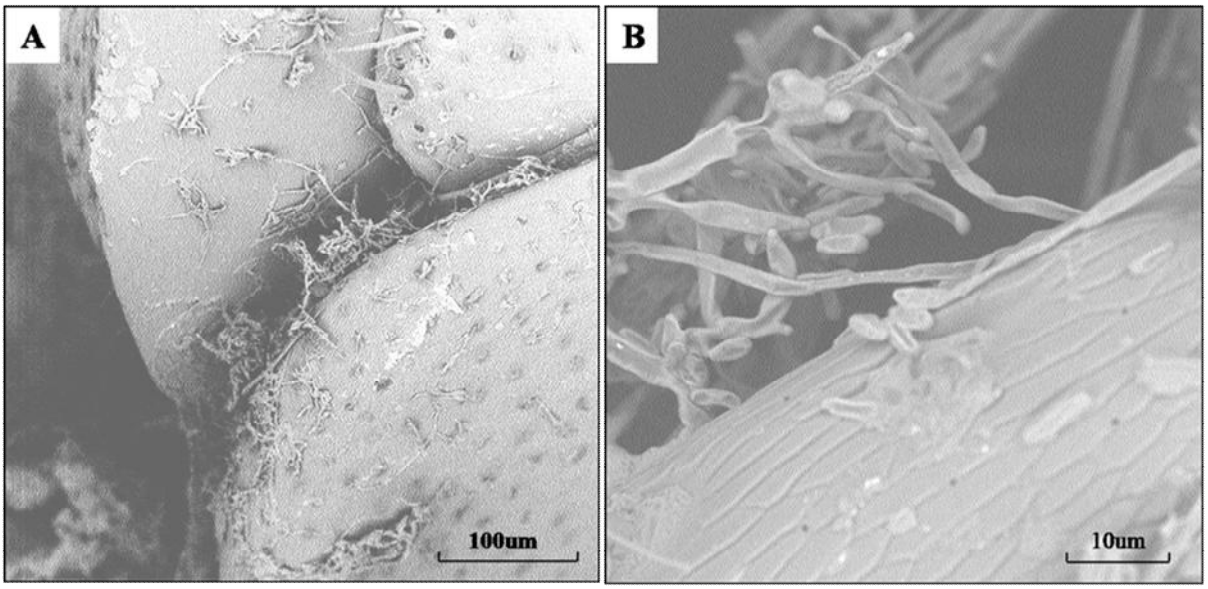

Fig. 1: *Fusarium* species mycelia and conidia attached on cuticle of *T. molitor* beetles. The scanning microscopy figures indicated that *F. proliferatum* conidia (A) and mycelia (B) attached on cuticle of *T. molitor* beetles.

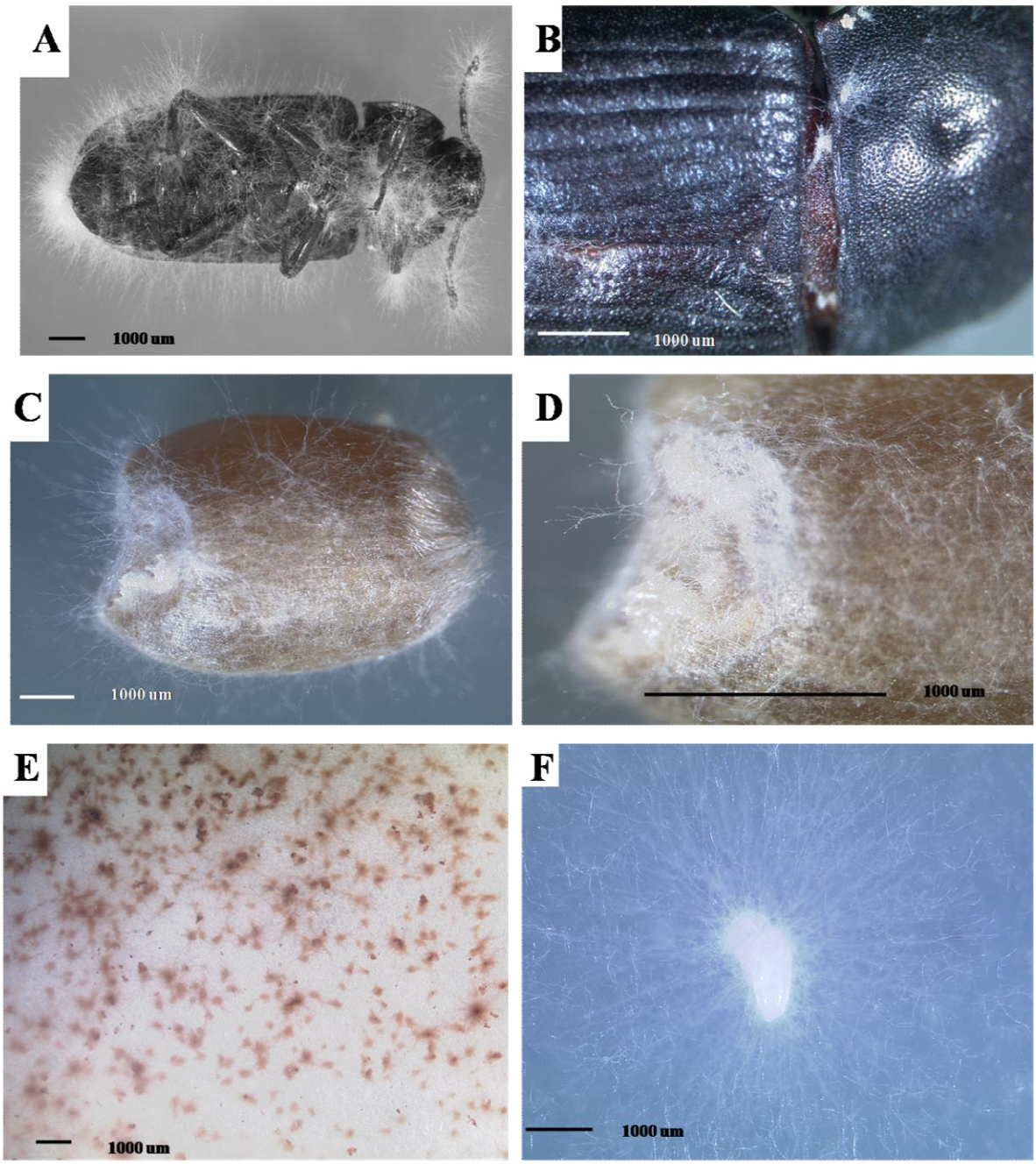

Fig. 2: *Fusarium* conidia germinated from dead beetles, contaminated kernels, beetles excreta and contaminated eggs, and cuticle friction. *Fusarium* conidia germination on dead beetles, beetles which died within 15 days after feeding on *F. proliferatum* mycelia and conidia (A and B). Autoclaved wheat kernels were contaminated by adult mouthparts and cuticle friction after beetles ingestion behavior (C and D). The conidia germination from excreta proved that the conidia survived from the gut passage (E). Fungal disseminated through copulation by male adult ingestion 72 h mycelia and conidia to female adults' eggs (F).

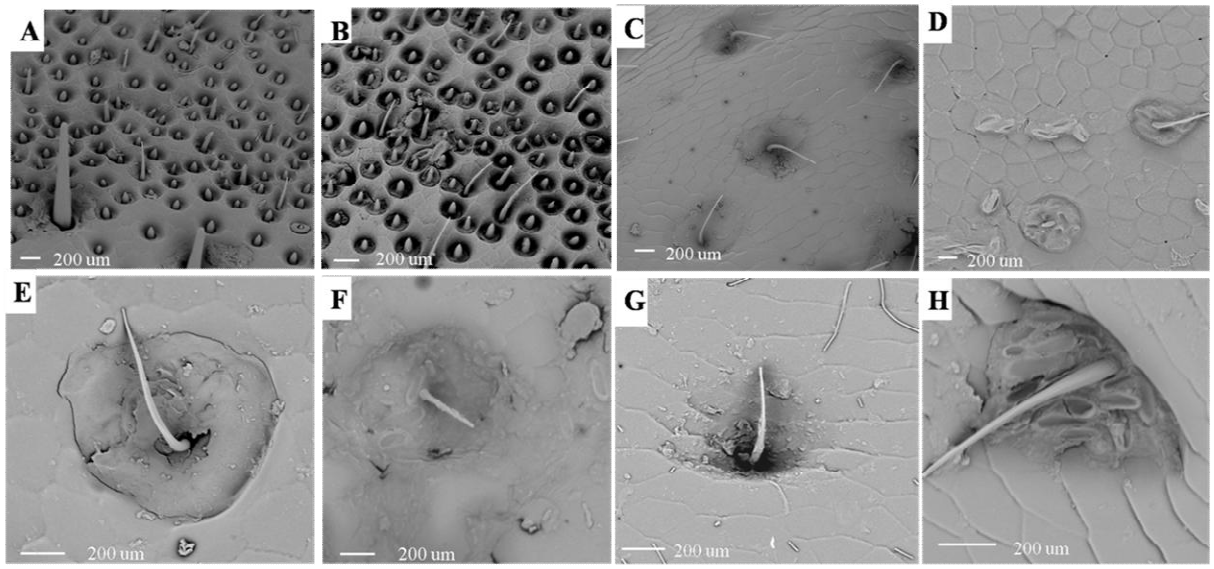

Figure S1: *Fusarium* species conidia and mycelia attached on different body parts of *T. molitor* beetles. The scanning microscopy figures indicated that *F. proliferatum* conidia attached on antennae (B, A as control), mouthpart (D, C as control), wings (F, E as control) and legs (G, H as control) of *T. molitor* beetles.

3.2 Fungal dissemination by beetles

To determine the potential contamination of naive kernels by insect feeding, autoclaved wheat kernels were placed into a Petri dish with *T. molitor* imagoes, which were feeding on *F. proliferatum* mycelium, and were changed every 24 h. This procedure was repeated for 20 days. Kernels collected on 1, 5, 10, 15 and 20 days were incubated on CZID in dark at the temperature 25 ℃. Mycelia grew out from the contaminated kernels which indicated the contamination by the mouthparts or the body friction by the beetles (Fig. 2：C and D).

We repeated this experiment for three further *Fusarium* species and *B. bassiana* as control to check whether there are differences on dissemination rates. The highest contamination rate was found with *F. proliferatum*. Even after 20 days the percentage of the contaminated kernels was still more than 90%. Of note, *F. culmorum* contamination percentage on 20 days samples were around 40% and for *F. avenacum* and *F. poae* there was no detectable contamination after 20 days (Fig. 3 A).

F. proliferatum 21.1 conidia survive beetle's gut passage for a long time (Fig. 2 E), *F. avenaceum* 1.27 and *F. poae* DSM 62376 conidia load decreased dramatically and were not detectable after 10 days (Fig. 3 B). *F. culmorum* 3.37 conidia load decreased to 40% compared to the initial value after 20 days, and *Beauveria bassiana* Bea2 conidia load was not

measurable since all treated beetles died within 5 days.

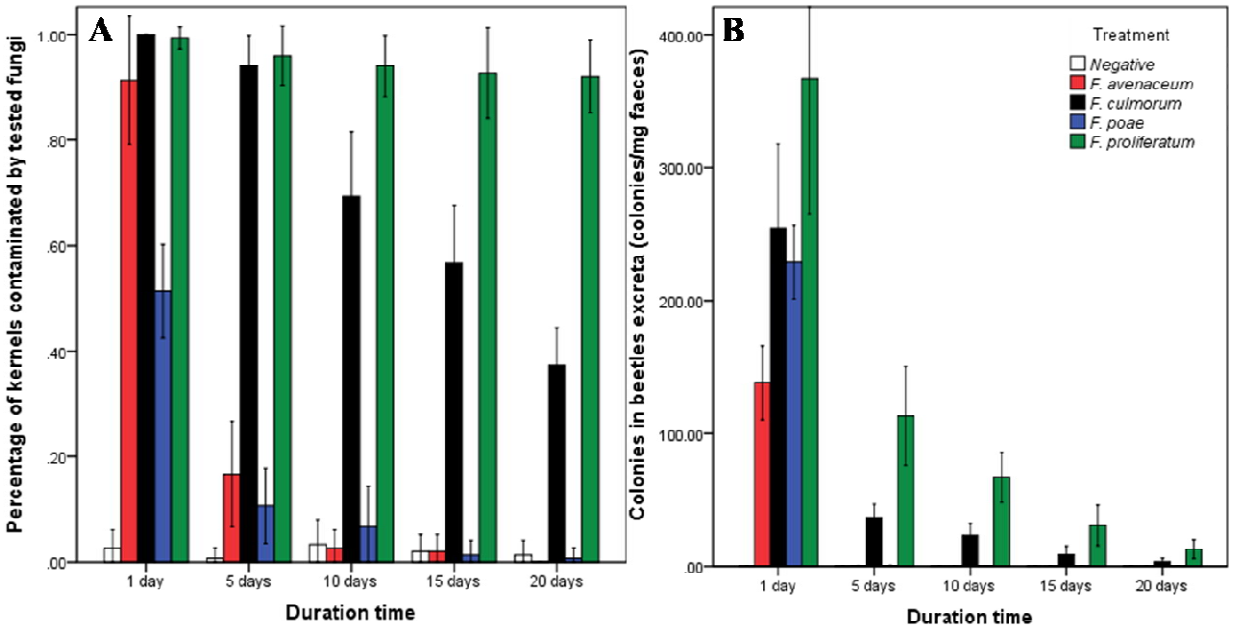

Fig. 3: Fungal dissemination by adults contaminated wheat kernels and conidia in excreta survival from gut passage. Percentage of mycelia germinated from contaminated kernels with the time points up to 20 days. Results were indicated mean ± S.D. N=525 (Fig. 3A). Fungal colony forming unit density existed in the excreta of *T. molitor* adults feeding on mycelia and conidia of *Fusarium* species. Results were indicated mean ± S.D. (Fig. 3B).

3.3 DNA quantification and mycotoxin analyses

The DNA content of *Fusarium* species in beetles grazing for 24 h on respective fungal mycelia were quantified by real time PCR at time points 1, 5, 10, and 15 whether the fungi can proliferate in living beetles. Fungal DNA of 6 tested species was detectable in beetles (Table 1). Fungal DNA biomass of the tested *Fusarium* species was not significantly different over tested time period. Fungal DNA biomass of *F. avenaceum* indicated at least no detectable proliferation in the beetles for up to 15 days after ingestion.

Table 1：Fungal DNA in beetles grazing on *Fusarium* spp. mycelia and conidia

Fungal species	Species-specific fungal DNA in beetles (ng/g) Days after grazing on fungi			
	1	5	10	15
Control	<LOD	<LOD	<LOD	<LOD
F. avenaceum	76±73* (7/10)	47±42* (5/10)	106±68* (9/10)	153±135 (4/4)
F. culmorum	233±250 (10/10)	90±120 (10/10)	197±201*(9/10)	<LOD* (1/1)

F. poae	3* (1/10)	5±5* (2/10)	41±70*(3/10)	3±3* (7/8)
F. proliferatum	218±132 (10/10)	69±105 (10/10)	101±92(10/10)	97±204(8/8)

Means and standard deviation of positive samples and proportions of positive sample per sampled beetles are shown.The value with asterisk * indicated not all the tested samples were positive.

According to the mycotoxin quantification, beauvericin, enniatin B, A_1, B_1 were detected in live beetles (Table 2). Enniatin A was only detectable in beetles grazing on *F. poae* and *B. bassinana*. Mycotoxin of fumonisin B_1 was not detected feeding on *F. proliferatum* mycelia and conidia. Deoxynivalenol, nivalenol and zearalenone were not detectable in the beetles ingesting *F. culmorum* and *F. graminearum*. Enniatin A_1, B_1, content in beetles grazing on *F. avenaceum*, *F. equiseti*, *F. poae* and *F. proliferatum* at time points 1 or 5 days was different. However, the mycotoxin content for enniatin A_1 and B_1 was higher at the time point 10 days.

3.4 Fungal dissemination by copulation of beetles

To examine whether beetles can transmit the fungus to female beetles as well as to the offspring, male imagoes ingested *Fusarium* were mated with naive female imagoes , After 7 days , the female adults were dissected and eggs were taken out of the cavity. The eggs were surface sterilized and incubated on CZID medium. 3-4 days later the *Fusarium* mycelia growth was recorded (Fig. 2F). Out of 50 eggs we detected 30 eggs were contaminated with *F. proliferatum.* As control, 50 eggs were treated similarly and no egg contamination with *F. proliferatum was detected. In one case we* observed a contamination with *Aspergillus* species. The results indicated that *Fusarium* contamination can occur during beetles' mating.

3.5 Fungal re-isolation and confirmation

To confirm the identity of isolated *Fusarium* mycelium was transferred to both PDA and CLA plates. Fungi were tested for specific morphological features of spores. In addition, DNA of isolated fungi from contaminated kernels, adults' excreta, eggs after mating, or beetles' guts were used in PCR with species-specific primers of e.g. *F. proliferatum*. A amplified DNA product resulting in a band with 585 bp size in agarose gel electrophoresis confirmed that the kernels, adults' excreta, eggs and adult gut were contaminated by *F. proliferatum*.

Table 2: Mycotoxin content in *T. molitor* live beetles

Fungi		Beauvericin	Fumonisin B_1	Enniatin A	Enniatin B	Enniatin A_1	Enniatin B_1	Deoxynivalenol	Nivalenol	Zearalenone
		(ng mycotoxin/g beetles meal)								
F. avenaceum	1 day	LOD	LOD	LOD	410±334*	275±12*	263±162	/	/	/
	5 days	LOD	LOD	LOD	382±152	261±32*	297±97	/	/	/
	10 days	LOD	LOD	LOD	510±181*	291±32*	355±111*	/	/	/
	15 days	LOD	LOD	LOD	216±28*	LOD	177±6*	/	/	/
F. poae	1 day	252±56*	LOD	LOQ	LOQ	203 ±33*	156±29	/	/	/
	5 days	176*	LOD	LOQ	366*	259±80	221±102	/	/	/
	10 days	1153±1114	LOD	LOQ	235 ±31*	337±82	305±104	/	/	/
	15 days	215±55*	LOD	212*	LOQ	345±352	292±332	/	/	/
F. proliferatum	1 day	1383±1656*	LOD	LOD	349±350*	265±108*	230±174	/	/	/
	5 days	499±466*	LOD	LOD	257±45*	207±15*	203±46	/	/	/
	10 days	LOQ	LOD	LOD	466±128*	209±25*	309±92*	/	/	/
	15 days	LOD	LOD	LOD	185±19*	225±11*	166±12	/	/	/
F. culmorum	1 day	/	/	/	/	/	/	LOD	LOD	LOD
	5 days	/	/	/	/	/	/	LOD	LOD	LOD
	10 days	/	/	/	/	/	/	LOD	LOD	LOD
	15 days	/	/	/	/	/	/	LOD	LOD	LOD
B. bassiana	1 day	161*	LOD	LOQ	349±43*	330±103	283±111*	/	/	/
	5 days	1759 ±1617	LOD	413±215*	232±81	279±48	224±46	/	/	/

Notes;

Enniatin B, enniatin A_1, enniatin B_1 were also detected in uninoculated control wheat kernels from field because of natural *Fusarium* infection.

The values indicate mean values and respective standard deviation. Oblique lines indicate that the mycotoxin was not analyzed.

The limits of quantification (LOQ) of beauvericin, enniatin A, enniatinB, enniatin A_1, enniatin B_1 were 155 ng/g while LOQ of fumonisin B_1 was 390 ng/g.

The asterisk * indicated not all 10 of the tested samples were positive.

4. Discussion

Here, we found evidence that *T. molitor* is capable of disseminating *Fusarium* species within grains. It is well reported that pest insects may increase mold in stored grain products by physical opening an entrance for fungi during feeding (Dunkel, 1988), however, to our knowledge it is the first time described that *F. proliferatum* may attract *T. moitor* to use these insects as vehicle to get dispersed between grains.

Adult *T. molitor* beetles ingested *F. proliferatum* mycelia and conidia ， were capable of contaminating wheat kernels at a rate of about 90% even 20 days later. The mouthparts and feces may represent predominant routes for this contamination (Hendrichs et al., 1991). Underlining our results, Teetor-Barsch also mentioned that some insects contribute to fungus dissemination by means of conidia passage through their guts (Teetor-Barsch & Roberts, 1983).

Mycotoxin of fumonisin B_1, deoxynivalenol, nivalenol and zearalenone were not detected in live beetles. One explanation could be that the insects were not an appropriate environment to synthesize these mycotoxins by fungi or that the insects detoxified the mycotoxin or excreted them (Abado‐Becognee et al., 1998). Enniatin A_1 and B_1 mycotoxin content were higher at time point 10 days compared that of 1 and 5 days time points, which indicates that mycotoxins were synthesized in live beetles after 5 days. Comparing the fungal DNA biomass and the mycotoxin content in beetles, we found a positive correlation.

Interestingly, we found *Fusarium* conidia transmission by beetles' mating in agreement with our study. Maniania et al. also found that male *Busseola fusca moths* successfully transmitted *Metarhizium anisopliae* or *Beauveria bassiana* fungi to females by mating (Maniania et al., 2011).

It is well documented that insects represent vectors for plant pathogenic fungi: based on a national survey of *Fusarium* species in Canada by Gordon (Gordon, 1959). *F. avenaceum*, *F. culmorum* and *F. poae* were isolated from following insects: common housefly (*Musca domestica*), clover leaf weevil (*Hypera punctata*) and grass hoppers (*Melanoplus bivittatus*). In addition, Windels et al. (Windels et al., 1976) isolated *F. solani*, *F. moniliforme*, *F. oxysporum* and *F. roseum* from picnic beetles (*Glischrochilus quadrisignatus*). These

observations suggest that other insects play also a role in the dissemination of *Fusarium* species as well.

In conclusion, our study illustrated ecological interactions of *T. molitor* beetles with tested *Fusarium* species. We found that *T. molitor* beetles may serve as vehicle for conidia transmission particularly for *F. proliferatum*. We believe that our present study provides valuable insights into the impact of insects on toxin-producing fungi dissemination within storage grains, which may help in the improvement of post-harvest management approaches for future food security.

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Chapter 4 Characterization of diverse *Fusarium proliferatum* strains and the pathogenicity on *Tenebrio molitor*

Abstract

Fusarium proliferatum (Matsushima) Nirenberg is an important pathogen infecting numerous crop plants thereby reducing yield and quality. In various climatic zones, *F. proliferatum* colonizes an extraordinarily broad range of host plants including maize, wheat, barley, rice, asparagus, banana, date palm, garlic, onion, miscanthus and more. In our previous experiments, we reported that the meal beetle *T. molitor*, was preferably feeding on *F. proliferatum* mycelia and conidia, and wheat kernels colonized with *F. proliferatum*. Our hypothesis is, utilizing behaviors property of the meal beetle *T. molitor* preference feeding on *F. proliferatum* mycelia and conidia, and *F. proliferatum* colonized wheat kernels, to find potential of *F. proliferatum* in biological control of storage pests as trap.

Radiate growth rate, sporulation, pathogenicity on 4^{th} instar *T. molitor* larvae of *F. proliferatum* single spore strains originally isolated from garlics, onions, asparagus, maize, miscanthus and dead larvae separately in Germany, France, Syria and Austria were recorded. Radiate growth rate and sporulation had weak negative correlation to the pathogenicity of *F. proliferatum* strains, and there was variance of pathogenicity among the tested *F. proliferatum* strains. DNA biomass in the larvae tissue between the cuticle and gut passage was quantified and illuminated that some of tested *F. proliferatum* strains can proliferate in the larvae.

1. Introduction

Fusarium proliferatum (teleomorph: *Gibberella intermedia*) is a worldwide pathogen distribution on a variety of agricultural plants, including maize, wheat, barley, rice, sorghum, oats (Marín et al., 1998, Park et al., 2005, Leslie et al., 1990, Wu et al., 2005, Edwards, 2009) and non-agricultural hosts, including banana (Jimenez et al., 1993), citrus fruits (Hyun et al., 2000), asparagus (Elmer, 1995), orchids (Benyon et al., 1996, Ichikawa & Takayuki, 2000), date palm (Abdalla et al., 2000), mango, and pine seedlings (Ocamb et al., 2002). *F. proliferatum* strains infect crops roots, seedlings, spikes, ears in the field as well as crops products postharvest. *Fusarium* mycelia colonization on postharvest kernels causes serious problem in storage grains and diminishes the quality of the crop products by secondary metabolites.

Fusarium species also infect animals, such as: nematodes, spiders, insects amphibians, reptiles, and mammals (Teetor-Barsch & Roberts, 1983). The research on pathogenicity of *Fusarium* species against insects was well studied. More than 13 *Fusarium* species are pathogenic to insects, and the group has a host range that includes Coleoptera, Diptera, Hemiptera, Hymenoptera, and Lepidoptera (Humber, 1992). *F. oxysporum* was effective against larvae of *Chilo auricilius*, *C. infuscatellus* and *Sesamia inferens*, and adults and nymphs of *Phytoscaphus perpusilla* (Varma & Tandan, 1996). Majumdar found that dense mycelia of *F. solani* grew inside puparia from transverse dissection of infected root maggot pupae, which indicated *F. solani* fungal penetration and pathogenicity (Majumdar et al., 2008). *F. avenacum* has been demonstrated its pathogenicity on greenhouse whitefly (*Trialeurodes vaporariorum*) (Rojas et al., 2003) in Colombia. *F. graminearum*, *F. culmorum*, *F. acuminatum*, *F. avenaceum*, and *F. equiseti* had entomopathogenic properties on wheat stem sawfly (*Cephus cinctus*) (Sun, 2008).

Many *Fusarium* spp. are known to produce a broad spectrum of protein and polysaccharide-hydrolysing enzymes, which could be useful in complete hydrolysis of complex organic substances, including both living and non-living plant cell walls and insect cuticles. This versatility of *Fusarium* spp. in transforming from plant pathogens to insect pathogens enables them to cause epizootics of both plant diseases and insect diseases in fields (St Leger et al., 1997). Venugopal et al. (Venugopalrao et al., 1989) observed that epizootics of *Fusarium* caused mortality levels that were equal to or even higher than predators and parasitoids in populations of the whitefly, *Bemisia tabaci* (Gennadius).

In addition to the insecticidal activities of extracellular hydrolytic enzymes, mycotoxins have been reported as important secondary metabolites of *Fusarium* species. Many mycotoxins and their derivatives, have insecticidal properties (Roberts, 1981). *F. proliferatum* is the well known producer of fumonisins production in food and feed products (Jurado et al., 2010, Desjardins et al., 2007). Besides, fumonisins, *F. proliferatum* synthesize beauvericin (Leslie et al., 2004, Logrieco et al., 1995, Plattner & Nelson, 1994), fusaproliferin (Leslie et al., 2004, Randazzo et al., 1993, Reynoso et al., 2004), fusarins (Miller et al., 1995), moniliformin (Logrieco et al., 1995, Miller et al., 1995). Gupta et al.(Gupta et al., 1991) isolated the toxin beauvericin, a cyclodepsipeptide, from *Fusarium*. Those authors showed that beauvericin is able to kill 50% of Colorado potato beetles, *Leptinotarsa decemlineata* (Say), larval test populations at a 633-ppm dose.

Tenebrio molitor is an important and globally distributed pest of stored products and sensitive to small quantitative variation in dietary composition and used as bioassay agent. *T. molitor* was demonstrated by Davis et al. (Davis et al., 1975) as bioassay agent to screen mycotoxins. Davis & Smith (Davis & Smith, 1977), who detected toxic metabolites from psychrophilic *Fusarium* pathogens in cereal grain, with larvae of *T. molitor*. Morales-Ramos et al. illustrated that *T. molitor* larvae have the ability to self-select optimal ratios of two dietary components for development and population growth (Morales-Ramos et al., 2011).

In our previous experiments, we reported that the meal beetle *T. molitor*, was preferably feeding on wheat grains colonized with *F. proliferatum* (Guo et al., 2014). In the present study, 31 single spore strains of *F. proliferatum* were isolated from garlics, onions, asparagus, maize, miscanthus and dead larvae separately in Germany, France, Syria and Austria were tested to find potential of *F. proliferatum* in biological control of storage pests as trap because of meal beetle *T. molitor* preference feeding behaviors property on *F. proliferatum* colonized wheat kernels. Our present study aims to illustrate that 1) whether radical growth rate and sporulation of tested *F. proliferatum* strains were correlated with pathogenicity on 4^{th} instar *Tenebrio molitor* larvae, 2) whether *F. proliferatum* mycelia can penetrate and propagate in the larvae tissue from cuticle or gut.

2. Materials and methods

2.1 Fungal strains cultivation

Single spore strains of *F. proliferatum* were isolated from garlics, onions, asparagus, maize,

miscanthus and dead larvae separately in Germany, France, Syria and Austria – these made up 31 strains as shown in Table 1. Species-specific PCR was also used to confirm the identification of *F. proliuferatum* strains based on morphological features by the light microscopy according to the characteristics on CLA and PDA. Forward primer PRO1: 5'-CTTTCCGCCAAGTTTCTTC-3' and reverse primer PRO2: 5'-TGTCAGTAACTCGACG TTGTTGTT-3' (Mulè et al., 2004). Species specific primers of *F. proliferatum* were designed based on partial sequence of the calmodulin gene (Waskiewicz et al., 2010). *Beauveria bassiana* (entomo-pathogenic fungi) was used as positive control.

Table 1: Strains codes, the years isolated , the host plants and the origin of *F. proliferatum* strains

Isolates	Host plants	Year	Origin
F. proliferatum 2-K	Garlic	2000	France
F. proliferatum 67-M	Miscanthus	1993	Brandenburg, Germany
F. proliferatum 78-M	Miscanthus	1993	Brandenburg, Germany
F. proliferatum 86-M	Miscanthus	1993	Brandenburg, Germany
F. proliferatum 2-Z	Onion	2008	Bad-wuertemburg, Germany
F. proliferatum 5-Z	Onion	2008	Rheinland-Pfalz, Germany
F. proliferatum 29-Mais	Maize	2005	Brandenburg,Germany
F. proliferatum 76-Mais	Maize	2008	Frei/Rust,Germany
F. proliferatum 219-S	Asparagus	2000	Rheinland-Pfalz,Germany
F. proliferatum 223-S	Asparagus	1997	Brandenburg,Germany
F. proliferatum 227-S	Asparagus	2003	Goldgeben 1, Lower Austria
F. proliferatum 231-S	Asparagus	2003	Goldgeben 2, Lower Austria
F. proliferatum 241-S	Asparagus	2003	Upper Austria
F. proliferatum 245-S	Asparagus	2003	Burgenland,Austria
F. proliferatum 259-S	Asparagus	2003	Lower Austria
F. proliferatum 3-B	Dead larvae	2005	Syria
F. proliferatum 14-F	Maize	2006	Germany
F. proliferatum 18-O	Maize	2006	Germany
F. proliferatum 20-J	Maize	2006	Germany
F. proliferatum 24-C	Maize	2006	Germany
F. proliferatum 27-K	Maize	2006	Germany
F. proliferatum 36-D	Maize	2006	Germany
F. proliferatum 42-D	Maize	2006	Germany
F. proliferatum 44-J	Maize	2006	Germany
F9	DSM62261		
F10	F. pro4		
F11	DSM840		
F12	DSM62267		
F13	DSM63267		
F14	DSM764		
F. proliferatum 21.1	Maize	2007	Hainichen, Germany

The meal beetle *T. molitor*, which is a common storage pest and destructive insect species, 4th and last instar (mature) larvae were used in our study. *T. molitor* larvae were reared on whole wheat flour with 5% yeast extract in a climate chamber in darkness at 27 ± 2 °C and a relative humidity of 65 ± 5%. 4th instar larvae were starved for 24 h for the pathogenicity assay and last instar (mature) larvae were starved for 48 h for *F. proliferatum* conidia germination and penetration experiments.Larvae were randomly selected prior use in experiments respectively.

2.2 Growth assessment of *F. proliferatum* strains in vitro

A 5.0 -mm-diameter agar disk filled with mycelia from the margin of a 3-day-old growing colony of each strain grown at 25 °C without light was used to centrally sub-inoculate on PDA. The plates were incubated at 25 °C condition for 7 days, and the experiment consisted of a fully replicated set of treatments with 3 repetitions. Experiments were done twice. Assessment of growth was recorded daily during the 7-day incubation period. The radical diameter of each growing colony was crisscrossed until the colony reached the edge of the PDA plate (Van Poucke et al., 2012, Jurado et al., 2008). The comparison of various isolates was done based on the area of colony ($S= \pi R^2 - \pi r^2$).

2.3 The sporulation variability

Mung bean medium was made according to recipe of Bai (Bai & Shaner, 1996). 1 Lwater was boiled in a flask on a hot plate and 40 g mung bean seeds were placed into boiling water and continue boiled for 10 min (make sure mung bean seeds are entire incase the seeds release the starch which inhibit the conidia growth). Mung beans broth was filtered through four-layer cheese cloth to remove mung bean and broth was harvested in clean flasks. Mung bean broth was subdivided 50 ml into 100 ml volume flask and then autoclaved at 121 ℃ for 20 min. Autoclaved mung bean broth was cooled down to room temperature.

The cryocultures of different isolates of *F. proliferatum* were inoculated on the PDA plates and incubated at 25 ℃ without light for 7 days. Later, three discs of 50 mm diameter PDA filled with mycelia were cut and transferred into 50 ml Erlenmeyer flask with mung bean medium under 150 rpm and 25 ℃ dark incubator condition for 4 days. Using four-layer cheese cloth for filtering the mycelia, conidia were collected in 50 ml tubes. Each isolate has 3 repetitions and the experiments were conducted twice. Harvest conidia were stored in -80 ℃ for plants inoculation. Conidia were counted directly in the suspension with haemocytometer cell.

2.4 The difference of pathogenicity on 4th instar *T. molitor* larvae

Agar disks filled with mycelia (as mentioned above) of each *F. proliferatum* strain were inoculated on PDA plates after 7-8 days growth, the mycelia reached to the edge of the plates. Each strain had 5 repetition PDA plates. Per PDA plate 10 *T. molitor* larvae at 4th-instar-age were reared on mycelia and conidia of diverse *F. proliferatum* strains, and survival rates were monitored within 15 days. Dead larvae were fetched out of the plates daily to avoid becoming putrid and contaminating other live larvae. The 4th instar larvae were starved for 24 h and were randomly selected prior use in experiments. Positive control treatment was PDA plates with *Beauveria bassiana* mycelia and conidia while negative control treatment was handled in the same way with uninoculated PDA agar. The experiment was repeated twice.

2.5 *F. proliferatum* conidia germination and penetration from gut passage

After mature larvae were starved 48 h and then placed on PDA plates filled with *F. proliferatum* strains mycelia and conidia for 15 days. Individual larvae was dissected and separated into three parts: the cuticle, the larvae tissue between cuticle and the gut, and the gut.10 individual larvae were done for each treatment and mature larvae ingesting *Beauveria bassiana* (an insect-pathogenic fungus) mycelia and conidia as positive, blank PDA plate without fungi infection as negative control.

The larvae tissue between cuticle and the gut samples were freeze-dried and ground into fine powder. Total DNA of *T. molitor* each larvae sample was extracted from ground sample fine powder using a CTAB method (Brandfass & Karlovsky, 2008). The DNA pellet was dissolved in 20 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). DNA of tissue was diluted fifty fold prior to PCR analysis. DNA of cuticle and gut was diluted a hundred fold prior to PCR analysis because of high lipids and chitin. Inhibition assay was done to test if any that matrix effects amplification of fungal DNA.

Thermocycler (CFX384TM, BioRad, USA) was used for real-time PCR analysis (quantitative PCR) in a total volume of 4 ul. DNA biomass in these sampels of tested *F. proliferatum* strains was compared. Primers Fp 3F/4R (Jurado et al., 2006) were used for the species-specific detection of *F. proliferatum*. The reaction mixture consisted of buffer (16 mM $(NH_4)_2SO_4$; 67 mM Tris-HCl; 0.01% (v/v) Tween-20, pH 8.8 at 25 ℃, Bioline GmbH, Luckenwalde, Germany), 0.15 mM of each dNTP (Bioline GmbH, Luckenwalde, Germany), 2.5 mM $MgCl_2$, 0.1 U of Taq DNA polymerase (BIOTaq, Bioline GmbH, Luckenwalde,

Germany), 0.3 mM of each primer, 0.1 x SYBR Green I (Invitrogen, Karlsruhe, Germany) and 1 mg/ml bovine serum albumin. The lowest standard set as limits of quantification (LOQ) was 2.09 fg/ul. DNA biomass in these samples of tested *F. proliferatum* strains was compared. Data analyses were conducted by SPSS (IBM SPSS Statistics 22, USA).

3. Results

3.1 Growth assessment of *F. proliferatum* strains

In a first step we determined the radical growth rate of various *F. proliferatum* strains within 7 days on PDA medium to monitor the mycelia growth speed contribution on pathogenicity of *F. proliferatum* to *T. molitor* larvae, which means if the radical growth was faster, the strains caused a higher mortality rate. We compared the colony area of thirty-one *F. proliferatum* strains according to the diameter of the colony.

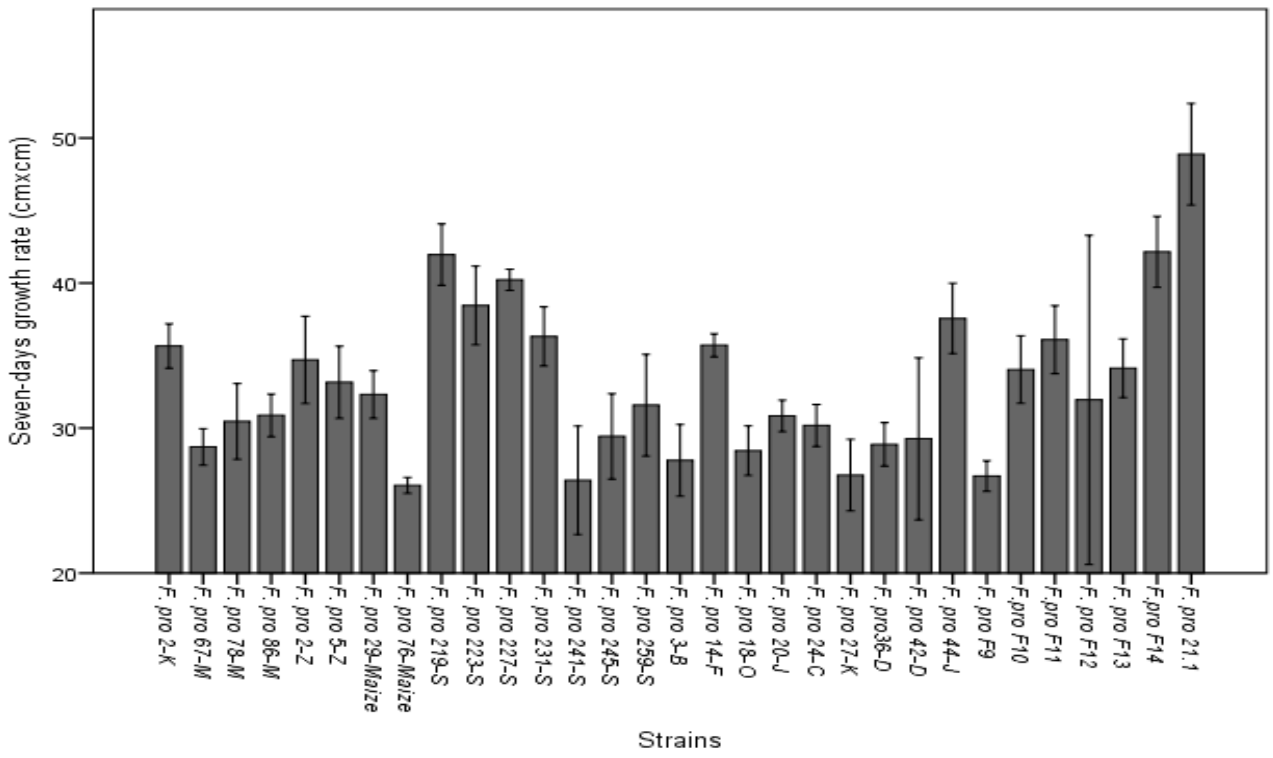

Fig. 1. Growth assessment of *F. proliferatum* strains on PDA plates ($S=\pi R^2-\pi r^2$). Radiate growth rates of various *F. proliferatum* strains isolated from divers host plants were significant different . Bars indicated mean ± S.D.

According to the data shown in Fig. 1, there was significant a difference among the strains. *F. proliferatum* 21.1, F14 and 219-S grew relatively faster compared to other tested strains whereas radical growth rates of *F. proliferatum* 76-Maize, 241-S, 3-B, 27-K and F9 were relatively lower. Even the *F. proliferatum* from the same host, the growth rate was variance.

For instance: *F. proliferatum* 219-S, 223-S, 227-S, 231-S, 241-S, 245-S and 259-S, were all isolated from asparagus. However, growth rate of *F. proliferatum* 219-S was significantly higher than that of *F. proliferatum* 241-S.

3.2 Sporulation variability in mung bean medium

To determine the sporulation contribution on pathogenicity of fungi to *T. molitor* larvae, the sporulation variability experiment of test isolates of *F. proliferatum* was conducted.

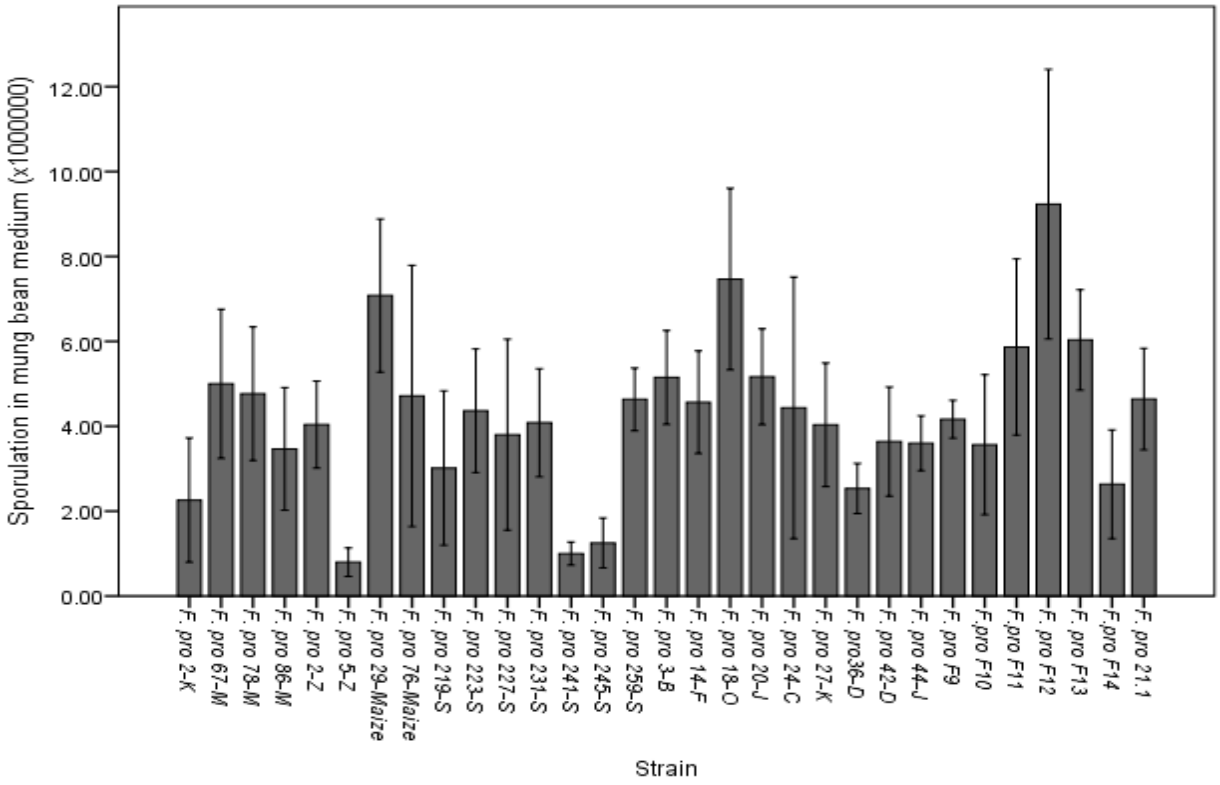

Fig. 2. Sporulation variability in mung bean medium. Sporulation ability of diverse tested *F. proliferaum* strains in mung bean medium was significantly different between each other. Bars indicated mean ± S.D. (N=186).

From the data above, sporulation variety existed among strains，sporulation value of *F. proliferaum* F12, *F. proliferaum* 18-O, and *F. proliferaum* 29-Maize were higher than the other strains, in contrast, conidia concentration of *F. proliferaum* 5-Z, *F. proliferaum* 241-S, and *F. proliferaum* 245-S was relatively lower. Though the strains were isolated from the same plant host, the sporulation ability was various, for example, *F. proliferaum* 2-Z and *F. proliferaum* 5-Z were isolated from onion, the sporulation ability of *F. proliferaum* 2-Z is significant higher compared *F. proliferaum* 5-Z. It also happened to *F. proliferaum* 219-S, 223-S, 227-S, 231-S, 241-S, 245-S and 259-S, which were isolated from asparagus.

3.3 The correlation radical growth rate, sporulation of different tested strains and mortality rate of 4th instar larvae

In a next step we determined the capability of *F. proliferatum* strains to induce mortality of larvae. We monitored the preference and avoidance feeding behaviors and also the mortality of 4th instar *T. molitor* larvae reared on PDA plates covered with mycelia of thirty-one *F. proliferatum* strains during a period of 15 days. Under these conditions fungal mycelium on PDA was the sole diet available for the 4th instar larvae.

Table 2: The preference and avoidance of 4th instar *T. molitor* larvae on the PDA covered with mycelia

Isolates	Host plants	Preference/Avoidance
F. pro 2-K	Garlic	-
F. proliferatum 67-M	Miscanthus	++
F. proliferatum 78-M	Miscanthus	++
F. proliferatum 86-M	Miscanthus	-
F. proliferatum 2-Z	Onion	++
F. proliferatum 5-Z	Onion	-
F. proliferatum 29-Mais	Maize	-+
F. proliferatum 76-Mais	Maize	-
F. proliferatum 219-S	Asparagus	-
F. proliferatum 223-S	Asparagus	++
F. proliferatum 227-S	Asparagus	-
F. proliferatum 231-S	Asparagus	++
F. proliferatum 241-S	Asparagus	++
F. proliferatum 245-S	Asparagus	-
F. proliferatum 259-S	Asparagus	-+
F. proliferatum 3-B	Dead larvae	++
F. proliferatum 14-F	Maize	-+
F. proliferatum 18-O	Maize	+
F. proliferatum 20-J	Maize	+
F. proliferatum 24-C	Maize	-
F. proliferatum 27-K	Maize	-
F. proliferatum 36-D	Maize	-
F. proliferatum 42-D	Maize	-
F. proliferatum 44-J	Maize	++
F9	DSM62261	++
F10	Fpro4	++
F11	DSM840	++
F12	DSM62267	-
F13	DSM63267	++
F14	DSM764	-+
F. proliferatum 21.1	Maize	++

Note: -- avoid feeding; -+avoid feeding to light preference; + light preference; ++ preference

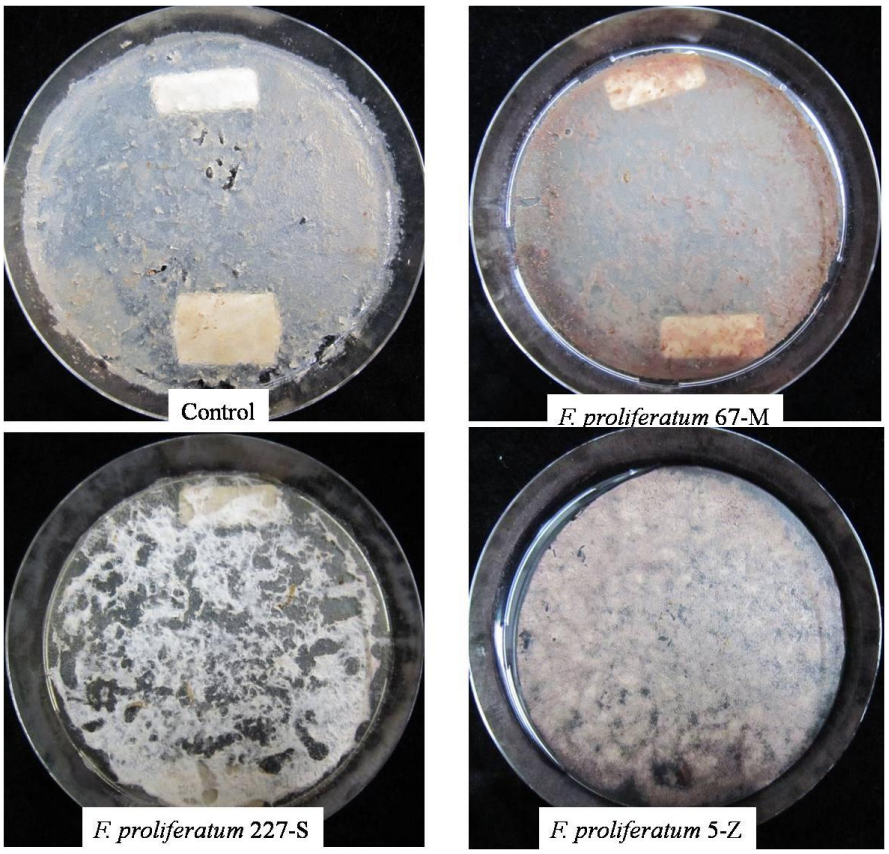

Fig. 3: the preference and avoidance feeding behavior of 4th instar *T. molitor* larvae on mycelia of representative strains. 4th instar *T. molitor* larvae preferred feeding on mycelia and conidia of *F. proliferatum* 67-M**,** whereas they avoided feeding on mycelia and conidia of *F. proliferatum* 67-M strain. For *F. proliferatum* 227-S, 30-40 % of the mycelia was grazed by the tested larvae.

The 4th instar *T. molitor* larvae were used in this experiments because of their sensitivity on different strains of *F. proliferatum*. As shown in the table 2 and Fig. 3, the 4th instar *T. molitor* larvae preferred feeding on the mycelia of *F. proliferatum* 67-M, 78-M, 2-Z, 223-S, 231-S, 241-S, 3-B, 44-J，F9，F10, F1, F13 and 21.1, which caused lower mortality rate. However, for *F. proliferatum* 86-M, 5-Z, 76-Mais, 219-S, 227-S, 245-S, 24-C, 27-K, 36-D, 42-D and F12, 4th instar *T. molitor* larvae avoided grazing on these strains mycelia, which caused a higher mortality rate. Interestingly, 4th instar *T. molitor* larvae preferred feeding on 44-J and 18-O which caused relatively high mortality.

We determined the mortality rate of larvae ingesting fungi colonized kernels for a period of 15 days.

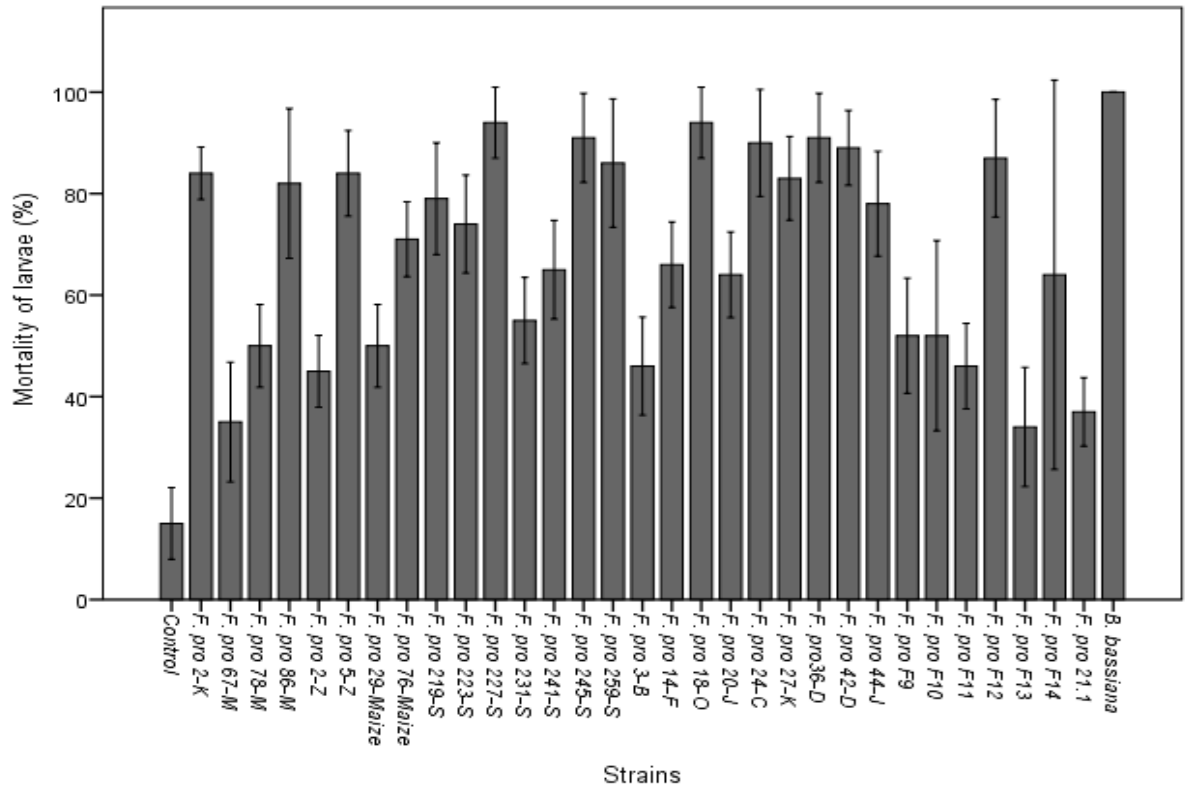

Fig. 4: the mortality of 4th instar *T. molitor* larvae feeding on mycelia of different *F. proliferatum* strains. Mortality of larvae reared on PDA plates covered with mycelium of diverse tested *F. proliferatum* strains within 15days. Results indicated mean ± S.D. (N=660)

Mortality of 4th instar *T. molitor* larvae within 15 days was recorded in Fig.4, which indicated the pathogenicity of different tested *F. proliferatum* strains. The 4th instar of *T. molitor* larvae were used in this experiment because of their sensitivity of the feed. *F. proliferatum* 67-M, 78-M, 2-Z, 29-Maize, 231-S, 241-S, 3-B, F9, F10, F11, F13 and 21.1 caused lower mortality, whereas *F. proliferatum* strains 2-K, 86-M, 5-Z, 227-S, 245-S, 259-S, 18-O, 24-C, 36-D and F12 resulted in relatively higher mortality.

To determine whether radical growth rate and sporulation contribute to the pathogenicity of different tested *F. proliferatum*, the correlation between them was analyzed and the results were shown as below:

Correlation coefficients (r) of radical growth rate, sporulation of different tested *F. proliferatum* strains and mortality rate of 4th instar larvae

Evaluable parameters	Mortality rate on 4th instar *T. molitor* larvae (N=310)
Radical growth rate (N=310)	r= -0.132 Sig.=0.020*<0.05
Sporulation (N=180)	r= -0.183 Sig.=0.014 *<0.05

*: Correlation is significant at the 0.05 level (2−tailed).

According to the data analyzed by SPSS 22.0 version, interestingly, we found a weak negative correlation (r= -0.132) between radical growth rate and mortality of larvae. Identically, a weak negative correlation (r= -0.183) was also detected between the sporulation and mortality of the *T. molitor* larvae.

3.5 *F. prolifertum* conidia germination and penetration from gut passage

To monitor if the tested *F. proliferatum* conidia ingested in gut passage of mature larvae can germinate and penetrate the tissue between the cuticle and gut and proliferate, we carried out this experiment.

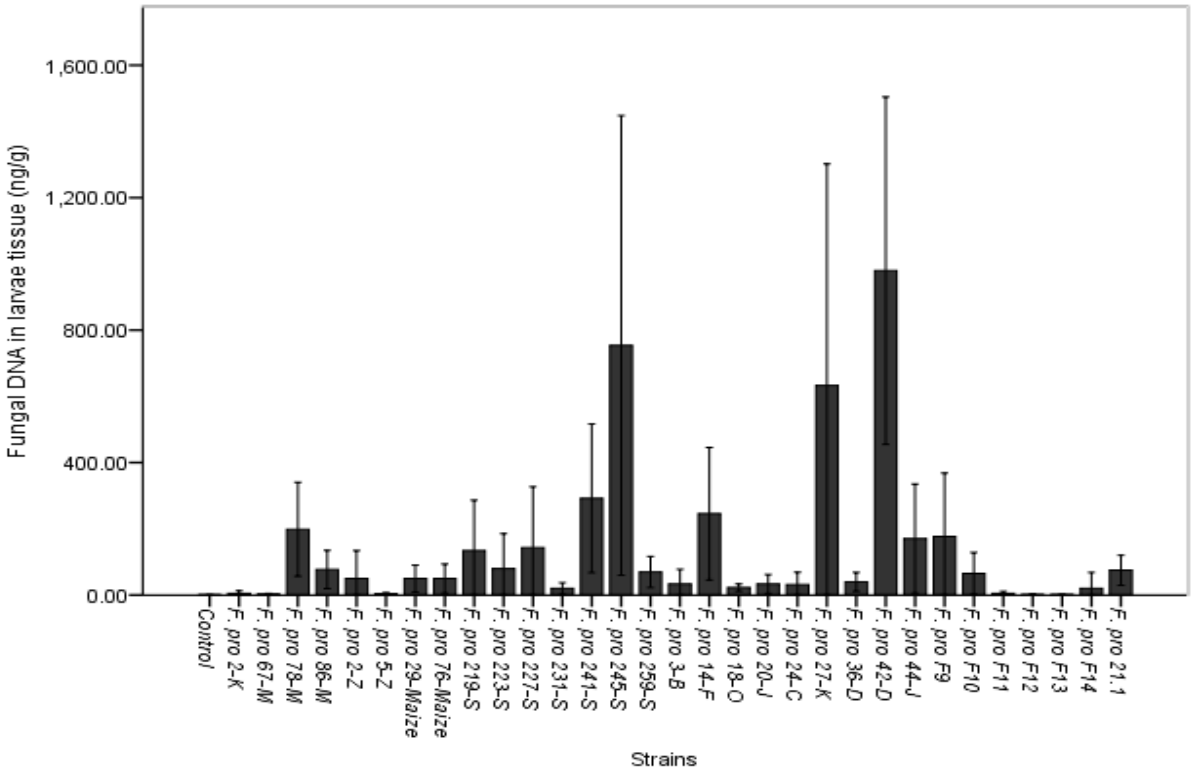

Fig. 5: Fungal DNA biomass of different *F. proliferatum* strains in tissue of mature *T. molitor* larvae. DNA of live larvae survived from feeding on mycelia and conidia of different *F. proliferatum* strains within 15 days was quantified. Results indicated mean ± S.D.

As shown in Fig. 5, DNA biomass of *F. proliferatum* was not all detected in the larvae tissue samples. DNA biomass in the tissue of the larvae ingesting on mycelia and conidia of *F. proliferatum* 2-K, 67-M, 5-Z, F11, F12, F13 was extremely low, whereas that of larvae tissue consumed mycelia and conidia of *F. proliferatum* 42-D, 27-K, 245-S, 14-F and 241-S was relatively low than the other tested strains. Surprisingly, the *F. proliferatum* strains 42-D, 27-K, or 245-S was avoided by the larvae feeding on.

4. Discussion

F. proliferatum strains tested in our present study from wide range of hosts (garlics, onions, asparagus, maize, miscanthus and dead larvae) and geographic areas showed that strains are highly diverse on growth rate. Even *F. proliferaum* strains isolated from the same host plant, for instance, *F. proliferaum* 219-S, 223-S, 227-S, 231-S, 241-S, 245-S and 259-S, all the strains were isolated from asparagus, there was difference among the strains on growth rates. *F. proliferaum* 219-S strain was the fastest whereas *F. proliferaum* 241-S was the slowest compared to the growth rate of the other strains. The radial growth indicated the speed of the mycelia spreading in the host. Marin reported the results that there was an inverse correlation between growth of *F. proliferatum* at optimum temperature condition and fumonisin B_1 production (Marin et al., 1999).

Sporulation of *F. proliferaum* indicated the ability of proliferation of the fungi on the host. Strains with high sporulation may colonize the host faster. Velluti investigated that there was no correlation between *F. proliferaum* sporulation and mycotoxin formation on colonized maize grains (Velluti et al., 2000). Fast growth and abundant sporulation are important characteristics for fungi to easily survive during the competition and occupy ecological niche (Ramakrishna et al., 1993).

Even the strains from the same host showed differences in their virulence on larvae. For example, *F. pro* 67-M and *F. pro* 78-M strains were not so toxic whereas *F. pro* 86-M was more aggressive than they others. *F. pro* 29-Maize did not cause a high mortality rate, by contrast, *F. pro* 76-Maize caused a high mortality rate. *F. pro* 3-B was isolated from dead larvae and didn't cause so high mortality on larvae indicating that *F. pro* 3-B may colonize larvae after they are dead as saprophytes. Radical growth rates and sporulation of the strains have no positive association with virulence on 4^{th} instar *T. molitor* larvae.

There are two possible pathways of the conidia penetrating into tissue between the gut and the cuticle. Firstly: the conidia attached on the cuticle germinated and penetrated into the tissue by the cuticle. This is also the hypothesis raised by Batta, who speculated that *F. avenacum* conidia can germinate and penetrate into the cuticle of rice weevil (*Sitophilus oryzae* L.) (Batta, 2012). Secondly, the conidia grazed by the mature larvae can germinate in the gut passage and subsequently penetrate the intestinal wall of the larvae. With time on, the mycelia colonize the tissue between the gut and the cuticle, finally, the whole insect. Hasan and Vago

proved that conidia of *F. oxysporum* germinated in the intestinal lumen after the fungi invaded mosquito host larvae via the oral route, subsequently, the ensuing mycelia invade all tissue (Hasan & Vago, 1972).

In conclusion, our research determined that the radical growth rates, sporulation, pathogenicity on 4th instar *T. molitor* larvae and fungi DNA biomass in the tissue between the cuticle and the gut difference among the tested *F. proliferatum* strains. This is the first time that DNA biomass in the tissue between the cuticle and the gut was reported, which can be the favorable evidence to prove *F. prolifertaum* conidia can germinate in the gut passage and penetrate through gut intestinal lumen to the tissue. Further experiments, GFP (green fluorescent protein) labeled *F. proliferatum* strains will be used to repeat the pathogenicity on the larvae to confirm the infect pathway.

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Chapter 5 Systemic infection characteristics of different strains of *F. proliferatum* on wheat

Abstract

Fusarium proliferatum is a *Fusarium* species worthy of investigation, as this fungus is frequently isolated from a wide range of host plants, causing crown, spears and root rot of asparagus, bulb rot in garlic and onion, fruit rot in date palms. In addition, *F. proliferatum* is also a main pathogen of maize worldwide and other small grains like: rice, sorghum, cause black point symptoms on wheat (rarely reported) and barley. Decreased yield as well as diminished quality of plant products due to *Fusarium* infection cause significant economic losses worldwide.

Single spore strains of *F. proliferatum* were isolated from garlics, onions, asparagus, maize, miscanthus and dead larvae separately in Germany, France, Syria and Austria. Greenhouse experiments were conducted to determine the occurrence of pathogenicity of diverse *F. proliferatum* isolates on wheat. Flag leaves, stems and kernels matrices of the *Fusarium* susceptible summer wheat cv. Taifun were used for in vitro studies on the production of mycotoxins and fungal biomass by different *F. proliferatum* isolates. According to the fungal biomass in different parts of infected wheat plants, in general, biomass in stem (the part from ear to last node) was the highest, and then the first flag leaf, and the kernels was the lowest.

Our present study illustrated that 1) Tested *F. proliferatum* strains from other host plants can colonize on wheat plants 2) *F. proliferatum* strains can infect systemically from stem to leaf and then to wheat kernels by soak-inoculation of mature seeds.

1. Introduction

Fusarium proliferatum (teleomorph: *Gibberella intermedia*) is a member of the mating population-D of *Gibberella fujikuroi*, which is a complex of eight mating population (MPs) and a number of asexual lineages, sexual compatibility, and DNA-based phylogenetic analysis (Britz et al., 1999, Leslie, 1991, Leslie et al., 2006), describes as distinct morphological species (Nirenberg & O'Donnell, 1998). Most representatives of *G. fujikuroi* complex have economic importance, *F. proliferatum* is specially worthy of investigation, as this fungi is frequently isolated from wide range of host plants, causing crown, spears and root rot of asparagus (Bargen et al., 2009), bulb rot in garlic and onion (Stankovic et al., 2007), fruit rot in date palms (Abdalla et al., 2000). *F. proliferatum* is also a main pathogen of maize worldwide and other small grains like: rice (Abbas et al., 1999), sorghum (Bacon & Nelson, 1994), cause black point symptoms on wheat (Desjardins et al., 2007) and barley (Marin et al., 1999). Decreased yield as well as diminished quality of plant products due to *Fusarium* infection cause significant economic losses worldwide (Placinta et al., 1999, Glenn, 2007).

Another detrimental effect of *F. proliferatum* infected grains is the accumulation of mycotoxins *F. proliferatum*, together with *F. verticillioides*, is the main producer of fumonisins, which is a health risk mycotoxin, contaminating crops products in the field and post-harvest. Fumonisins can be detected in symptomless infected kernels (Bacon & Hinton, 1996, Bullerman & Tsai, 1994). Beside fumonisins, *F. proliferatum* synthesize beauvericin (Leslie et al., 2004, Logrieco et al., 1995, Plattner & Nelson, 1994), fusaproliferin (Leslie et al., 2004, Randazzo et al., 1993, Reynoso et al., 2004),fusarins (Miller et al., 1995) and moniliformin (Logrieco et al., 1995, Miller et al., 1995).

Plant systemic infection has been reported by several *Fusarium* species. The entire maize plant can be systemically colonized without causing symptoms by *F. verticillioides*, and with this method, *F. verticillioides* can be transmitted from seeds to whole plant to kernels (Munkvold & Carlton, 1997, Munkvold et al., 1997). *F. subglutinans* was also proven to infect maize kernels similar as *F. verticillioides*. The pathway of the infection pathogens initiated from the colonized maize seeds, to the seedling roots and then the entire plant, and at last the kernels (Wilke et al., 2001). Bacon & Hinto have reported that *F. moniliforme* caused systemic infections of maize kernels, subsequently, colonized maize kernels served as dissemination vehicles and new inocula sources. The authors also illustrated the essential

difference between symptomless infected plants and plants showing disease symptoms. In plants without visual signs, mycelia were intercellular only and distributed through the whole plant, whereas in plants presenting disease symptoms, the mycelia were both intercellular and intracellular (Bacon & Hinton, 1996).

F. proliferatum predominantly found in infected maize (*Zea mays* L.) and associated with maize ear rot and, to a lesser extent, in maize seeds (Logrieco et al., 1995). In previous studies, *F. proliferatum* was reported as an endophytic fungus isolated from the stem bark of *Dysoxylum binectariferum* Hook. f (Kumara et al., 2012). *F. proliferatum* was also described to be endophytic under non stress conditions in wheat (Bishop, 2002), and alters the hosts defense response (Bishop et al., 2002). However, *F. proliferatum* infecting wheat as a pathogen and causing disease were rarely studied. The objectives of this study: we conducted experiments with *F. proliferatum* strains isolated from different hosts to assess variation in colonization at wheat flag leaves, stems (the internode between ear to last node), and kernels matrices of wheat plant (cultivar: Taifun) inoculated (soak-inoculation of mature seeds). Colonization was quantified using species-specific DNA based on quantitative PCR.

2. Materials and methods

2.1 Fungi strains cultivation

Sixteen single spore strains of *F. proliferatum* were isolated from garlics, onions, asparagus, maize, miscanthus and dead larvae separately in Germany, France, Syria and Austria (Table 1). Species-specific PCR was also used to confirm the identification of *F. proliferatum* strains based on morphological features by the light microscopy according to the characteristics on CLA and PDA. Forward primer PRO1: 5'-CTTTCCGCCAAGTTTCTTC-3' and reverse primer PRO2: 5'-TGTCAGTAACTCGACGTTGTTGTT-3' (Mulè et al., 2004). Species specific primers of *F. proliferatum* were designed based on partial sequence of the calmodulin gene (Waskiewicz et al., 2010).

Table 1 shows the isolates codes, the host plants, the years and the origins of *F. proliferatum* strains

Isolates	Host plants	Year	Origin
Fpro-2-K	Garlic	2000	France
Fpro-67-M	Miscanthus	1993	Brandenburg, Germany
Fpro-78-M	Miscanthus	1993	Brandenburg, Germany
Fpro-86-M	Miscanthus	1993	Brandenburg, Germany
Fpro-2-Z	Onion	2008	Bad-wuertemburg, Germany
Fpro-5-Z	Onion	2008	Rheinland-Pfalz, Germany
Fpro-29-Maize	Maize	2005	Brandenburg,Germany
Fpro-76-Maize	Maize	2008	Frei/Rust,Germany
Fpro-219-S	Asparagus	2000	Rheinland-Pfalz,Germany
Fpro-223-S	Asparagus	1997	Brandenburg,Germany
Fpro-227-S	Asparagus	2003	Goldgeben 1, Lower Austria
Fpro-231-S	Asparagus	2003	Goldgeben 2, Lower Austria
Fpro-241-S	Asparagus	2003	Upper Austria
Fpro-245-S	Asparagus	2003	Burgenland,Austria
Fpro-259-S	Asparagus	2003	Lower Austria
Fpro-3-B	Dead larvae	2005	Syria

2.2 Plant material

The main experiments were conducted using the summer wheat cultivar “Taifun” (KWS, Germany). The susceptible score of cv. Taifun was 6 (ranking from 1 to 9 presents from resistant to susceptible). Stem, flag leaf and wheat kernels matrices of the cv. Taifun were used for in vitro analyses on fungal biomass and mycotoxin production (Wagacha Maina, 2008).

2.3 Inocula preparation

Mung bean medium was made according to recipe of Bai (Bai & Shaner, 1996) and optimized according to our laboratory equipments. Mung bean broth subdivided into 50 ml in 100 ml volume flask and then autoclaved at 121 ℃ for 20 min. Autoclaved mung bean broth was cooled down to room temperature. The cryo-cultures of sixteen different isolates of *F. proliferatum* were inoculated on the PDA plates and incubated at 25 ℃ without light for 7 days. Later, three discs of 50 mm diameter PDA filled with mycelia were cut and transferred into 50 ml Erlenmeyer flask with mung bean medium under 150 rpm and 25 ℃ in darkness for 4 days. Using four-layer cheese cloth for filtering the mycelia, conidia were collected in 50 ml tubes. Conidia concentration was enumerated with haemocytometer cell and then harvest conidia were stored in -80 ℃ for wheat seeds inoculation.

2.4 The pathogenicity diversity of *F. proliferatum* strains on wheat

Wheat seeds (Taifun) were surface-disinfested for 3 min in 1.2% sodium hypochlorite, subsequently rinsed 3 times with sterile water. Water was soaked up with the autoclaved paper under clean bench. 50 entire wheat kernels were immersed in 10 ml 10^4 spore/ml conidial suspension for 48 h on a shaker to make the conidia suspended (Glenn, 2006). Infected wheat seeds were sowed in the twice-autoclaved soil, 10 kernels per pot. Uninoculated control seed were reserved for germination in sterile water to assess sterilization effectiveness. Each strain had five repetitions. The experiments were carried out twice. The replicates were arranged in a complete randomized design in which fungal strains represented the pots.

In greenhouse, the temperature cycled between 26 °C (6:00 a.m. to 22:00 p.m. daytime) and 22 °C (22:00 p.m. to 6:00 a. m. night) in a day-night rhythm with a relative humidity of 50%. Pots were watered every other day after planting till the kernels were ripe. The wheat plants without watering two weeks after ripe, the ear, first flag leaf and the stem between first flag leaf and ear were collected.

2.5 Fungi re-isolation from soak-inoculation-seeds infected wheat plants

The stem tissues (the interdode from ear to the last node) were collected. Stem tissue was cut into 2~3 cm small sections and surface sterilized with 1.2% sodium hypochlorite for 3 min and then rinsed 3 times in the autoclaved distilled water. Stem sections were placed on CZID (a selective medium priority for *Fusarium* species growth) and incubated in 25 ℃ incubator. 3-4 days later, the result was recorded. Single mycelium tip was transferred to PDA plated, and mycelia and conidia were collected for DNA extraction. PCR was carried out with *F. proliferatum* species-specific primers PRO1:5'-CTTTCCGCCAAGTTTCTTC-3'and reverse primer PRO2: 5'-TGTCAGTAACTCGACGTTGTTGTT-3' (Mulè et al., 2004) to confirm the re-isolated fungi .

2.6 Fungal DNA quantification in stem, flag leaf and harvest kernels

Total DNA was extracted from inoculated wheat kernels, flag leaf and stem fine powder using a CTAB method (Brandfass & Karlovsky, 2008). The pellet was dissolved in 50 ul TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). DNA was diluted fifty fold prior to PCR analysis. Inhibition assay was done to test if any wheat matrix effects on the amplification of fungi DNA.

Thermocycler (CFX384TM, BioRad, USA) was used for real-time PCR analysis (quantitative PCR) in a total volume of 4 ul. Primers Fp 3F/4R (Jurado et al., 2006), were used for *F. proliferatum* fungal biomass detection. The reaction mixture consisted of buffer (16 mM $(NH_4)_2SO_4$; 67 mM Tris-HCl; 0.01% (v/v) Tween-20, pH 8.8 at 25 ℃, Bioline GmbH, Luckenwalde, Germany), 0.15 mM of each dNTP (Bioline GmbH, Luckenwalde, Germany), 2.5 mM $MgCl_2$, 0.1 U of Taq DNA polymerase (BIOTaq, Bioline GmbH, Luckenwalde, Germany), 0.3 mM of each primer, 0.1 x SYBR Green I (Invitrogen, Karlsruhe, Germany) and 1 mg/ml bovine serum albumin. The lowest standard set as limits of quantification was 2.09 fg/ul for *F. proliferatum*.

2.7 Mycotoxins analyses in kernels[3]

100 mg freeze-dried fine kernels powder was weighted and transferred into 2 ml new eppis. 1 ml acetonitrile:water (84:16) was added. After thoroughly mixing the samples, they were shaken overnight at 180 rpm. Next day the samples were centrifuged at 14,500 rpm for 10 min. 950 ul supernatant was transferred into 2 ml eppis and stored in -20 ℃ for mycotoxins analyses. For the analyses of beauvericin, fumonisin B_1 and enniatin A, A_1, B and B_1 HPLC separation was performed on a RP column Kinetex C18 at 40 °C followed by electrospray ionization in positive mode connected to an ion trap 500 MS (Varian, Darmstadt, Germany). For each mycotoxin detected, three mass transitions were used.

Calibration curves were constructed using analytical standards dissolved in methanol/water (1:1) with a correction for recovery and matrix effects. The limits of quantification for beauvericin, enniatin A, B, A_1, B_1 and fumonisin B_1 were 155 and 390 ng/g separately in wheat kernels.

3. Results

3.1 Fungal re-isolation from infected wheat stem

To confirm that *F. proliferatum* strains can systemically infect the wheat plants, fungi were re-isolated from stem (the internode between the ear and last node) of infected wheat plants (Fig.1 A). Re-isolated fungi were confirmed by the species-specific PCR (Fig.1 B).

[3] The mycotoxins analyses experiments were carried out by Dr. Katharina Pfohl.

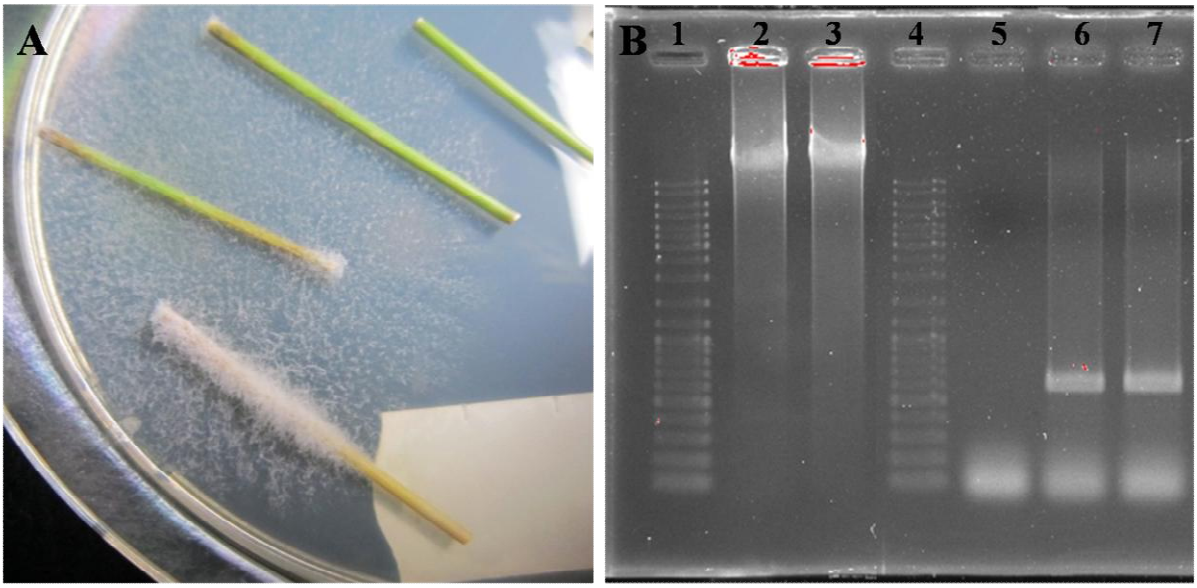

Fig.1: Fungi re-isolation from colonized wheat stem and confirmation. Fungi grew out from seed -inoculated wheat stem on CZID medium (A).Two single colonies were randomly selected and fungi DNA was extracted lane 2, lane 3. Fungi DNA was confirmed with *F. proliferatum* species-specific primers lane 6 and 7; lane1and lane were DNA ladders and lane 5 was negative control of PCR (B).

3.2 DNA biomass in stems, flag leaves and harvest kernels

To determine whether *F. proliferatum* strains systemically infect wheat from seeds to the top of wheat plant, fungal DNA biomass in stem, flag leaf and harvest kernel were quantified.

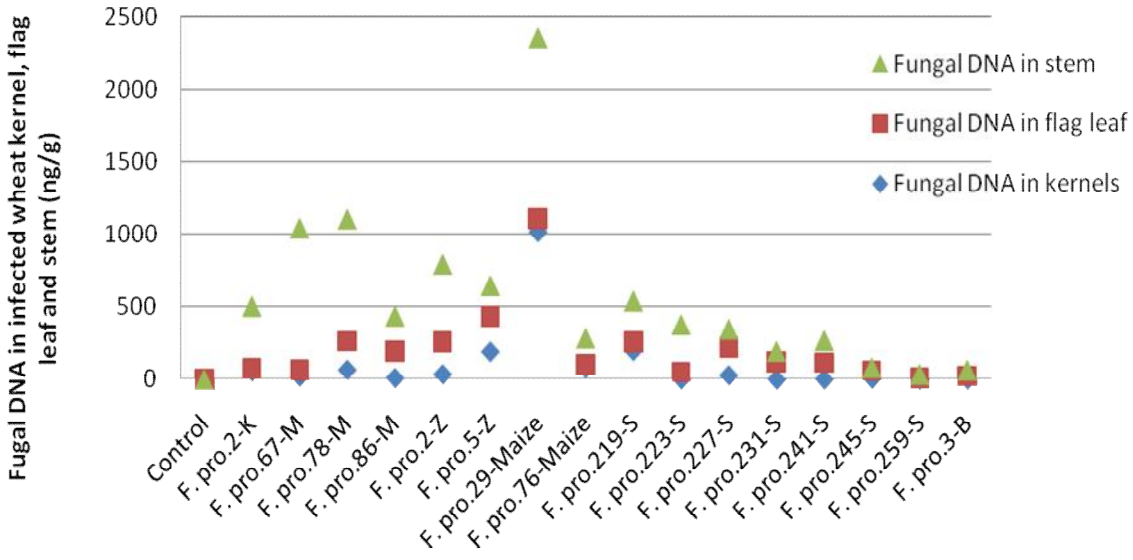

Fig. 2: DNA biomass in seed soak-inoculated wheat plant stem, flag leaf and kernels. DNA of *F. proliferatum* strains in wheat stem (the inter-node between the last node and ear), the 1st flag leaf, and kernels was quantified by quantitative real time PCR.

As the graph shown (Fig. 2), fungal DNA in inoculated wheat stem, flag leaf and harvest kernel were compared. Which indicated tested *F. proliferatum* strains systemically infected

wheat plants. Several *F. proliferatum* strains from different hosts had colonized wheat plants. According to the data from the quantitative PCR, fungal DNA of *F. pro* 5-Z, *F. pro* 29-Maize and *F. pro* 219-S were higher than other tested strains. Especially, wheat plants inoculated with strains *F. pro* 29-Maize contaminated 1000 ng/g and 2500 ng/g fungal biomass in infected kernel and stem separately. However, *F. proliferatum* strains isolated from the same host Miscanthus in the same year, for instance, *F. pro* 67-M, *F. pro* 78-M, *F. pro* 86-M differed also in their fungal biomass production; the amount of fungal DNA of *F. pro* 86-M was much lower than the other two strains. The same phenomenon also happened to *F. pro* 29-Maize and *F. pro* 76-Maize, fungal DNA biomass of *F. pro* 29-Maize was obviously higher than *F. pro* 76-Maize. *F. pro* 3-B was isolated from colonized dead larva, the DNA biomass was relatively lower among the tested strains. Fungal DNA from the stem was much higher than flag leaf and kernels as tendency.

The graph (Fig. 3) indicated fungal DNA in kernels with two replicates, though the value was little difference, the trend was the same. The agreement between the two replicates was also analyzed by SPSS (IBM SPSS statistics, USA) with Kendall's tau_b method. The result was shown in Table 2. Kendall's tau factor =0.647 indicated that there was agreement between two replicates

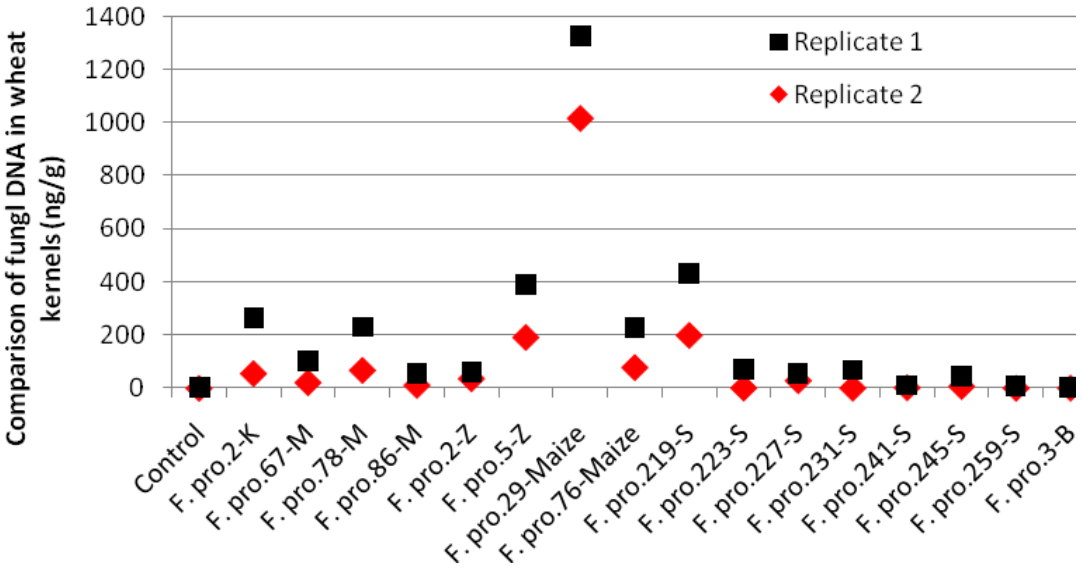

Fig. 3: the variance of the DNA biomass between the two replicates. The tendency was the same. The amount of fungal DNA of *F. pro* 29-Maize inoculated wheat kernels was higher than other tested strains.

Table 2 the agreement of the two replicates on fungal DNA in wheat kernel

			Replicate 1	Replicate 2
Kendall's tau_b	Replicate 1	Correlation Coefficient	1.000	.647**
		Sig. (2-tailed)	.	.000
		N	17	17
	Replicate 2	Correlation Coefficient	.647**	1.000
		Sig. (2-tailed)	.000	.
		N	17	17

**. Correlation is significant at the 0.01 level (2-tailed).

3.3 Fumonisin B_1, beauvericin production and their correlation with DNA biomass in colonized wheat kernels

Based on the mycotoxin quantification data by HPLC, mycotoxins were not detected in all the tested wheat kernels samples. Fumonisin B_1 was detected in *F. proliferatum* 2-K, 29-Maize, or 219-S infected wheat kernels, whereas beauvericin was quantified in *F. proliferatum* 2-K, 78-M, 29-Maize, 76-Maize or 219-S inoculated wheat kernels. For *F. proliferatum* 2-K, 29-Maize or 219-S infected wheat plants, both fumonisin B_1 and beauvericin were detected, moreover, fumonisin B_1 production is relatively higher than that of beauvericin (Fig. S1).

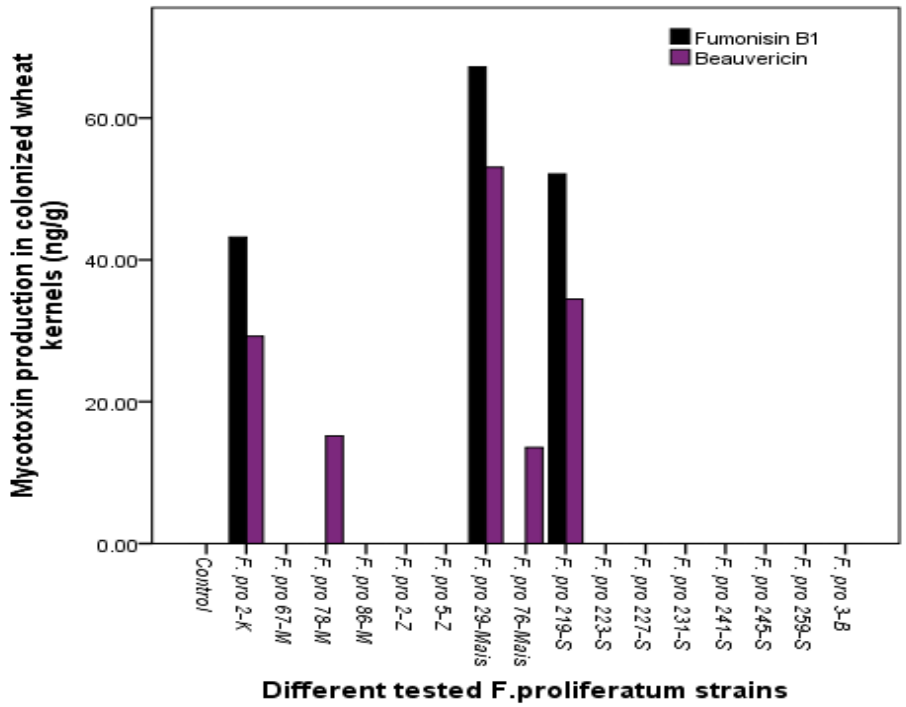

Figure S1: Fumonisin B_1 and beauvericin production in colonized wheat kernels. Fumonisin B_1 and beauvericin production in colonized wheat kernels infected by different tested *F. proliferatum* strains with soak-inoculation mature seeds method.

To determine whether DNA biomass correlated to fumonisin B_1, or beauvericin production, the correlations between them were analyzed. According to the data analyzed by SPSS (IBM SPSS statistics, USA), interestingly, we found that there was strong positive correlation (r= 0.817) between DNA biomass and fumonisin B_1 in colonized wheat kernels. Identically, strong positive correlation (r= 0.871) existed between amount of fungal DNA and beauvericin in colonized wheat kernels (Table 3).

Table 3 Correlation coefficients (r) of DNA biomass and fumonisin B_1, beauvericin production in colonized wheat kernels

Mycotoxin production	DNA biomass in colonized wheat kernels (N=17)	
Fumonisin B_1 (N=17)	r=0.817	Sig.<0.001**
Beauvericin (N=17)	r= 0.871	Sig.<0.001**

**: Correlation is significant at the 0.01 level (2-tailed).

4. Discussion

According to results in our research, we confirmed the ability of *F. proliferatum* strains infecting wheat plants grown in greenhouse condition systemically from seed to stalk to wheat kernels. However there is significant difference among the tested isolates. Quantification of fungal DNA seen as parameter for biomass in plant material is a useful approach to trace the colonization of the fungi in whole plant (Schaad et al., 2003). Quantitative real- time PCR is convenient technique to amplify and detect the tested fungal DNA in one step (Schena et al., 2004). Quantification of DNA of diverse strains in different parts of host wheat plants indicated the ability to infect the wheat plants. Which was not determined by hosts (garlic, onions, asparagus, maize, miscanthus and dead larvae) that the strains were isolated. There was no association found between the geographic origin of the strains and the variance of colonization on wheat plants.

In the present experiments, the first flag leaf and the stem between the ear and the last node were chosen for DNA biomass quantification because these parts are closest to the kernels. Based on the quantification data, fungal DNA in stem was the highest followed by flag leaf whereas the lowest amount was detected in kernels. There is a speculation, during the evolution of the wheat plant, that wheat plant has the mechanism to prevent transporting adverse nutrients to kernels. The explanation that the fungal DNA biomass was higher in the stem could be that *F. proliferatum* mycelia grew and spread in the pith parenchyma (including

the vascular system), which has no structure limitations and abundant nutrients and proper humidity for fungal dispersal. This hypothesis was already proven with *F. graminearum* and *F. culmorum* (Mudge et al., 2006, Guenther & Trail, 2005, Kang & Buchenauer, 1999).

Conidia concentration of 10^4 spores/ml suspension was used for the greenhouse experiments. The concentration was not high, by using higher concentrations, the amount of fungal biomass in the wheat plant last node, flag leaf and kernels could probably be higher. Giving the results from greenhouse extended application, *F. proliferatum* is a soilborne fungi species, the conidia can infect the seeds in the field and finally the entire plants. Wheat seeds can also be infected by *F. proliferatum* strains that originate from soil. The procedure of infection is probably a symptomless (or with symptoms) systemic infection of the plants, initiated through the seedling roots to the stem, leaf and the kernels. The same infection pathway of *F. verticillioides* has been demonstrated in experiments (Desjardins et al., 1998).

In conclusion, our study firstly proved that *F. proliferatum* strains can systemically infect wheat plants form wheat seeds to kernels. Due to the production of mycotoxins by this fungus, symptomless infection of wheat grains used for human and animal consumption poses a significant hazard to human and animal health. In addition, the vegetative parts: roots, leaves and stems during wheat growth infected by *F. proliferatum* can disperse the pathogen by the residues. The research broadened our understanding of the seeds infection pathway and heightened our awareness of the vegetative plants inoculums from symptomless infected wheat plants. Proper actions should be taken between the cropping seasons in field managements to avoid the fungus transmission.

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Chapter 6 General discussion

According to our research results, *T. moliotr* performed preference feeding on *F. prolifertaum* and *F. poae* colonized wheat kernels whereas avoided grazing on *F. avenaceum* colonized wheat kernels or selectively consumed the inner part of *F. culmorum* colonized wheat kernels. Olfactory receptors (Harbach & Larsen, 1977) from *T. moliotr* antennae are speculated to sense the signal from mycotoxins and organic volatile compounds (Eifler et al., 2011) and perform feeding behaviors. This characteristic can be used as a biology trap to control the storage insects. Further studies are necessary to determine which organic volatile compounds (besides mycotoxins) play major roles on influence the feeding behavior of the *T. molitor* larvae on feeing *Fusarium* colonized wheat kernels.

Two main possibilities are responsible for the mortality of *T. moliotr* larvae, firstly, the mycotoxins which have the insecticidal property like beauvericin (Xu et al., 2008) and the analogues of beauvericin, enniatins (Grove & Pople, 1980). Secondly, the pathogenicity of different *Fusarium* species to the larvae, mycelia or conidia ingested by *T. moliotr* larvae oral route, the conidia can germinated and penetrated through the intestinal lumen and ensued all the tissue.

Fusarium species affected not only the storage insects *T. molitor* feeding behaviors and causing mortality on the larvae. Some *Fusarium* species such as *F. proliferatum*, which was attractive to the *T. molitor* larvae for fungal dispersal. *T. molitor* larvae or beetles internally and externally disseminated fungi conidia as vehicle. Conidia dispersed by copulation between contaminated male and uncontaminated female to their offspring was firstly illustrated in our research. Contamination of the eggs by the copulation may be caused by the fungal penetration through the egg integument in female beetles' body or by the conidia on the cuticle of the male beetles as inoculums. To exclude the contamination from the surface contact with the conidia, the female beetle dissection experiment was conducted, and eggs were taken out from ovary. After transferred the eggs on the CZID medium, mycelia grew out, which denied the hypothesis that the eggs contaminated by the cuticle contamination.

After the beetles fed on different *Fusarium* species mycelia and conidia for 24 h, the duration of beetles disseminating fungi to autoclaved wheat kernels was surprising: even after 20 days,

more than 90% of the wheat kernels were contaminated by the beetles attached fungi conidia. *Fusarium* conidia can survive through the gut passage and germinated from the feces was firstly illustrated in detail in our research. DNA quantification was confirmed the precise DNA amount at different time points. DNA biomass in beetles grazing on different *Fusarium* species at 5 days were lower compared that of at 1 day time point, the predominant factor is that conidia (DNA) were excreted out of the beetles. Windels et al. (Windels, Windels, & Kommedahl, 1976) also proved that some of the internal *Fusarium* propagules could be lost through the feces or destroyed in the gut of the insect.

F. proliferatum species drew our research focus, because both mycelia and conidia of *F. proliferatum*, and *F. proliferatum* colonized wheat kernels were attractive to *T. molitor* larvae and beetles. Pathogenicity variance of different *F. proliferatum* strains from diverse plants and geographic origins on *T. molitor* larvae was determined. Moreover, fungi DNA of *F. proliferatum* strains were detected in the tissue in the larvae tissue. It was firstly reported that *F. proliferatum* strains DNA biomass was detected in the larvae's tissue. Mycelia and conidia of *F. proliferatum* strains like *F. proliferatum* 67-M and *F. proliferatum* 21.1 were surprisingly attractive to *T. molitor* larvae, which caused lower larval mortality. However, *T. molitor* larvae avoided feeding on *F. proliferatum* strains like *F. proliferatum* 227-S and *F. proliferatum* 5-Z, which caused relative higher mortality of the larvae.

F. proliferatum strains caused higher mortality of the *T. molitor* larvae positively associated with high fungal DNA biomass in leaves tissue. e.g. *F. pro* 245-S, *F. pro* 27-K and *F. pro* 42-D DNA biomass was higher than that of other tested strains and also caused higher mortality of *T. molitor* larvae. Using this characteristic, these *F. proliferatum* strains can be used as endophyte or biology to control the insects in the field. Further experiments should be conducted to study the mycelia from the tissue between the gut and cuticle, or to confirm establishing a biotrophic or pathogenic relationship within larval tissue (Bushnell, Hazen, Pritsch, & Leonard, 2003). *F. proliferatum* species is soil-borne pathogen, its entomo-pathogenicity can be used to control larvae or pupae of the insects in the soil (Majumdar, Boetel, & Jaronski, 2008).

The geographic origin and hosts of the strains were found to have no association with the variance of colonization on wheat plants. *F. proliferatum* strains caused higher mortality of the *T. molitor* larvae did not associate with high fungal DNA biomass in wheat stem, leaves

and kernels. The pathogenicity of *F. proliferatum* strains on larvae was not positively correlated with the pathogenicity on wheat (cultivar: Taifun). Fungal DNA biomass of *F. proliferatum* strains in larvae tissue and inoculated wheat kernels were variable. For example, the highest DNA biomass in larval tissue was *F. proliferatum* 42-D with 1,500 ng/g fungal DNA in larval cavity tissue. While highest DNA biomass was *F. pro* 29-Maize1000 ng/g found in inoculated kernels and 2500 ng/g in colonized stem separately, which indicated that the pathogenicity of the same isolates was different on different hosts and the pathogenicity mechanisms were different.

The occurrence of fumonisin B_1 was low on wheat and barley in the nature (Marin et al., 1999). This is consistent with our mycotoxin quantification result that fumonisin B_1 was detected only in *F. proliferatum* 2-K, 29-Maize, 219-S strains. The mycotoxins production greatly differed as a result of different nutrition matrix and temperatures (Marin et al., 1999; Samapundo, Devliehgere, De Meulenaer, & Debevere, 2005).

The systemic infection of the wheat plants can be two pathways: conidia from the soil germinated and infected the plants roots, subsequently, the mycelia spread in the vascular system and went with the water evapotranspiration to the stem to the leaves and to the wheat kernels. Alternatively, conidia proliferated from infected leaf or the stem and become the new inocula to the upper parts of the wheat plants till the kernels.

Therefore, cereal straw may provide a source of *Fusarium* mycotoxins into the animal feed chain. Moreover, *F. prolifertaum* is frequently isolated from a wide range of host plants, e.g. asparagus (Bargen et al., 2009), onion (Stankovic, Levic, Petrovic, Logrieco, & Moretti, 2007), date palms (Abdalla, Al-Rokibah, Moretti, & Mule, 2000). *F. proliferatum* is also a main pathogen of maize (Marin et al., 1999) worldwide and other small grains like: rice (Abbas et al., 1999), sorghum (Bacon & Nelson, 1994), wheat (Desjardins, Busman, Proctor, & Stessman, 2007) and barley (Marin et al., 1999). Public awareness of food contamination with trichothecenes and other mycotoxins is low.

The findings of our research emphasized ecological interactions between *Fusarium* species, different strains of *F. prolifertaum* and storage insects *T. molitor*. The mycelia and conidia of tested *Fusarium* species, and colonized wheat kernels influenced the feeding behavior, weight gain and survival rate of *T. molitor* larvae. *T. molitor* beetles dissemination was tested with

Fusarium fungal conidia internally and externally and the DNA biomass and mycotoxin were quantified in the beetles at different time points. Radiate mycelia growth rate, sporulation ability of *F. prolifertaum* strains, caused mortality on *T. molitor* 4th larvae and DNA biomass in the larval tissue were treated as parameters to determine the variance among *F. proliferatum* strains. *F. proliferatum* strains systemic infection on wheat plants with seed-inoculation method illustrated further information of the pathogenicity variance among the strains.

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Chapter 7 Summary

This dissertation has elucidated the ecological interactions of *Fusarium* species and *Tenebrio molitor*. The results obtained in my present research can be summarized in detailed as follows:

- We found that *Fusarium* species (*F. avenaceum*, *F. culmorum*, *F. poae* and *F. proliferatum*) colonized wheat kernels affected the feeding behaviors of *T. molitor* mature larvae. Wheat kernels colonized by *F. proliferatum* and *F. poae* attracted *T. molitor* larvae significantly more than untreated (control) kernels, whereas kernels colonized with *F. avenaceum* or *Beauveria bassiana* (entomo-pathogenic fungi as positive control) were avoided by the larvae. Interestingly, larvae selectively fed on the inner part of *F. culmorum* colonized wheat kernels. The selective feeding behaviors (avoidance or preference) correlated with larval weight gain within 15 days. Correspondingly, larvae consumed *F. proliferatum* or *F. poae* colonized kernels had similar survival rates as control. However, larvae fed on *F. culmorum*, *F. avenaceum* or *B. bassiana* colonized kernels had enhanced larvae mortality. Our results also indicated that *T. molitor* larvae have the ability to sense potential survival threats from kernels colonized with *F. avenaceum* or *B. bassiana*.

  Mycotoxins production both in *Fusarium* colonized wheat kernels and dead larvae grazing on *Fusarium* colonized wheat kernels were quantified. Although fumonisins, enniatins and beauvericin were detected in *F. proliferatum* or *F. poae* colonized kernel, the larvae were able to ingest those kernels as diet without exhibiting increased mortality. Consumption of *F. avenaceum* colonized kernels, however, increased larval mortality. These colonized kernels had higher enniatins levels than the ones from *F. proliferatum* or *F. poae* colonization, which suggested that *T. molitor* mature larvae can tolerate or metabolize those toxins.

- After feeding on *Fusarium* (*F. avenaceum*, *F. culmorum*, *F. poae*, *F. proliferatum* and *B. bassiana*) mycelia and conidia for 24 h, live conidia were traceable in beetles' excreta and beetles were capable of contaminating a high proportion of wheat kernels. Fungal dissemination duration by beetles and fungal colony forming unit density in beetles

excreta were recorded for up to 20 days. Kernels contamination and fungal colony forming unit density of *F. proliferatum* were higher than other tested *Fusarium* species. *T. molitor* beetles disseminated tested *Fusarium* fungal conidia internally and externally. The detailed description of the conidia attachment on antennae, mouthpart, wings and legs were observed under scanning electronic microscopy. And colonies from beetles excrete were observed on selective medium. Fungal contamination by copulation was first described in our present research.

F. proliferatum DNA were positively detectable in living beetles collected at 1 day, 5, 10 and 15 days time points. HPLC-MS was performed to detect beauvericin, fumonisins, enniatins in live beetles. Beauvericin was found in beetles feeding on *F. poae*, *F. proliferatum*, or *Beauveria bassiana* mycelium. Enniatins were detected in beetles grazing on *F. avenaceum*, *F. poae*, and *F. proliferatum* mycelia.

- Various tested *F. prolifertaum* strains caused mortality on *T. molitor* 4th larvae indicated pathogenicity difference among the tested trains. According to the data, pathogenicity difference was neither related to the hosts *F. prolifertaum* strains isolated from garlic, onion, asparagus, maize, miscanthus or dead larvae separately nor associated with the origin in Germany, France, Syria and Austria. Radial mycelia growth rate and sporulation ability were treated as parameters to evaluate the difference among the tested strains, however, the pathogenicity was not corresponding to these two parameters.

  DNA biomass in the larvae tissue was quantified as parameter to assess to the variance among *F. proliferatum* strains. Surprisingly, fungal DNA was detected in the tissue between the cuticle and the gut passage. Fungal DNA biomass in the tissue of the larvae feeding on *F. proliferaum* 42-D, 27-K and 245-S mycelia and conidia were significantly higher than the others. However, the DNA biomass in the larvae tissue was not positively correspondingly with the mortality of the larvae.

- By soak-inoculation of mature wheat seeds, diverse *F. proliferatum* strains (isolated from garlics, onions, asparagus, maize, miscanthus and dead larvae) pathogenicity on wheat plants under greenhouse condition was assessed. *F. proliferatum* infected wheat plants showed symptomless. However, fungal DNA was detected in flag leaf, stem and kernels matrics of the *Fusarium* susceptible summer wheat cv. Taifun. According to the fungal

DNA biomass, in general, DNA biomass in stem (the part from ear to last node) was the highest, and then the first flag leaf, and the kernels was the lowest.

The results showed that *F. proliferatum* strains can systemically infect wheat plants from seeds soaked in conidia suspension to stem, leaf and then to wheat kernels, which indicated systemic infection to wheat. DNA biomass was quantified as marker to assess the aggressiveness of *F. proliferatum* strains, which was not corresponding to the mortality of the larvae. The explanation suggested the difference between mechanisms of *F. proliferatum* on insects and plants.

To sum up, our present research illustrated the ecological interactions of the meal beetle *Tenebrio molitor* with diverse *Fusarium* species from many aspects. Different *Fusarium* species affected *T. molitor* larvae; *T. molitor* beetles disseminated different *Fusarium* species conidia internally and externally; Pathogenicity assessment of different *F. proliferatum* strains on *T. molitor* and wheat plant according to several parameters and different mechanisms. The results gave a deeper understanding of the association of insects and toxin-producing fungi within storage grains, which may raise awareness of food security and in the improvement of management approaches in post-harvest cereals.

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