Fungal Secondary Metabolites:
Fusarium spp. and *Microdochium* spp.

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Chapter 1. General introduction

1. Fungal secondary metabolites

The terms "primary" and "secondary" metabolites were coined by Albrecht Kossel (1891): "*I suggest to call the essential components of the cell as primary ones, however those which cannot be found in every growing cell we shall call secondary ones*". In contrast to primary metabolites, secondary metabolites (natural products) are nonessential for the survival and reproduction of their producers. They are diverse in their function and chemical structure and produced by many organisms (Goyal et al., 2017). Furthermore, genes that encode enzymes catalyzing secondary metabolite biosynthesis are often arranged in gene clusters. In comparison, genes responsible for synthesizing primary metabolites are distributed over the fungal genome (Keller, 2019).

The isolation of the fungal natural products commenced in the first half of the 20th century with the work of Harold Raistrick and colleagues, who isolated almost 200 compounds. However, determining their structure was (and is) a challenge (Bennett & Bentley, 1989). Fungal secondary metabolites represent double-edged weapons. The contamination with mycotoxins can cause detrimental effects, including cytotoxic (Koyama et al., 1988) and carcinogenic (Nesbitt et al., 1962) effects. Conversely, they also represent a source for potential pharmaceutical drugs, such as antibiotics (Lalchhandama, 2020; Ravichandiran et al., 2019), anticancer agents (Demain & Sánchez, 2009; Wang & Lin, 2012; Zhao et al., 2004), and anti-HIV drugs (Singh et al., 2003). Estimates point out that fungi have supplied about one-third of all secondary metabolites with notable biological activities. (Demain & Sánchez, 2009).

Historically, events associated with mycotoxin consumption have led to the recognition of many fungal-caused disease symptoms. A very famous case was the poisoning caused by ergot alkaloids (known as Saint Anthony's Fire) that followed the consumption of grains infected with sclerotia of *Claviceps purpurea*. Sclerotia contained high amounts of ergot alkaloids was part of grain mixtures used to bake bread or prepare porridge. As a result, thousands of people died (Kück et al., 2014). Moreover, in the 1960s, turkey X disease became famous after the birds consumed aflatoxin-contaminated peanuts (Wannop, 1961). In contrast, the discovery of penicillin in 1928 helped save humankind from various lethal bacterial infections (Lalchhandama, 2020).

A broad spectrum of isolated bioactive substances has been classified according to their mode of action (Ghannoum & Rice, 1999). In comparison, the ecological role of only a few of them has been proven (Cary et al., 2014; Xu et al., 2019; Zhao et al., 2017).

1.1. Classification of fungal secondary metabolites

Fungal secondary metabolites are classified based on their biosynthetic origin. Several thousands of them are formed from a limited number of primary metabolite precursors (Keller et al., 2005). Three major biosynthetic pathways (Figure 1) are known that include (1) the mevalonic acid pathway (terpenes), (2) the shikimic acid pathway (aromatic amino acids and alkaloids), and (3) the acetate pathway (polyketides, fatty acids) (Goyal et al., 2017; Krause et al., 2018). Classes of fungal secondary metabolites have been defined according to the involved enzymes (backbone enzymes) as polyketides (aflatoxin, lovastatin, and aurofusarin), peptides (penicillin, gliotoxin, cyclosporine A, and xanthocillin), terpenes (trichothecene, gibberellins, and steroids*), and* alkaloids (ergoline, fusaric acid, lycomarasmine, and tryptamine) (Keller, 2019; Mahmood et al., 2010; Quin et al., 2014). The polyketides are derived from acetyl coenzyme A (acetyl-CoA) and malonic acid that catalyzed by polyketide synthases (PKSs) (Bentley & Bennett, 1999; Fujii et al., 2001; O'Hagan, 1992; Shen, 2003). The regulation of nonribosomal peptides from proteinogenic amino acids (protein-building) and non-proteinogenic amino acids is achieved via multimodular enzymes known as nonribosomal peptide synthetases (NRPSs) (Finking & Marahiel, 2004). Commonly, terpene cyclase (Caruthers et al., 2000; Rynkiewicz et al., 2001; Tudzynski et al., 2001) along with the building blocks dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP), which are produced from acetyl-CoA via the mevalonate pathway in fungi are fundamental for terpenes biosynthesis. The products are known as sesquiterpenes (C_{15}), diterpenes (C_{20}), and triterpenes (C_{30}) according to the length of the precursor molecule (Cen et al., 2020; Keller et al., 2005; Schmidhauser et al., 1990). The biosynthesis of indole alkaloids starts with amino acids utilizing NRPS enzymes (Gao et al., 2011). Hybrid metabolites (such as a polyketide-nonribosomal peptide) produce diversity in secondary metabolisms (Kück et al., 2014).

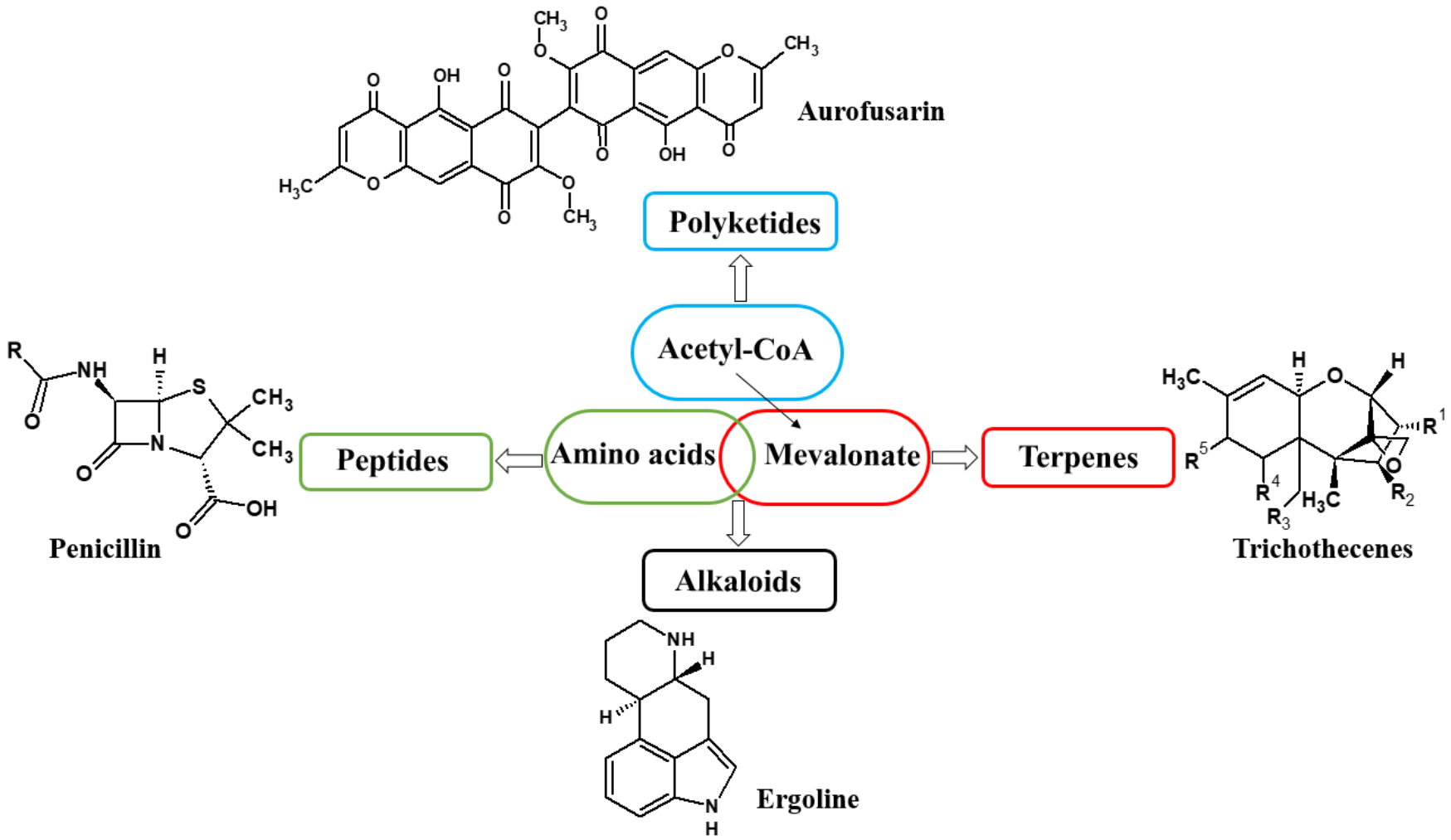

Figure 1. Fungal secondary metabolites pathways. Most secondary metabolites can be grouped into four classes: polyketides derived from acyl-CoA, terpenes derived from acyl-CoA via the mevalonate pathway, peptides derived from amino acids, and hybrid alkaloids derived from polyketide–terpene according to (Nielsen & Nielsen, 2017)

1.2. Bioactivity of fungal secondary metabolites

1.2.1. Stress-induced bioactivity

Fungi can adapt to biotic and abiotic stress that is directly influence the quantity and quality of fungal metabolites (Goyal et al., 2017; Lebeau et al., 2017; Vandermolen et al., 2013). This adaptation is facilitated by particular mechanisms protecting fungi (Convey, 2005; Duarte et al., 2019). Pigment formation, especially by surface-dwelling fungi, protects the producers from the high solar ultraviolet radiation levels (Wynn-Williams et al., 2002). On the other hand, the predation of fungal mycelium by fungivores (e.g., springtails) induced the release of bis-naphthoquinones (Xu et al., 2019). Notably, in nature, associated fungal communities diversify depending on their plant or animal host (Abdelhalem et al., 2020; Hoeksema et al., 2018; Tardy et al., 2015; Velluti et al., 2000). Co-cultivation of several fungi on the same host can stimulate or inhibit the release of specific fungal metabolites. For instance, secretion of fusaric acid by *Fusarium oxysporum* altered the levels of volatile secondary metabolites emitted by *Trichoderma atroviride* (Stoppacher et al., 2010).

1.2.2. Bioassays, chemical, and genetic regulations-determined bioactivity

Bioassays (cell culture, organ, or the whole organism) are important to prove the bioactivity of a substance (Furey, 2010). However, looking for affordable, fast, reproducible, and reliable bio-tests is crucial (Espinel-Ingroff, 1998; McLaughlin et al., 1993). Several organisms are used as a model for the bioassays. Common examples are brine shrimps *A. salina* (Meyer et al., 1982), mealworms *T. molitor* (Abado-Becognee et al., 1998), springtails *F. candida* (Fountain & Hopkin, 2004), radish seeds *R. sativus* (Chiapusio et al., 2004), duckweed *L. minor* (McLaughlin et al., 1993) and antimicrobial bio-tests (Espinel-Ingroff, 1998). Even bioassays on rodents have been well established (Morris, 2003), although their use is ethically a subject of controversy (Furey, 2010). Several methods are used to estimate the obtained results. For example, estimation of the LD_{50} (the concentration of target compound adequate to kill 50% of the tested organisms) is a common method to characterize toxic effects of a compound (Meyer et al., 1982).

Isolation procedures include fractionation and purification, guided by flash chromatography, structure elucidation by mass spectrometry (MS) and nuclear magnetic resonance

(NMR), and purity control by LC linked to UV, MS, and ELSD (evaporative light scattering) detectors (Mahato et al., 2019; Searle et al., 2004).

Gene knockout represents an elegant technique to explore the participation of an enzyme in the biosynthesis of a particular secondary metabolite, or to obtain pairs of organismic clones that produce or lack a specific metabolite (Keller, 2019; Malz et al., 2005). Genetically engineered fungal strains have been used widely in the bioassays experiments, such as *PKS6*-deficient *Neurospora crassa* mutant, to assess the biological role of furocoumarin (Zhao et al., 2017). Moreover, genetically modified fungi can be invested in commercial purposes (Leger et al., 1996; Westphal et al., 2018). Further enhancement can be provided via manipulation of the surrounding conditions (Goyal et al., 2017), such as metabolites stimulation by mechanical damage (Xu et al., 2019).

1.3. Bis-naphthopyrone aurofusarin

Aurofusarin is a dimeric naphthopyrone polyketide (Figure 1) produced by several fungal species in the genus *Fusarium* (Munkvold, 2017). Aurofusarin was first isolated as small yellow crystals from *Fusarium culmorum* (Ashley et al., 1937) and was successfully crystallized in ethanol as deep red prisms (Gray et al., 1967). Later, its chemical structure was identified by nuclear magnetic resonance (NMR) and the infrared spectroscopy (IR) to be $C_{30}H_{12}O_{18}$ (Birchall et al., 1966). Aurofusarin is water-insoluble and sparingly soluble in many organic solvents (Ashley et al., 1937; Birchall et al., 1966; Gray et al., 1967; Shibata et al., 1966) and degraded at room temperature and when exposed to light (Jarolim et al., 2018). The color of aurofusarin is a function of the pH since it appears yellow in acidic pH and turns red in alkaline pH (Ashley et al., 1937; Gray et al., 1967). Aurofusarin-producers can synthesize a considerable amount of aurofusarin, reaching 12% of the mycelial dry weight (Gray et al., 1967), depending on the ambient conditions (Medentsev & Akimenko, 1998). The biosynthesis pathway of aurofusarin and the participated enzymes have been meticulously identified. The biosynthesis starts intracellularly, and rubrofusarin is generated as an intermediate converted to aurofusarin extracellularly (Frandsen et al., 2006, 2011). Recently, a comprehensive study about the ecological role of aurofusarin proved that aurofusarin is non-toxic to arthropods but displayed a deterrent effect against several predators (Xu et al., 2019) (more details about aurofusarin are provided in Chapter 2).

1.4. Secondary metabolites in *Microdochium* spp.

M. nivale and *M. majus* are distributed worldwide, harming winter crops (Ponomareva et al., 2021; Tronsmo et al., 2001), turfgrasses (Abdelhalim et al., 2016; Hofgaard et al., 2006), causing a dramatic yield losses every year (Humphreys et al., 1997). Several mycotoxins had been isolated from *M. nivale* (Tatsuno et al., 1968). However, recent reports demonstrated that *M. nivale* is a non-toxigenic species (Chełkowski et al., 1991; Gagkaeva et al., 2020). (Chapter 5 provides more details).

2. Research objectives

In this research, we focused on studying two topics: the properties and functions of aurofusarin; and the investigation of secondary metabolites in *Microdochium nivale* and *M. majus*.

Consumers tend to trust naturally derived substances rather than synthetically originated ones. Therefore, many polyketide naphthoquinone-based compounds are considered promising natural dyes sources (Dufossé et al., 2017). Although fungi produce a wide range of pigments (Avalos et al., 2017), the concerns of toxic properties of fungal metabolites (Goyal et al., 2017), as well as aqueous insolubility combined with light and heat sensitivity (Wissgott & Bortlik, 1996) impedes the development of their industrial utilization. We think that the non-toxic red pigment aurofusarin could be used as an alternative natural source for synthetic colorants in the textile industry in the future. However, the preferable cultural conditions, the purification, and other physical and chemical properties of aurofusarin are still unexplored.

On the other hand, *M. nivale* was known as a producer of nivalenol and its derivative fusarenon X (Tatsuno et al., 1968). In contrast to this, recent studies unanimously reported that *M. nivale* is a non-toxigenic fungus (Chełkowski et al., 1991; Gagkaeva et al., 2020).

Depending on the previously mentioned facts, the aims of this work are:

- Finding out the proper media for stimulation the biosynthesis of aurofusarin by *Fusarium* spp.
- Determination of the solubility of aurofusarin in various organic solvents.
- Calculation of the molar extinction coefficient of aurofusarin.

- Evaluation of aurofusarin yield purified in several organic solvents and validation the efficiency of the optimal extraction method.
- Estimation of the stability of aurofusarin in water-containing solvents.
- Definition of the preferable temperature *in vitro* for growing *Microdochium majus* and *M. nivale*.
- Examination of the toxicity (influence on the fitness) of *M. majus* and *M. nivale* against springtail (*F. candida*), mealworm (*T. molitor*), brine shrimp (*A. salina*), radish seed (*R. sativus*), and duckweed (*L. minor*).
- Purification of the bioactive compounds from the efficient *Microdochium* strains by flash chromatography and preparative HPLC based on bioassays-guided fractionation.
- Structure elucidation of the isolated bioactive compounds.

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Chapter 2. Aurofusarin as a model for bis-naphthopyrone antifeedants protecting fungi from predators: A comprehensive review

Albatol Alsarrag, Petr Karlovsky

1. Abstract

Aurofusarin is a dimeric naphthopyrone produced by ascomycetous fungi, mainly species in the genus *Fusarium*. Aurofusarin was isolated as red prisms several decades ago. The biosynthesis pathway of aurofusarin has been elucidated stepwise by identifying the catalyzing enzymes and concluding that the last steps of the biosynthesis take place extracellularly. Recently, an extensive study proved that aurofusarin deterred fungivores and displayed a non-toxic effect against arthropods. In this review, we shed light on the properties and functions of aurofusarin. However, assessing the solubility and the molar extinction coefficient of aurofusarin remains unexplored. Here, we determine the solubility of aurofusarin in several solvents at 20°C, identifying *m*-cresol as the best solvent (~ 5 g L^{-1}). Furthermore, the molar extinction coefficient of 21,600 M^{-1} cm^{-1} was determined at λ_{max} [266] nm and 17.6 µM. We also investigated the influence of the nutrient composition on the biosynthesis of aurofusarin by *F. graminearum*, concluding that commercial potato−dextrose broth (PDB) inhibits aurofusarin production. By contrast, aurofusarin was generously secreted in self-made PDB.

Keywords: solubility, stability, potato−dextrose broth, media inhibitory effect, the molar extinction coefficient biosynthesis pathway.

2. History of aurofusarin discovery

Medium color serves as character for species *Fusarium* identification (Brown & Horne, 1924). In 1904, Bessy isolated a crude pigment from *F. culmorum* that appeared yellow in the acidic conditions and turned red in the alkaline (Bessey, 1904). However, many attempts to isolate

these fungal pigments failed until 1937, when Ashley and colleagues successfully purified red, yellow, and colorless crystals named rubrofusarin, aurofusarin, and culmorin, respectively (Figure 1), from two strains of *F. culmorum*. At that time, the chemical formula of aurofusarin was proposed to be $C_{30}H_{20}O_{12}$, which includes two hydrogens more than the current formula (Ashley et al., 1937).

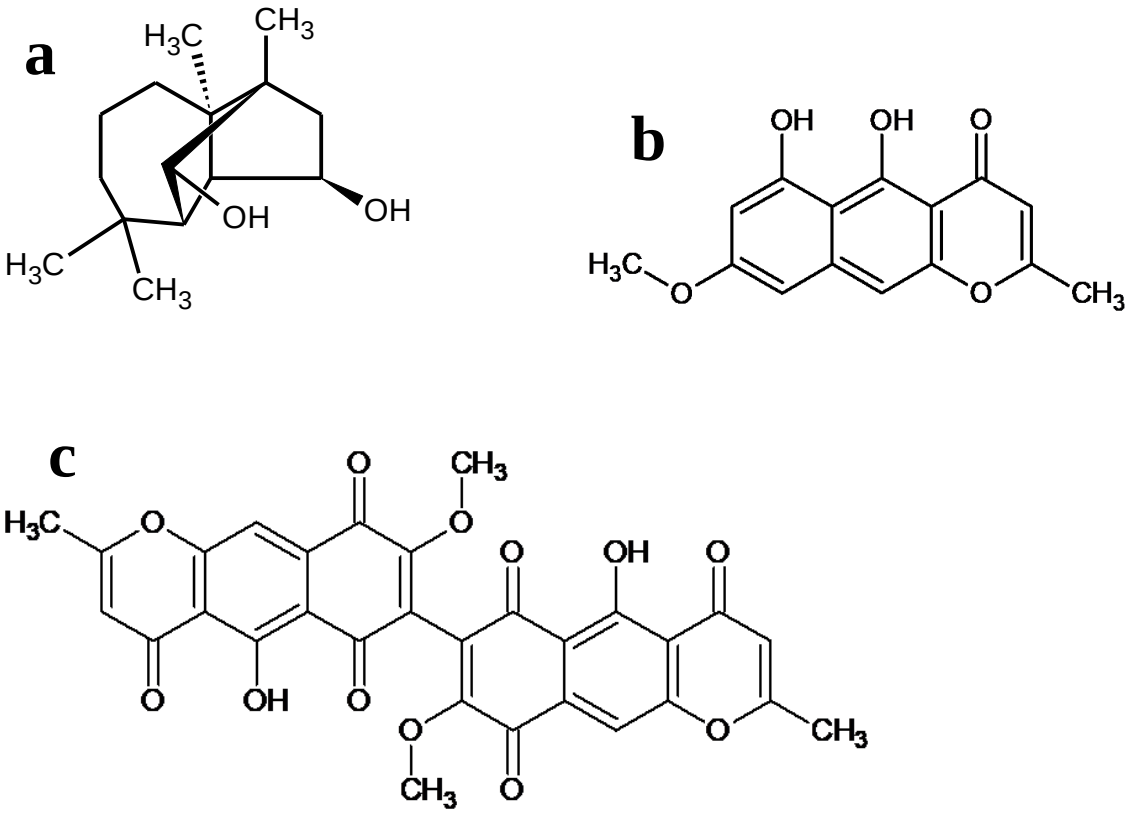


Figure 1. Structures of a) culmorin, b) rubrofusarin, and c) aurofusarin

2.1. Structure and Purification of aurofusarin

2.1.1. Structure elucidation

In the 1960s, efforts increased to elucidate the correct structure of aurofusarin. Although the structure of aurofusarin was correctly elucidated to be $C_{30}H_{18}O_{12}$ via a combination of the data from nuclear magnetic resonance (NMR) and infrared spectra (IR), the allocation of the hydroxyl and keto groups on the rings and the monomer connection mode remained unclear. One proposal suggested that the C_{30} structure is derived from two C_{15} molecules that could be modified rubrofusarin moieties. This formula was identical to that published by Raker and Robert (Birchall

et al., 1966). Previously, the structure of the rubrofusarin was identified as a stable linear naphtho-γ-pyrone (Stout & Jensen, 1962; Tanaka et al., 1966). Further analysis of the pigment produced by *Fusarium culmorum* (W.G. Smith) Sacc. by NMR confirmed the previously proposed formula of aurofusarin and assessed identical double bonds in the two rings, which favored the hypothetical conjugation of two identical rubrofusarin monomers. Noteworthy, the alkaline degradation of aurofusarin generates acetone and acetic acid, which was considered as evidence for the presence of an α-methyl-γ-pyrone ring in the structure of aurofusarin (Shibata et al., 1966). In 1967, Gray and coworkers unambiguously proved that the red pigment was aurofusarin (Gray et al., 1967). Comparing the UV and NMR spectra of oxidized rubrofusarin monomethyl ether with aurofusarin dimethyl ether revealed that both compounds have identical spectra and that the last compound is a dimer (C_7-$C_{7'}$) of the first one. In conclusion, the structure of aurofusarin was elucidated as 7,7´-bis[5-hydroxy-8-methoxy-2-methyl-4*H*-naphtho[2,3-*b*]pyran-4,6,9(6*H*,9*H*)-trione] (Morishita et al., 1968).

2.1.2. Purification

Organic solvents, such as methanol, ethyl acetate, acetone, methylene chloride, and chloroform/methanol mixtures were used to extract several dimeric and mono-naphthopyrones from *Aspergillus* spp. (Ehrlich et al., 1984; Li et al., 2016; Song et al., 2004; Xiao et al., 2014). Similar solvents were used to extract aurofusarin from other fungi (Birchall et al., 1966; Gray et al., 1967; Medentsev et al., 2005; Shibata et al., 1966). Contrary to the highly water-soluble mycotoxins, such as NIV and DON (Lauren & Ringrose, 1997), the hydrophobic nature and the dimeric shape of aurofusarin affects its solubilization behavior in different solvents. Aurofusarin is water-insoluble or only sparingly soluble in many organic solvents (Ashley et al., 1937; Birchall et al., 1966; Gray et al., 1967; Shibata et al., 1966). However, it dissolves readily in chloroform, dioxin, phenol (Ashley et al., 1937; Gray et al., 1967), glacial acetic acid, nitrobenzene, and *m*-cresol (Ashley et al., 1937). Aurofusarin was successfully crystallized in ethanol as deep red prisms. Reduced aurofusarin is slightly soluble in alcohol (Gray et al., 1967). Generally, aurofusarin was isolated from TLC as one spot in chloroform, chloroform/methanol (50:1, v/v), and acetone (Birchall et al., 1966). Aurofusarin is readily eluted from silica columns, in which the silica was saturated with oxalic acid when chloroform and benzene/acetone mixtures of 9:1 (v/v) and 4:l (v/v) are used (Shibata et al., 1966). The color of aurofusarin associates with the pH value,

and this color appears yellow in acidic pH and changes to red in alkaline pH (Ashley et al., 1937; Gray et al., 1967).

3. Chemical properties of aurofusarin

3.1. Stability

Temperature and light are crucial factors that determine the stability of aurofusarin. A remarkable amount of aurofusarin was lost when it was stored in dim vails at room temperature. At the same storage conditions, but in transparent vails, aurofusarin was completely degraded. At − 20°C, aurofusarin was relatively stable for several days in the water/acetonitrile (7:3, v/v) or dimethylsulfoxide. Interestingly, repeated cycles of thawing and freezing had a negligible effect on the stability of aurofusarin (Jarolim et al., 2018). As aurofusarin is unstable in liquid culture, several decomposition products were detected (Westphal et al., 2018). We quantified aurofusarin content at several time intervals and found that atropisomers of aurofusarin are stable in pure methanol but degraded rapidly in 1:1 (v/v) mixtures with water (chapter 3, Figure 4).

3.2. Solubility

According to the “Like dissolves like” concept, miscible solvents share the same structural properties as the solute (Hansen, 2004).

We determined the solubility of aurofusarin in a wide range of polar and non-polar solvents. It was soluble in several non-polar solvents as a red solution. In contrast, in polar solvents, the solution was pale pink to light red, depending on the solvent’s properties, in line with previous findings (Ashley et al., 1937; Birchall et al., 1966; Gray et al., 1967). Taking into account the remarkable impact of the surrounding temperature and pressure on the solubility of the natural pigments (Rodrigues et al., 2006).

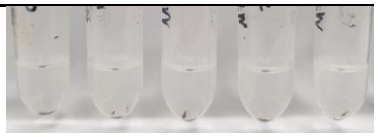
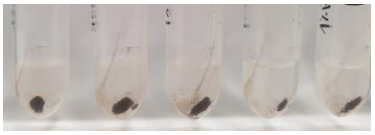
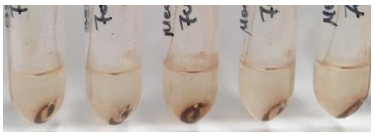
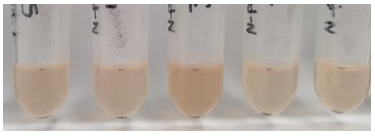
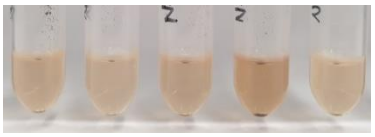
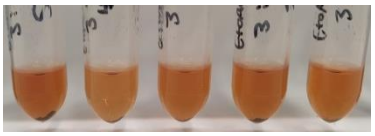
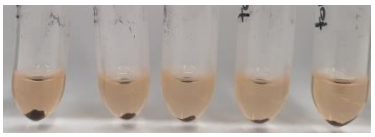
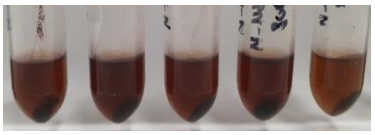
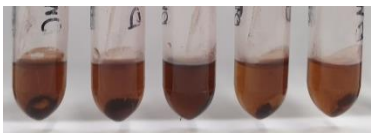
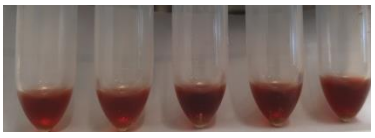
Table 1 summarizes the solubility of aurofusarin in different solvents and selected solvent mixtures at 20°C. Aurofusarin is water-insoluble. Even with ultra-sonication, the soluble amount did not exceed 1 mg L^{-1} (Chapter 3, Table 1). Therefore, many methods were developed to enhance the solubility of the substances in water, such as the addition of small amounts of other water-miscible solvents (Jouyban, 2010) or by forming a soluble derivative of the target solute (Censi &

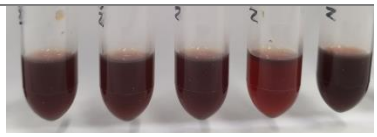
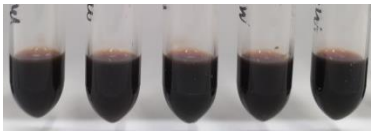
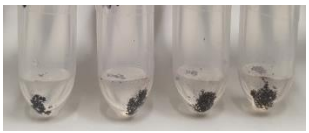
Di Martino, 2015). For example, aurofusarin content increased in the binary solvents 1% dimethyl sulfoxide and methanol/ water (7:3, v/v) to 4 and 10 mg L^{-1}, respectively.

In polar and H-bonding solvents like methanol and ethanol (Chapter 3, Table 1), *n*-propanol, and *n*-butanol, the solubility of aurofusarin improved slightly and increased in tandem with the number of the solvent´s carbon atoms. Accordingly, the highest aurofusarin content was recorded in *n*-butanol (36 mg L^{-1}). Remarkably, the solubility of aurofusarin improved in the polar-aprotic solvents dimethylsulfoxide and dimethylformamide, which dissolved 100 and 500 mg $L^{-1,}$ as well as in ethyl acetate, acetone, and acetonitrile, in which 53, 143, and 170 mg L^{-1} dissolved (Chapter 3, Table 1). The electron affinity of the naphthoquinone ring system is increased by conjugation with the electronigative groups, such as chloro- and nitro-(Chatterjee, 1971).

Solubility of aurofusarin in phenol and *m*-cresol was recognized in 1937 during the first structure elucidation attempts. As expected, aurofusarin was better soluble in heated solvents, boiling chloroform, or nitrobenzene (Ashley et al., 1937). We obtained deep red solutions when aurofusarin was dissolved in chloroform/methanol (8:2, v/v) and chloroform (Chapter 3, Table 1), dichloromethane, nitrobenzene, and *m*-cresol, the content ranged between 2 and 5 g L^{-1}. Surprisingly, aurofusarin was slightly soluble in toluene (41 mg L^{-1}). The solution color intensity provided a good indicator for aurofusarin solubility (Table 1).

Table 1. The solubility of aurofusarin in different solvents. Conc. Mean ± SD in various solvents (n = 5).

Solvent	**mg L^{-1}**	
Tert-butyl-methyl ether	< 1	
Dimethyl sulfoxide/water (1:99, v/v)	4 ± 1	
Methanol/water (7: 3, v/v)	10 ± 2	
n-propanol	30 ± 6	
n-butanol	36 ± 7	
Ethyl acetate/dichloromethane/methanol (3:2:1, v/v/v)	370 ± 45	
Toluene	40 ± 2	
N-*N*-dimethylformamide	500 ± 122	
Dimethyl sulfoxide	110 ± 30	
Dichloromethane	3,400 ± 700	

Nitrobenzene	3,000 ± 400	
m-Cresol	6,000 ± 600	
Choline chloride/urea (5:4, v/v)	na	

An undetermined amount of purified aurofusarin was dissolved in 0.5 mL of different solvents. The samples were shortly sonicated and vortexed, then incubated overnight at 20°C, 130 rpm, and centrifuged at 20°C. An aliquot from every sample was diluted in pure methanol analyzed by LC−QQQ/MS.
na: not detected

3.3. Extinction coefficient

The molar extinction coefficient (ε) has been used to efficiently quantify the concentration of several pigments, proteins, and organic compounds (Müh & Zouni, 2005; Singh et al., 2004; Singh et al., 2018). So far, the extinction coefficient of aurofusarin is not known. We applied a quantitative spectrophotometric method to calculate the molar extinction coefficient of purified aurofusarin at 28 ± 2°C. Table 2 summarizes the result. The absorption spectra of aurofusarin in methanol at four concentrations, 17.6, 8.8, 4.4, and 2.2 µM, were recorded (n = 3-5). A linear regression analysis of the data ($R^2 > 0.997$) was performed (Table 2). The maximum absorption peak λ_{max} was recorded at 266 nm (Figure 3). Since the light absorption at the highest concentration of aurofusarin was consistent and reliable, two more replicates were prepared to get a mean A^{266} of a total of five replicates.

According to the Beer-Lambert Law, the molar extinction coefficient ε was calculated using the following equation:

$A = \varepsilon \times L \times C$

A, light absorbance at a particular wavelength (unitless)

ε, molar extinction coefficient ($M^{-1} cm^{-1}$)

L, cuvette diameter (cm)

C, aurofusarin conc. (M)

At the second maxima 378 nm and 17.6 µM, the absorption was 0.10 and according to Beer-Lambert Low the molar extinction coefficient ε is 6,100 $M^{-1} cm^{-1}$

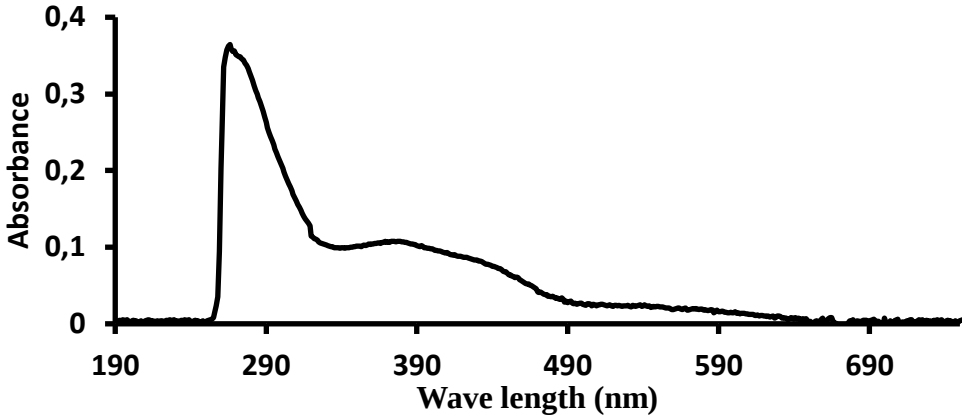


Figure 3. The light absorption of aurofusarin. The maximum absorption was recorded at 266 nm. A further maximum was found at 378 nm

Table 2. Data for determination of the molar extinction coefficient of aurofusarin in methanol at λ_{max} of 266 nm and 28 ± 2°C

Aurofusarin µM	A^{266}	n	Cycle	ε^{266} $M^{-1}cm^{-1}$	CV %	Correlation Determination R^2	CV %	Slope	CV %
2.2	0.04	3	10	17,800 ± 140	1	0.997 ± 0.001	0.1	0.0378 ± 0.001	3.3
4.4	0.10	3	10	23,400 ± 700	3				
8.8	0.18	3	10	20,800 ± 450	2				
17.6	0.38	5	10	21,600 ± 500	2				

A stock solution of purified aurofusarin in dimethyl sulfoxide was diluted in methanol to get several concentrations in µM, n= 3-5, each repetition was measured ten times (cycle). A^{266}, the light absorbance occurred at 266 nm; ε^{266}, the molar extinction coefficient calculated according to the Beer-Lambert law, illustrated in the equation $A = \varepsilon . L . C$. Both values are shown as mean ± s.d. correlation Determination. R^2 and slopes are shown as mean ± s.d. of three calibration curves; CV%, the coefficient of variation of A^{266}, ε^{266}, R^2 and slopes are in good agreement

4. Biosynthesis of aurofusarin:

4.1. Aurofusarin biosynthesis pathway

Several studies report on identifying a gene cluster that encodes the genes required for aurofusarin biosynthesis. Malz and coworkers (2005) identified *PKS12* as the essential enzyme using a *PKS12*-deficient mutant strain. Remarkably, disruption of *PKS12* caused downregulation of ten other genes of the cluster, including the putative transcription factors *aurR2* and *aurJ*, pump (*aurT*), *aurO, aurF, gip1* and *aurL2,* and two open reading frames (ORFs). The crucial role of *PKS12* in the biosynthesis of aurofusarin was confirmed via a combination between a molecular analysis, which confirmed the abortion of *PKS12* in the aurofusarin-deficient mutant, and the chemical analysis, which detected the absence of aurofusarin in the mutant strains. Concomitantly, albino phenotype cultures were obtained from a *PKS12*-disrupted *F. graminearum* mutant (Malz et al., 2005). Kim and co-authors (2006) carried out their study on *Gibberella zea*. They identified *PKS12* and the putative laccase *gip1* as required genes for the biosynthesis of aurofusarin. Target deletion of both enzymes resulted in a colorless phenotype in both cases, although the ectopic type produced a red-colored pigment on PDA. Aurofusarin reappeared in the mutants that were restored with *PKS12* and *gip1* (Kim et al., 2005). Similar strategies were applied to prove the participation of *gip2*–the same gene *aurR1* was assigned by Frandsen et al. (2006)–as putative transcription regulator of the aurofusarin biosynthesis pathway (Kim et al., 2006). The genome analysis of *G. zeae* led to the identification of fifteen *PKS* genes. Among them, *aurR1* was proposed to be responsible for the biosynthesis of aurofusarin (Gaffoor et al., 2005). A comprehensive study of the aurofusarin biosynthesis determining the genes and the involved domains was provided by Frandsen and co-authors (Figure 3), among of which were the polyketide synthase *PKS12*, transcription factors *AurR1* and *AurR2*, pump *aurT*, the dehydratase *AurZ*, the *O*-methyltransferase *AurJ*, the laccase *aurL2*, and the extracellular enzyme complex *GIP1* (laccase), the monooxidase *AurF*, and the oxidoreductase *AurO*. The biosynthesis commences when the building blocks of one acetyl-CoA and six malonyl-CoA are condensed to a linear heptaketide. The condensation is catalyzed by PKS12. YWA1 is formed via a Claisen cyclase domain in PKS12. Thioesterase (TE) domains present in the C-terminus region of the enzyme may also serve as Claisen cyclases responsible for cyclization of the second aromatic ring of naphthopyrone (Fujii et al., 2001). Then,

AurZ catalyzes the dehydration of YWA1 to the heptaketide naphthopyrone nor-rubrofusarin. The hydroxyl group on C_8 in nor-rubrofusarin is methylated by AurJ, which yields rubrofusarin. Later, rubrofusarin is translocated outside the cell across the endoplasmic reticulum to the plasma membrane, a process which is facilitated by aurT as a transporter. The conversion of the naphthopyrone rubrofusarin to the naphthopyrone-quinone aurofusarin is catalyzed by an extracellular enzyme complex containing aurO, aurF, gip1, and aurS. First, the insertion of the hydroxyl group on the C_9 by AurF (flavin-dependent monooxygenase) turns rubrofusarin to 9-hydroxy-rubrofusarin. The last molecule is activated via Gip1 laccase that generates a free radical group–one hydroxyl group on the fifth or sixth carbon is deprotonated and oxidized. As a result, a C_7–$C_{7'}$ bond is formed. The last chemical reaction is catalyzed by the dehydrogenase AurO (dehydrogenase), which oxidizes the hydroxyl groups on the ring to keto groups. AurR1 is a transcription factor that upregulates the transcription of *PKS12*, *aurJ*, *aurF*, and *gip1*. Δ*aurR1* and Δ*PKS12* mutant colonies remained colorless; the Δ*aurZ* strain was green, and in all of them, neither aurofusarin nor rubrofusarin was detected, only YWA1 accumulated. Colonies of Δ*aurS*, Δ*GIP1*, and Δ*aurF* strains appeared yellow as rubrofusarin was detected in them. Interestingly, Δ*aurT* and Δ*aurR2* mutant colonies appeared red, though the rubrofusarin content was higher than that of aurofusarin. The aurofusarin content in Δ*aurL2* strains was comparable to the wild type (Frandsen et al., 2006, 2011). Recently, Westphal and colleagues enhanced the production of aurofusarin in *F. graminearum* via overexpression of the transcription factor *aurR1*. A proteome comparison between the ectopic and the wild type revealed that all detected proteins related to the aurofusarin gene cluster were upregulated in the overexpression mutant. Of those, only five genes, *aurO*, *aurJ*, *aurF*, *PKS12*, and *gip1* increased significantly (Westphal et al., 2018).

Disruption of the genes responsible for the biosynthesis of aurofusarin in *F. graminearum* accompanied with a robust growth rate and conidia production in comparison to the wild type (Kim et al., 2005; Malz et al., 2005). Furthermore, we found that aurofusarin content corresponded proportionally with the weight mycelium biomass in *F.* spp. Exceptions are one strain of *F. avenaceum* and *F. sporotrichioides* (Supplementary Table 1).

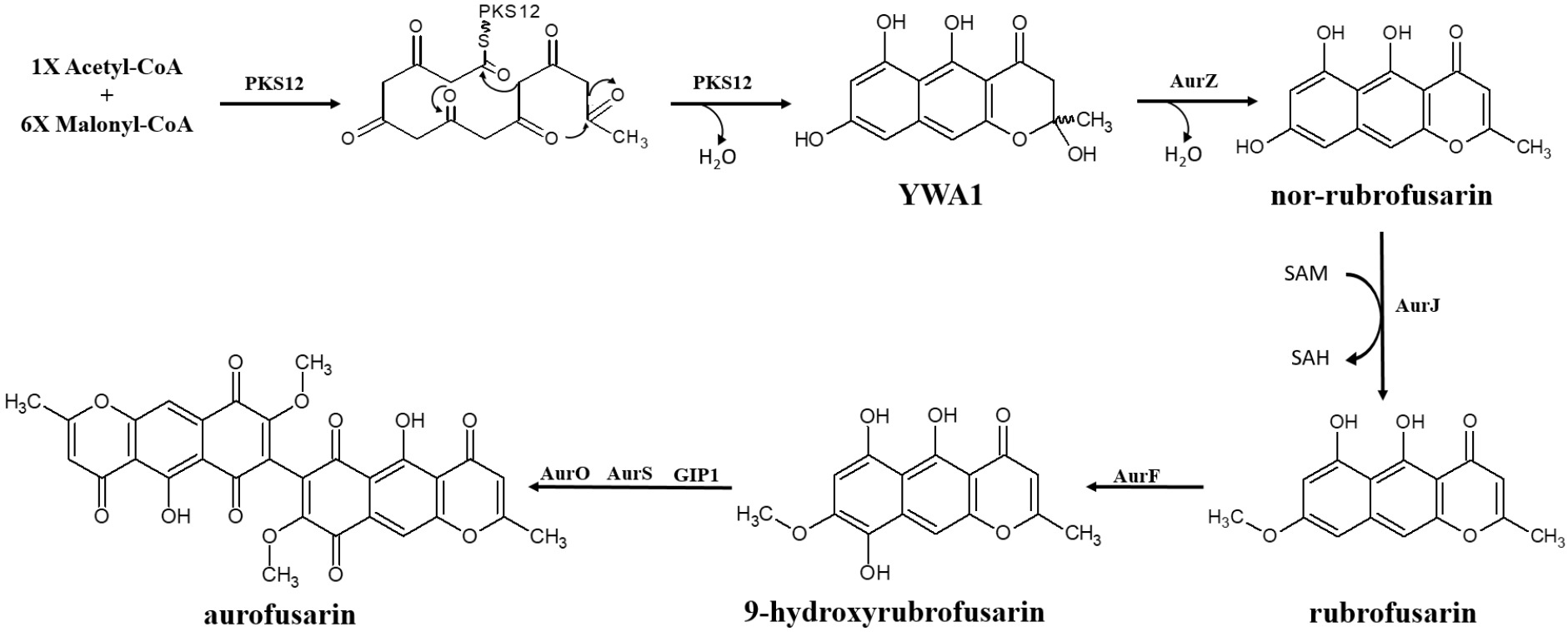


Figure 3. The biosynthesis pathway of aurofusarin as described by Frandsen et al. (2006, 2011). The polyketidesynthase *PKS12*, the dehydratase *AurZ*, the *O*-methyltransferase *AurJ*, the laccase *GIP1*, the monooxidase *AurF*, and the oxidoreductase *AurO*. with fasciclin domains AurS

4.2. Aurofusarin producers

Aurofusarin was first isolated as small yellow crystals from strains related to *F. culmorum* (Ashley et al., 1937; Shibata et al., 1966). It was successfully purified from *Hypomyces rosellus* as dark red prisms (Birchall et al., 1966; Gray et al., 1967). Several *Fusarium* spp. have been listed to produce aurofusarin (Medentsev & Akimenko, 1998; Munkvold, 2017), of which Table 3 provides a summary.

Table 3. Aurofusarin fungal producers

Producer	Usage	References
F. culmorum	Aurofusarin isolated as small yellow crystals	Ashley et al., 1937; Shibata et al., 1966
Dactylium dendroides ***Hypomyces rosellus***	Aurofusarin purified as dark red prisms	Birchall et al., 1966; Gray et al., 1967
F. acuminatum ***F. sporotrichioides*** ***F. crookwellense*** ***F. langsethiae*** ***F. tricinctum*** ***F. decemcellulare*** ***F. sambucinum***	Aurofusarin producers	Medentsev & Akimenko, 1998; Munkvold, 2017
F. decemcellulare ***F. bulbigenum***	Aurofusarin producers	Medentsev et al., 2005
F. graminearum	A model in genetically engineering studies determining the biosynthesis pathway and the ecological role of aurofusarin	Frandsen et al., 2006, 2011; Kim et al., 2006; Malz et al., 2005; Westphal et al., 2018; Xu et al., 2019
Cladobotryum* spp.** ***F. avenaceum, ***F. poae*** ***F. venenatum***	Aurofusarin producers	Põldmaa, 2011; Xu et al., 2019
F. pseudograminearum	Mutants used to assess the role of aurofusarin on the fungal pathogenicity	Malz et al., 2005

4.3. Aurofusarin in plant tissues

In nature, the red pigment aurofusarin has been detected and quantified in plant tissues that were infected with *Fusarium spp*. For example, in Thailand, aurofusarin content in naturally contaminated wheat with *Fusarium* sp. reached 0.01−2.46 mg kg^{-1} (Tola et al., 2015). Similar content was detected on maize kernels between 2008−2010 in Italy (Blandino et al., 2015). In Croatia (Spanic et al., 2020), aurofusarin was one of the most abundant mycotoxins on wheat (10−140 mg kg^{-1}). Aurofusarin was detected in all naturally infected maize samples collected from several rural areas in Malawi and was particularly prevalent in the cooler zones (Matumba et al., 2015). We quantified aurofusarin content *in vitro* in *Fusarium* spp. cultures that were grown on wheat grains (Figure 4). The highest aurofusarin content was detected in cultures of *F. sporotrichioides*.

Aurofusarin fungal producers can synthesize a considerable amount of aurofusarin depending on culture conditions and fungal species. For example, aurofusarin content in *Hypomyces rosellus* reached 12% of the mycelial dry weight (Gray et al., 1967). In *F. culmorum*, aurofusarin content reached 4.5% of the dry mycelium weight, and comparable results were obtained in *F. graminearum* (Ashley et al., 1937). Likewise, aurofusarin content reached 7% in *F. graminearum* and *F. venenatum* cultured on shaken and still home-made potato-dextrose broth (Xu et al., 2019).

We confirmed the previous results in the same *Fusarium* species (Supplementary Figure 1).

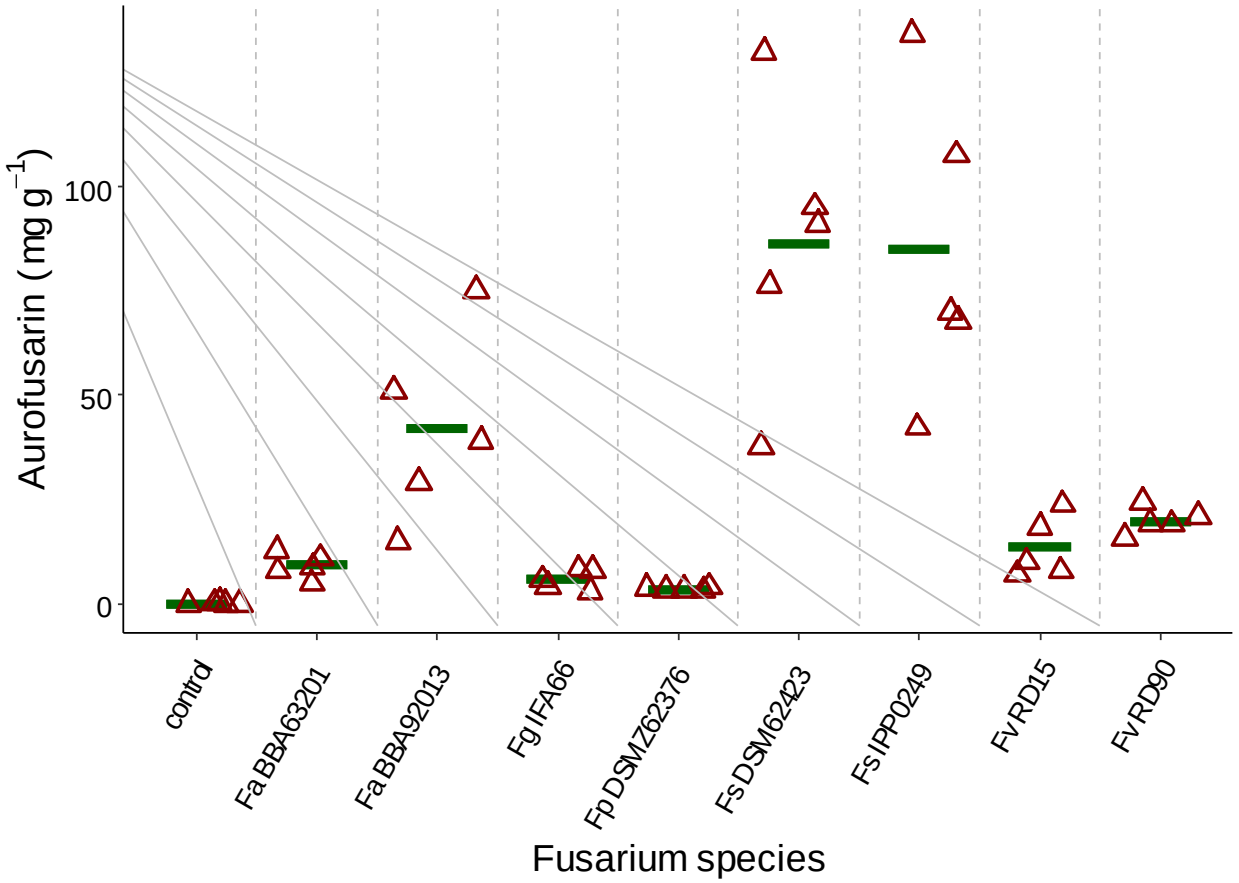


Figure 4. Aurofusarin content per gram wheat grains infected with different *Fusarium* sp. *F. avenaceum* (BBA63201, BBA92013), *F. graminearum* IFA66, *F. poae* DSMZ62376, *F. sporotrichioides* DSM62423, IPP0249), *F. venenatum* (RD15, RD90). Intact wheat grains were considered as control. All strains were cultured on wheat grains for two weeks at 23°C in the dark. Samples were freeze-dried, extracted with chloroform/methanol (8:2, v/v), then aurofusarin content was determined by LC-QQQ/MS. Flask weight was measured before (including the grains) and after (empty) the extraction. Horizontal green lines represent means (n= 5).

4.4. Does shaking induce aurofusarin synthesis in liquid media?

It was supposed that aurofusarin is stimulated in shaken cultures. We found that aurofusarin content in shaken cultures of *F. graminearum* and *F. culmorum* was higher than the content in the static cultures. However, in cultures of *F. venenatum* and *F. avenaceum*, the shaking did not enhance the production of aurofusarin over the still cultures (Xu et al., 2019). To confirm the effect of the shaking on the secretion of aurofusarin, we assessed the aurofusarin content in some *Fusarium* species, including the previously tested strains (Supplementary Figure 1).

The new results are in line with the previous results. Aurofusarin content differed through the strains regardless the stability of the cultures, and the strains of the same fungal species reacted differently to produce aurofusarin at identical growing conditions. Does the aeration catalyze the

biosynthesis of aurofusarin? Aurofusarin is an oxidized dimer of rubrofusarin, and the ratio between aurofusarin and rubrofusarin in the liquid cultures depends on culture status (Leeper & Staunton, 1984). It was predicted that a protein complex is responsible for the extracellular conversion of rubrofusarin to aurofusarin and is related to the trans-plasma membrane redox system. The dimerization of two rubrofusarin monomers occurred after the generation of free radicals catalyzed by laccase followed by the oxidation of hydroquinones to quinones (Frandsen et al., 2006, 2011; Kim et al., 2005). The shaken fungal mycelia gather, forming clumps where the outside surfaces were red, and the internal surfaces were yellow. In the static cultures with high aurofusarin content as in *F. vevenatum* RD90, the upper and lower mycelial surface were red (Supplementary Figure 2).

4.5. Induction the biosynthesis of aurofusarin

4.5.1. Influence of the media type

Providing micro-organisms with favorable conditions, particularly nutrients, is challenging (Basu et al., 2015; Pasanen et al., 1991). Defining the proper media composition and cultural conditions is crucial in identifying the quality and quantity of fungal secondary metabolites such as aurofusarin (Ashley et al., 1937; Mentges et al., 2020; Sørensen & Sondergaard, 2014; Vandermolen et al., 2013). For example, a remarkable yield of aurofusarin by *F. culmorum* was obtained in the Raulin-Thom medium compared to the Czapek-Dox medium (Ashley et al., 1937). Likewise, sufficient nitrogen and phosphorous sources can suppress the fungal growth and enhance the aurofusarin yield (Medentsev & Akimenko, 1998). Taking into account that the same media type from different origins possesses different chemical and physical properties, and even the media of the same origin could be structurally non-identical depending on the preparation process and the harvest time of the natural components involved in this medium (Booth, 1971). Aurofusarin was preferably produced on rice culture rather than the liquid media (Vandermolen et al., 2013). However, we recorded a considerable content of aurofusarin in the self-made potato broth. In contrast, the amount was negligible in the commercial PDB.

4.5.2. Influence of the artificial-PDB media on the biosynthesis of aurofusarin

Pigment-producing fungi turn colorless on commercial media (Brierley, 1917). A culture of *F. graminearum* turned deep red on shaken self-made PDB medium. However, the culture turned yellow on artificial PDB. The color intensity was associated with the portion of self-made PDB in the mixed cultures (Supplementary Figure 3), and the quantitative analysis was comparable to the visual result. This observation led us to the hypothesis that, production of aurofusarin is inhibited by artificial-PDB. Our hypothesis has been proved by preparing mixed media with a final volume, including the full portions of the ingredients in amounts equal to that of the pure single medium. The chemical analysis of the extracted samples revealed that the highest aurofusarin content was detected in the unpeeled self-made PDB. Whereas in artificial-PDB, the content was negligible (Figure 5). In mixed cultures, the amount of aurofusarin was reversely associated with the artificial PDB content. An additional experiment using potato peel-dextrose broth (Peel-DB) pure and mixed with artificial-PDB was achieved to confirm the inhibition effect of artificial-PDB on aurofusarin biosynthesis. Aurofusarin was detected in the peel-PDB cultures in small amounts that decreased when the artificial PDB was included (Figure 5).

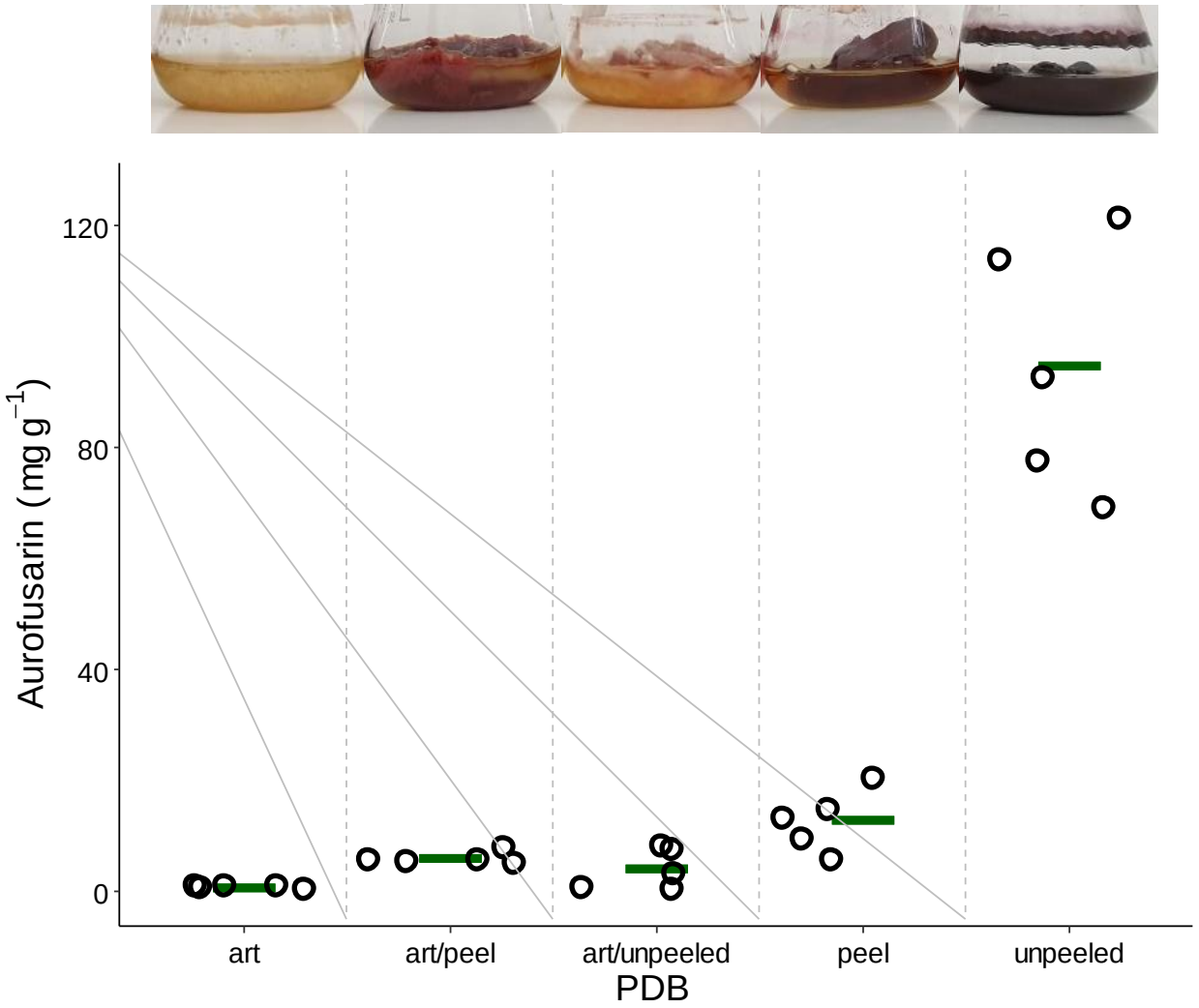


Figure 5. Aurofusarin content in the dry fungal mycelium. *F. graminearum* was cultured on PDB artificial (art) and self-made (unpeeled-PDB and peel-DB) separately and combined. The fungi were cultured for 14 days at 23°C and 130 rpm, and mycelia were freeze-dried after filtration, then extracted with chloroform/methanol (80:20). Aurofusarin content was determined by HPLC-MS/MS (horizontal lines represent the means, n= 5).

4.5.3. Influence of culture conditions on the accumulation of aurofusarin

Cultural conditions and periods influence the metabolic state of the fungal agents (Heide et al., 1985). The acidic state of the medium determines the growth rate and the metabolism of fungi. Generally, the fungal growth is enhanced in the acidic pH (Rousk et al., 2009). Anyway, the pH values of 5.5−6.6 (Medentsev et al., 2005) and < 4 (Ashley et al., 1937) stimulated the production of aurofusarin, which in another study was also enhanced when the pH value of the culture was increased (Medentsev & Akimenko, 1998).

The color of aurofusarin (Gray et al., 1967) and other dimeric naphthopyrones such as xanthomegnin, viopurpurin, and vioxanthin produced by *T. rubrum* (Blechert et al., 2019) is a function of the culture pH. Aurofusarin appears yellow in the acidic medium and changes to red in the alkaline medium. Generally, the production of aurofusarin is stimulated at 20−25°C and in the darkness (Ashley et al., 1937; Birchall et al., 1966; Shibata et al., 1966).

We estimated the pH of unpeeled self-made PDB, potato peel, and artificially PDB prepared in pure and mixtures inoculated with *F. graminearum* for two weeks at 23°C and in the uninoculated cultures. Notably, after two weeks, the pH value was neutral in unpeeled-PDB and alkaline in peel-DB. The alkalinity was higher in the cultures supported by artificial media and pure artificial cultures (Supplementary Table 2).

Westphal and co-authors recognized the potential importance of aurofusarin as a natural pigment. Therefore, they enhanced aurofusarin yield in *F. graminearum* mutant via overexpression of the transcription factor *aurR1*. Unexpectedly, increasing the concentration of copper ions in the culture medium (the multicopper oxidase gip1 is essential for the biosynthesis of aurofusarin) did not increase the biosynthesis of aurofusarin (Westphal et al., 2018).

In previous work, we found that *F. graminearum* and *F. culmorum* were stimulated to produce aurofusarin as a defense mechanism against springtails *F. candida* and two nematodes species (Xu et al., 2019). After predation, the transcriptome analysis of the extracted fungal culture revealed that most genes in the aurofusarin gene cluster were upregulated, and the chemical analysis confirmed the high content of aurofusarin in the damaged cultures. To conclude the influence of the mechanical damage on the secretion of aurofusarin, *F. graminearum* was mechanically injured with a razor blade array causing accumulation of aurofusarin in the damaged mycelium (Xu et al., 2019). Interestingly, stress does not always inhance the secretion of aurofusarin. Treatment with the bacterial secondary metabolite antimycin A or H_2O_2 suppressed the biosynthesis of aurofusarin (Medentsev et al., 2005). However, the visual observation of neutral *Fusarium*-PDB artificial cultures untreated and treated with α-solanine and α-chaconine individually and combined at levels that mimic their natural concentration in potato tuber (Bushway & Ponnampalam, 1981) revealed no color contrast between the treatment and the controls.

5. Biological activities of aurofusarin

Aurofusarin is commonly associated with *Fusarium*-infected foodstuffs and is usually detected in a complex with other mycotoxins in the infected crops. Therefore, its potential toxicity was the focus of many investigations. Some studies considered aurofusarin as cytotoxic (Uhlig et al., 2006) and toxic to mitochondria (Vejdovszky et al., 2016). The toxic effects on cell line proliferation were ascribed to the increased oxidative stress as glutathione oxidation, and reactive oxygen species (ROS) levels were increased (Jarolim et al., 2018).

In contrast to many solar radiation-protective fungal pigments, aurofusarin showed no conclusive protective effect against UV radiation. Still, a comparison of aurofusarin producers with aurofusarin-deficient mutant strains suggested that aurofusarin has some mycotoxin properties (Malz et al., 2005).

We recently explored the ecological role of aurofusarin as a non-toxic defensive agent that repels fungivores and protects its producer from being grazed (Xu et al., 2019).

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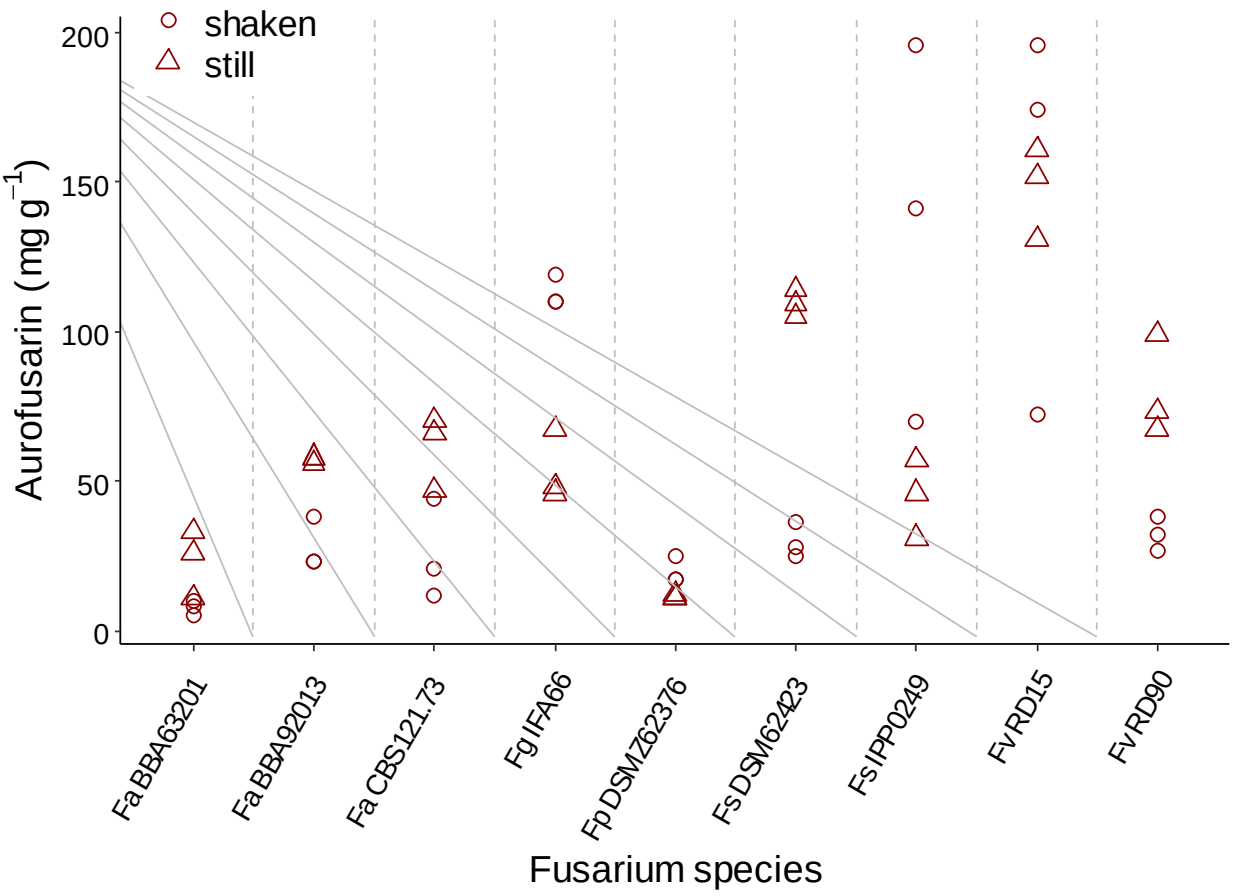
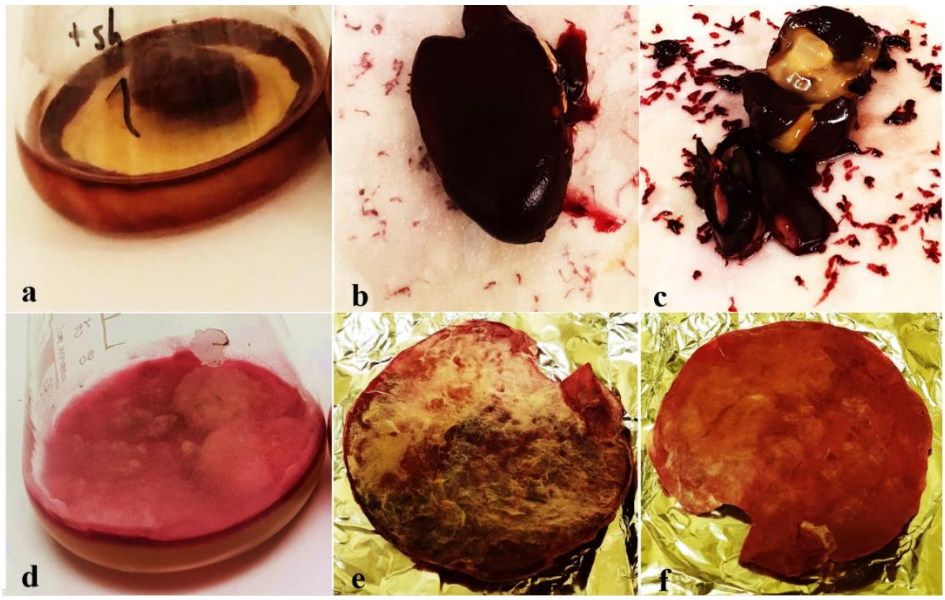
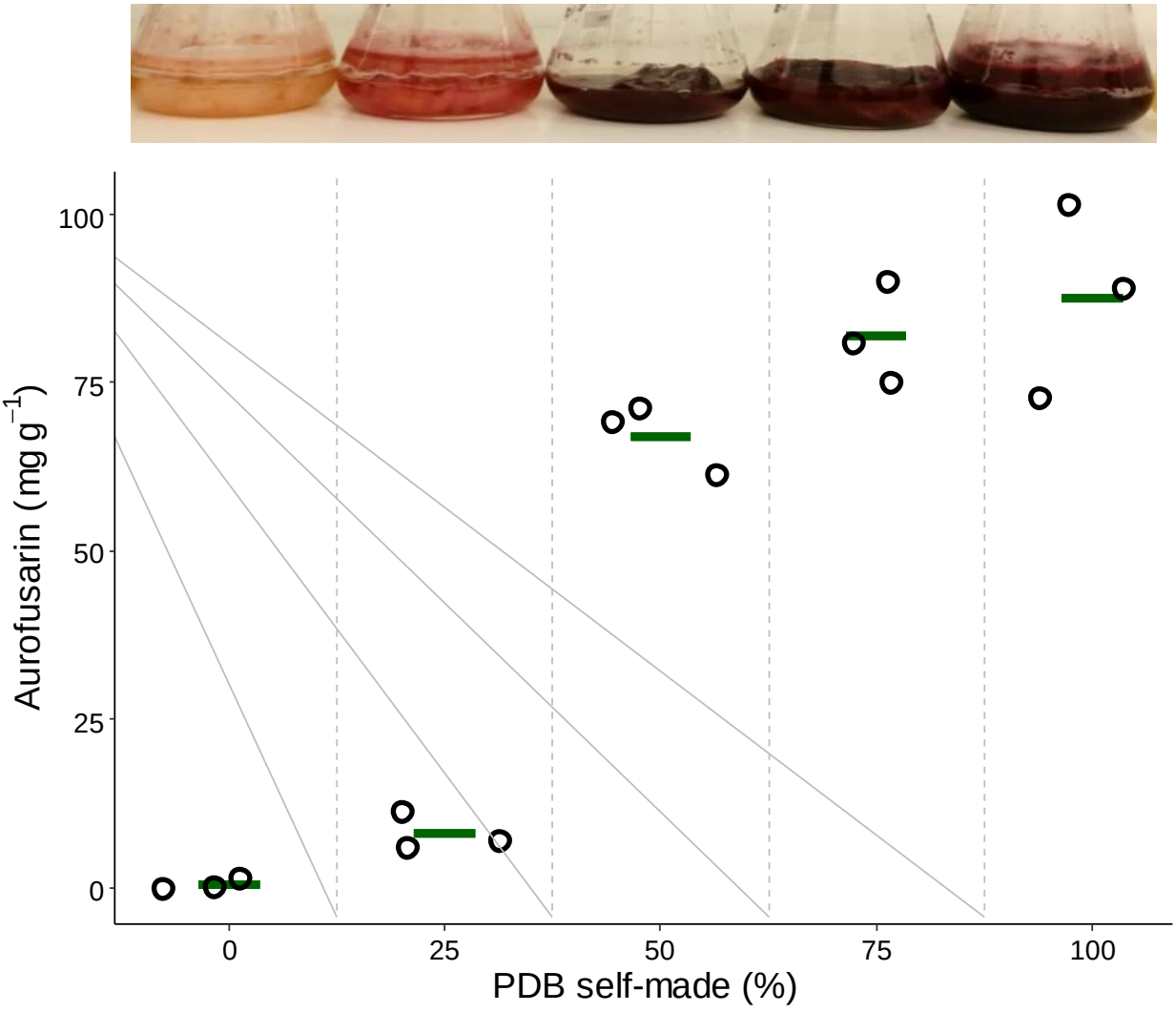
7. Supplementary information



Supplementary Figure 1. Aurofusarin content on self-made PDB still and shaken. In fungal cultures of *F. avenaceum* (BBA63201, BBA92013, and CBS121.73), *F. graminearum* IFA66, *F. poae* DSMZ62376, *F. sporotrichioides* (DSM 62423, IPP0249), *F. venenatum* (RD15, RD90). All strains were cultured on self-made PDB still and shaken at 130 rpm for two weeks at 23°C. Mycelia were freeze-dried after filtration, then extracted with chloroform/methanol (8:2, v/v). Aurofusarin content was determined by LC-QQQ/MS. Values represent means (n= 3).



Supplementary Figure 2. Shaken and static cultures. *F. venenatum* was cultured at 23°C for two weeks a), b), and c) with shaking. d), e), and f) without shaking



Supplementary Figure 3. Aurofusarin content in the dry fungal mycelium. *F. graminearum* was cultured on PDB for 14 days at 23°C and 130 rpm on commercial and self-made PDB individually and as a mixture at different ratios. The mycelia were freeze-dried after filtration, then extracted with chloroform/methanol (8:2, v/v). Aurofusarin content was determined by HPLC-MS/MS (horizontal lines represent the means, n= 3).

Supplementary Table 2. pH-values of fungal cultures. Different media types inoculated and non-inoculated with *F. graminearum* shaken for two weeks at 23°c in the dark Mean ± SD (n= 5).

	Control	Treatment
unpeeled	5.5 ± 0.1	7.0 ± 0.1
unpeeled/ artificial	5.4 ± 0.0	8.6 ± 0.2
peel	5.7 ± 0.0	8.0 ± 0.6
peel/artificial	5.5 ± 0.0	8.8 ± 0.2
artificial	5.3 ± 0.0	8.4 ± 0.2

Chapter 3. Aurofusarin analysis by HPLC-MS/MS: challenges of limited solubility and stability of standards in water-containing solvents

Albatol Alsarrag, Mohammad Alhussein, Petr Karlovsky

1. Abstract

Naphthopyrones are a class of secondary metabolites that includes a wide range of bioactive compounds. They are also considered an important source for bio-pigments. The bis-naphthopyrone derivative aurofusarin exhibited a repellent but non-toxic effect against various fungivores. Theoretically, aurofusarin could be utilized as a natural pigment in the future. However, the aqueous-insolubility and the poor solubility of aurofusarin in many organic solvents and its instability during the chemical analysis present the main burdens that prevent aurofusarin from being applicable. In this study, we tested the solubility of aurofusarin in several organic solvents and some solvent mixtures and found that aurofusarin was consistently soluble in a mixture of chloroform/methanol (8:2, v/v), yielding a concentration of 4 g L^{-1}. The same solvent mixture was used to extract a considerable amount of aurofusarin from *F. graminearum* mycelium exceeding 85 mg g^{-1} dry mycelium. The extraction efficiency was assessed using HPLC−QQQ/MS by determining the recovery, matrix effect, and process efficiency. The analytical method was validated by examining selectivity, specificity, accuracy, precision, linearity, the limit of detection (LOD), and quantification (LOQ). We also showed that methanol protects aurofusarin from being degraded during measurement overnight.

Keywords Aurofusarin, bis-naphthopyrone, fungal pigment, organic solvents, extraction efficiency, validation parameters

2. Introduction

Dimeric naphthoquinone-pyrones (shortly bis-naphthopyrones) are produced by many fungal species (Donner 2015; Põldmaa 2011). They serve as a base structure for many bioactive compounds (Lu et al., 2014), including such with antitumor (Koyama et al., 1988), anti-HIV (Singh et al., 2003), cytotoxic (Li et al., 2016), and anti-oxidant activity (Song et al., 2004). Several dimeric- and mono naphthopyrones have been identified successfully (Bouras et al., 2005; Campos et al., 2005; Ehrlich et al., 1984; Lee et al., 2006; Li et al., 2016; Schüffler et al., 2009). Linear or angular mono-naphthopyrones can form dimers (Chiang et al., 2011; Frandsen et al., 2006). Fungal secondary metabolites, particularly those produced by filamentous fungi, represent an important source for drug leads from nature (Caro et al., 2015). Though extraction and identification methods are more or less established (Lebeau et al., 2017), the potential function in an ecological context still warrants further exploration (Bennett, 1995; Grace, 2013).

The red pigment aurofusarin is a dimeric naphthopyrone produced by several fungal genera, among which *Fusarium* is considered the predominant (Munkvold, 2017). In 1937, aurofusarin was successfully isolated from *F. culmorum* (Ashley et al., 1937). A few decades later, its molecular formula was established (Birchall et al., 1966), and some of its chemical and physical properties were investigated, such as its melting point and solubility in some organic solvents (Gray et al., 1967). No further prominent studies focused on aurofusarin until the beginning of the current century. However, aurofusarin has received more attention recently. The biosynthesis pathway of aurofusarin and its gene cluster was identified (Frandsen et al., 2011; Malz et al., 2005). Also, analytical methods were optimized to quantify aurofusarin in naturally infected wheat tissues (Neuhof et al., 2008). Moreover, aurofusarin was successfully synthesized from pyrone and quinone through a Dikeman condensation reaction (Qi et al., 2018). The application of aurofusarin in bioassays is hampered by several issues: (1) aurofusarin is insoluble in water (Ashley et al., 1937); (2) knowledge about its solubility in organic solvents is limited; and (3) aurofusarin is unstable at room temperature and sensitive to ambient light (Jarolim et al., 2018). Aurofusarin is an antifeedant against fungivores that does not associate with a toxic effect (Xu et al., 2019).

Many bioassays require adding the test compound as a solution to adjust its homogeneity, for which organic solvents can be used. Therefore we explored several organic solvents and mixtures regarding extraction efficiency, solubility, and solution stability of aurofusarin. Aurofusarin concentrations in different solvents were analyzed by LC−QQQ/MS and

LC−TOF/MS. There is an increasing awareness to use dyes of natural origins, to which filamentous fungal pigments can contribute substantially (Kalra et al., 2020).

3. Materials and methods

3.1. Media and fungal samples

Potato Dextrose Broth (PDB) was prepared by boiling 200 g unpeeled potato in tap water for 20 minutes. The extract was filtered, and the volume adjusted to 1 L. Twenty grams of glucose were added. The mixture was autoclaved at 121°C for 20 min. Two different *F. graminearum* strains were used, a wild type *F. graminearum* strain IFA66 (DON chemotype) was obtained from Dr. Marc Lemmens (BOKU, Tulln, Austria) via Professor Thomas Miedaner (University of Hohenheim, Germany), and *F. graminearum* aurofusarin-deficient mutant was generated by disrupting the polyketide synthase gene . Spores suspensions were inoculated on 30 ml PDB media in 100 ml Erlenmeyer flasks, then incubated on a laboratory shaker at 23°C and 130 rpm in the darkness. Five biological replicates were prepared. After two weeks, the fungal mycelium was filtered using a mini-membrane-vacuum pump and filter paper (MN615 size 55mm diameter). The filtered mycelium was stored at −20°C for 24 h, then lyophilized at −57°C and 0.046 mbar for 72 h. The mycelium was ball-milled and stored at −20°C for later use.

3.2. Analytical standard and purification of aurofusarin

A BVT-0392 aurofusarin standard of purity > 98% was supplied by Hans Peter Kroll, Bio Viotica (Dransfeld, Germany). A stock solution in dimethyl sulfoxide was prepared, divided into aliquots of 200 µL, and stored at −80°C for later use. The purification process of aurofusarin from *F. graminearum* is briefly described; *F. graminearum* mycelium was extracted with chloroform/ methanol (8:2, v/v). The crude extract was dissolved with phenol and precipitated with ethanol (Gray et al., 1967; Xu et al., 2019).

3.3. Chemicals and solvents

All solvents were purchased from CHEMSOLUTE® (Th. Geyer, Höxter, Germany). The purity of the analytical solvents was ≥ 99.5%. Ultra-pure water was prepared with an Arium pro-VF (Sartorius, Göttingen, Germany), bi-distilled water with a GFL water distillation unit (Burgwedel, Germany).

3.4. Extraction of aurofusarin

Ten milligrams of fungal mycelium were each extracted with 1 mL of either chloroform, methanol, ethanol, acetone, ethyl acetate, acetonitrile, acetonitrile/water (84:16, v/v), and chloroform/ methanol (8:2, v/v). All extractions were performed in five replicates. The extraction solutions were shaken overnight at 23°C with 130 rpm, then centrifuged at 13000 rpm. Aliquots of 10 µL were separately diluted in pure methanol before LC−QQQ/MS and LC−TOF/MS analyses.

3.5. Solubility of Aurofusarin

Dry purified aurofusarin was dissolved in 0.5 mL of each solvent mentioned above and bidistilled water in five replicates. All solutions were sonicated for a few seconds, then incubated at 20°C and shaken at 130 rpm in the dark to obtain saturated solutions. Subsequently, the solutions were centrifuged at 20°C and 13000 rpm for ten minutes to remove unsolubilized aurofusarin. Saturation was controlled visually by inspecting the precipitation of aurofusarin at the bottom of the Eppendorf tubes. Then, aliquots were diluted with pure methanol for LC−QQQ/MS and LC−TOF/MS analyses.

3.6. Optimization of the extraction method

Ten milligrams of the milled red wild-type *Fusarium* mycelium were first defatted in 1 mL light petroleum by shaking for 30 min (130 rpm). The supernatant was transferred to a new Eppendorf tube and stored at −20°C for later analysis. After the defatting step, the remaining mycelium was extracted three times successively with chloroform/ methanol (8:2, v/v). The first extraction process lasted for 12 h, the second and third for one hour each (depending on a previous exploration, the most extractable aurofusarin is obtained in the first extraction, therefore no need for a long extraction time posteriorly). During extraction, the samples were constantly shaken at

200 rpm. After every extraction process, the samples were centrifuged, and the supernatants were transferred to new Eppendorf tubes. All supernatants were stored at −20°C for separate later analyses. Every extraction variant was executed in a set of three replicates.

3.7. Degradation of aurofusarin

An aliquot of the analytical standard in dimethyl sulfoxide was re-dissolved in pure methanol and considered a stock solution for further dilutions performed in ultra-pure water, bidistilled water, and pure methanol separately to get final concentrations of 1 µg mL^{-1} (in methanol/water v:v and pure methanol). The samples were prepared at a constant temperature of 20°C, then were directly measured at the same temperature by LC−QQQ/MS at gradually increased time intervals for 30 h. The accurate masses and the proposed formula of the degradants were assessed by LC−QTOF/MS.

3.8. Analytical method validation and performance criteria

3.8.1. Material

Purified aurofusarin and mycelium from an aurofusarin-deficient mutant of *F. graminearum* as matrix were used in the analytical experiments.

3.8.2. Triple quadrupole (QQQ/MS)

HPLC-system was linked via an ESI interface (positive mode, capillary voltage 4,000 V) to an Agilent QQQ/MS 6460 detector (Agilent Technologies, Waldbronn, Germany), which was adjusted in the multiple reaction monitoring (MRM) mode. The column was C18 (Polaris Ether, 100 x 2.0 mm; Varian, Darmstadt, Germany), the flow rate 0.2 mL min^{-1} that was thermostatted at 40°C. Solvent A was water with 0.1% formic acid (v/v), B was methanol with 0.1% formic acid in (v/v). The solvent gradient was applied as follows: 0.2 min 40% B, 6.8 min 40–98% B, 2 min 98% B. Ionization was implemented by electrospray in a positive mode, and a capillary voltage of 4,000 V. The acquired data were processed using Mass Hunter Workstation, Version B.08.02 (Agilent Technologies, Waldbronn, Germany). The quantitative analysis was carried by QQQ/MS in MRM-mode monitoring the transition *m/z* 571.3 → 556.0, which displayed the maximum

efficiency as a quantifier, and the transitions *m/z* 571.3 → 511.0. and *m/z* 571.3 → 541.0 as additional qualifiers (Figure. 2).

3.8.3. Analysis by LC–TOF/MS

An Agilent 1290 Infinity II HPLC system (Agilent Technologies, Waldbronn, Germany) was coupled to an Agilent 6545 QTOF-MS detector (Agilent Technologies, Waldbronn, Germany). The separation was performed on a Zorbax Eclipse Plus C18 column (50 x 2.1 mm, 1.8 µm particle size, Agilent Technologies, Waldbronn, Germany). The column was thermostatted at 40°C; the flow rate was 0.4 ml min^{-1}, the injection volume 5 µl. Solvent A was water with 0.1% formic acid (v/v), solvent B was methanol with 0.1% formic acid in (v/v). The solvent gradient was applied as follows: 0 to 0.2 min, 40% B; 0.2 to 7 min, 40% to 98% B; 7 to 10 min, 98% B; 10 to 10.50 min. The eluent was ionized using a dual Agilent Jet Stream Electrospray Ionization source (Dual AJS ESI). The source conditions were as follows: the sheath gas temperature 350°C with a flow rate of 11 L/min, capillary voltage 3,500 V, nozzle voltage 1,000 V, nebulizer pressure of 35 psi, drying gas temperature 320°C with a flow rate of 8 L min^{-1}, fragmentor voltage 175 V, skimmer voltage 65 V, and octopole RF peak 750 Vpp. MS1 data were acquired in full scan mode and positive ionization. The mass range was from 100 to 1,700 *m/z,* and the acquisition rate was two spectra/s. The MS2 spectra of aurofusarin were acquired in the targeted mode with a collision energy of 10 V. The acquired data were processed using Mass Hunter Workstation, Version B.08.02 (Agilent Technologies, Waldbronn, Germany).

3.8.4. Sample preparation

A stock solution of purified aurofusarin in chloroform/methanol (8:2, v/v) at a concentration of 1 mg ml^{-1} was added to the aurofusarin-deficient mutant extract. Set A (n = 6) comprised samples for which an aliquot of the standard stock solution was dried, then dissolved in chloroform/methanol (8:2, v/v). Set B (n = 5) comprised samples for which the fungal mycelium was spiked with the aurofusarin stock solution, dried, and then extracted with chloroform/methanol (8:2, v/v) till the solvent was colorless. Set C (n = 5) was an aurofusarin stock solution spiked onto the mycelial extract in chloroform/methanol (8:2, v/v). Aliquotes of the samples were diluted in pure methanol and analyzed by LC−QQQ/MS.

3.8.5. Validation parameters of the extraction process

The extraction method of aurofusarin from the fungal mycelium with chloroform/methanol (8:2, v/v) was validated by assessing the percentage of process efficiency, matrix effect, recovery, accuracy, and precision (Matuszewski et al., 2003).

Process efficiency quantifies the difference of the aurofusarin signal intensity between the sample (mycelium of the mutant) spiked before the extraction and the extracted internal standard solution Process efficiency (%) = (meanB/ meanA) x 100. The matrix effect was estimated by quantifying the difference of the aurofusarin signal intensity between the sample (mycelium of the mutant) spiked after the extraction (spiked extract) and internal standard solution Matrix effect (%) = (meanC/ meanA) x 100; The difference in the aurofusarin response of the spiked fungal extract and the internal standard corresponds to the contribution of the co-extracted matrix ingredients on aurofusarin ionization. A value of 100% points to the absence of any matrix effect. Large or lower percentages indicate either a positive or negative effect. The recovery of our method was calculated by quantifying the difference of the aurofusarin signal intensity between the sample (mycelium of the mutant) spiked before, and after the extraction, Recovery (%) = (meanB/ meanC) x 100; any variation reflects the potential influence of analytes on the extraction process.

The method accuracy was estimated in the spiked sample before the extraction by quantifying the difference of the aurofusarin signal intensity between the extracted internal standard solution, and the sample (mycelium of the mutant) spiked before the extraction, Accuracy = (meanA / meanB) x 100. The deviation of the observed signals from the mean values in every sample set determine the precision of the method, the Precision = Standard deviation within on sample set.

meanA: mean peak area of internal standard solution

meanB: mean peak area of the sample that was spiked before the extraction

meanC: mean peak area of the sample that was spiked after extraction

3.8.6. Performance characteristics of analytical determination

Assessment selectivity was evaluated by comparing extracts of non-spiked *F. graminearum* mutant samples with ones that were spiked with an aurofusarin standard (Matuszewski et al., 2003). The specificity of the method was determined by extracting the mycelium of the aurofusarin deficient mutant with aurofusarin (Bartolomeo & Maisano, 2006). The presence or absence of that

followed the intensity of the aurofusarin specifier signal providing evidence for defining the selectivity and specificity of the method. Calibration curve linearity was tested on the basis of standard concentration that included 3, 6, 12, 25, 50, 100, and 150% of the aurofusarin stock solution. Aurofusarin content was calculated using the equation:

$$y = ax + b$$

Level of detection (LOD) and level of quantitation (LOQ) were calculated as follows (Wenzl et al., 2016):

$$\text{LOD} = 3.9 \times \text{SD (slope)}$$

$$\text{LOQ} = 3.3 \times \text{LOD}$$

4. Results

4.1. Aurofusarin solubility in different solvents

Table 1 summarizes the solubility of aurofusarin in a wide range of polar and non-polar solvents. Aurofusarin dissolved better in non-polar solvents. A mixture of chloroform/methanol (8:2, v/v) proved as the best solvent for aurofusarin (> 3000 mg L^{-1}). Although aurofusarin was readily soluble in chloroform, the high volatility of chloroform at room temperature hampered the precision of the preparation process. Aurofusarin was insoluble in water. Only ultra-sonication and stirring facilitated a dissolution of a limited portion of aurofusarin in water (< 1 mg L^{-1}). Applying a mixture of water with compatible organic solvents enhanced the solubility of aurofusarin in acetonitrile/water/acetic acid mixture (84:15:1, v/v/v). This combination helped to increase the dissolution to 53 mg L^{-1}. Aurofusarin is only sparingly soluble in polar organic solvents such as methanol and ethanol, 16 and 20 mg L^{-1}, respectively. In ethyl acetate, aurofusarin dissolved twice as efficiently. However, acetone and acetonitrile proved to be three times as efficient, dissolving 143 and 170 mg $L^{-1,}$ respectively.

Table 1. The solubility of aurofusarin in different solvents. Values represent means ± SD of 5 replicates

Solvent	**mg L^{-1}**
bidist. water	< 1
ethyl acetate	50 ± 5
ethanol	20 ± 6
methanol	16 ± 4
acetonitrile/water/acetic acid (84:15:1)	50 ± 7
acetonitrile	170 ± 20
acetone	140 ± 35
chloroform	5,500 ± 1,000
chloroform/methanol (8:2, v/v)	4,000 ± 364

4.2. Extraction of aurofusarin with different solvents

Aurofusarin was extracted with nine different solvents from fungal mycelium (Figure 1). Of all the nine solvents and solvent mixtures, chloroform and chloroform/ methanol (80:20, v/v) were the most efficient to extract aurofusarin. Aurofusarin was efficiently extracted with chloroform. However, an apparent variation of aurofusarin ranging from 50−86 mg g^{-1} dry mycelium was observed. Similarly, applying a mixture of chloroform/methanol (8:2, v/v) resulted in yields of > 85 mg g^{-1} dry mycelium, albeit more consistently. On the contrary, the extraction efficiency of cyclohexane was extremely low, about 900-times lower than the yield in chloroform/methanol. Aurofusarin was moderately extractable in the rest of the examined solvents, ranging from 5−20 mg g^{-1} dry mycelium.

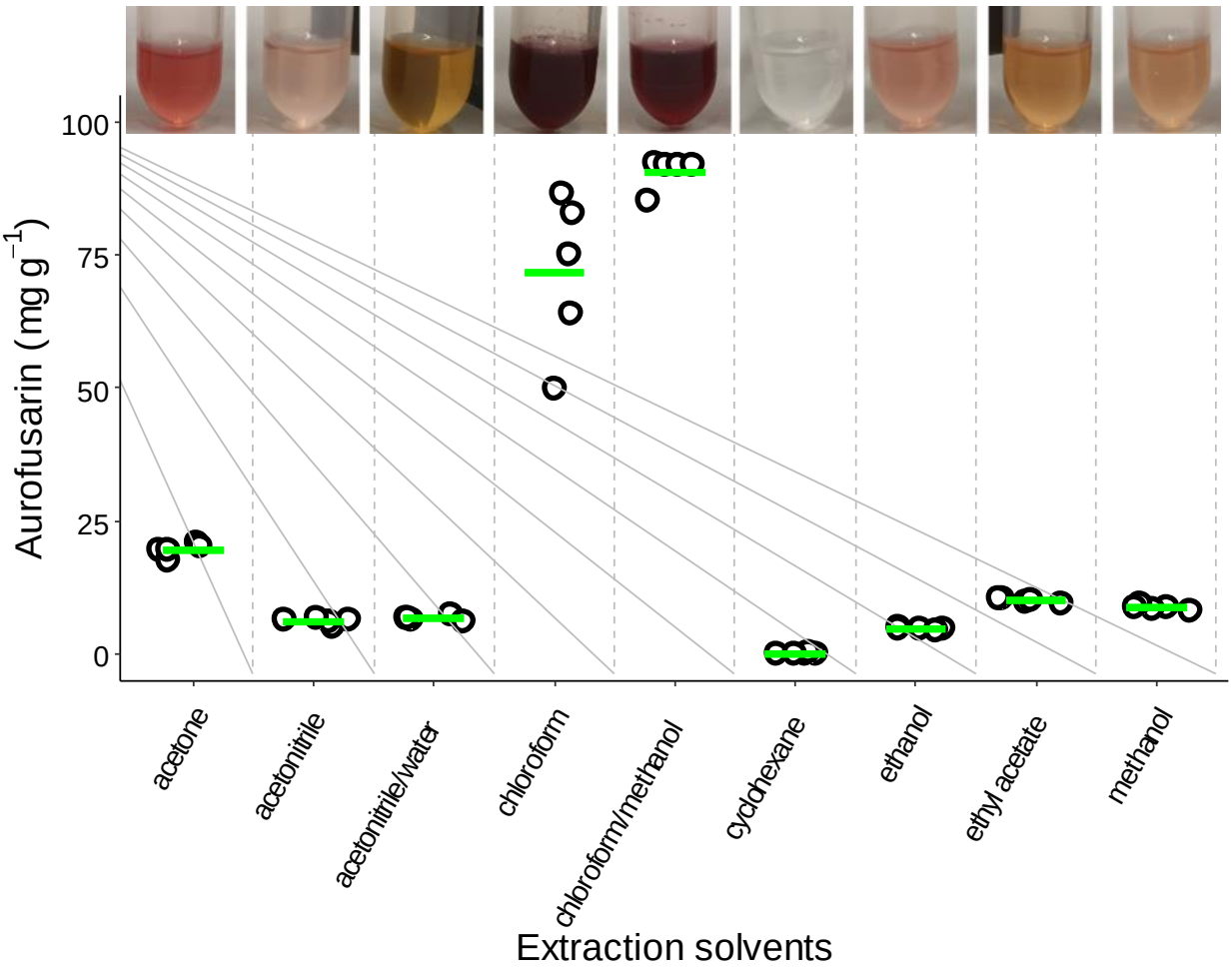


Figure 1. Aurofusarin content in *F. graminearum* IFA66 mycelia that were extracted with different solvents and solvent mixtures (n= 5).

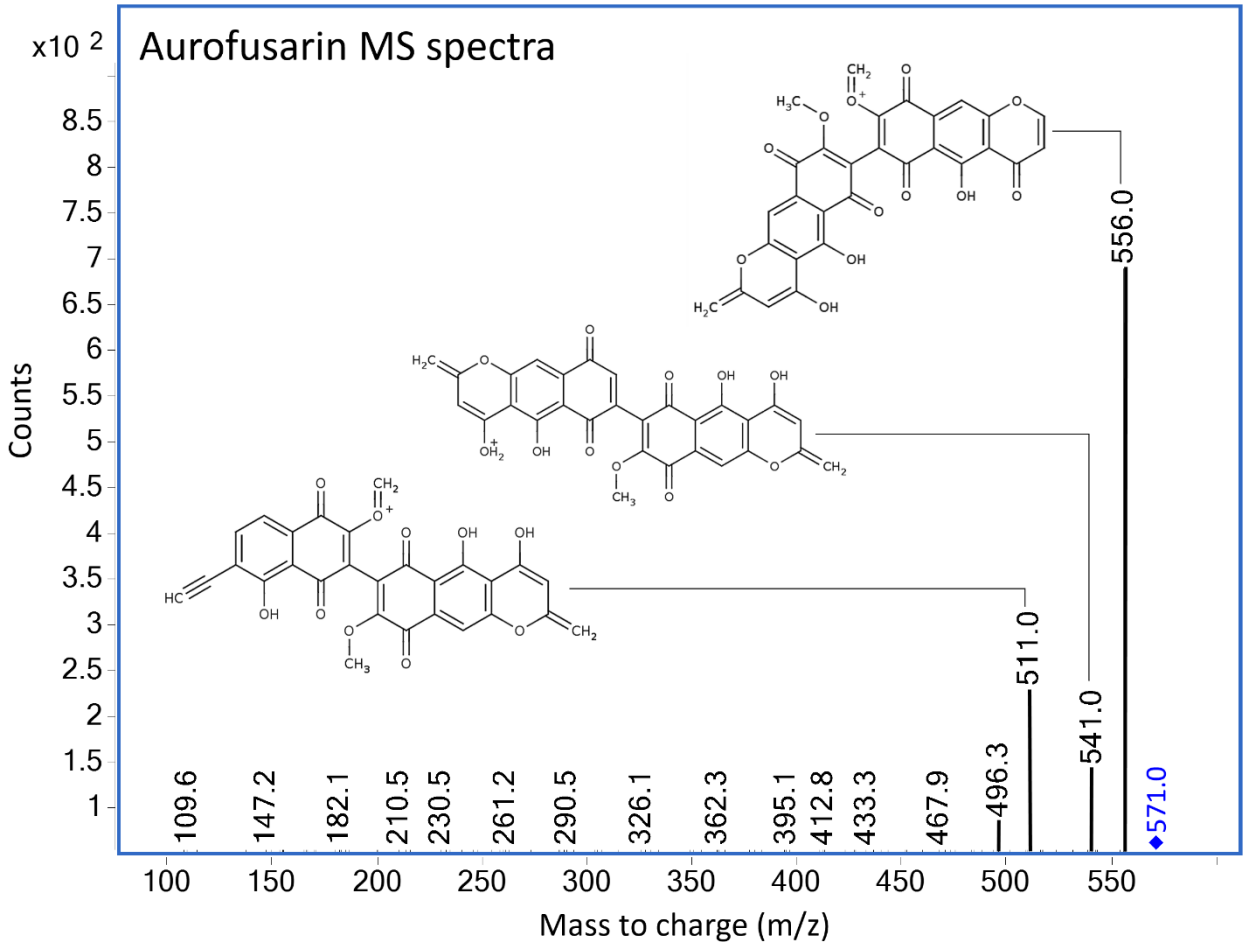


Figure 2. Aurofusarin analysis by LC−QQQ/MS, The chemical structure of the fragments was predicted using CFM-ID 3.0 web tool; for details, see 3.8.2.

The petroleum ether fractions that were used for defatting contained no aurofusarin. The color intensities varied between the extraction repetitions, the first one being the most colorful, the third being more or less colorless, similarly to the petroleum ether fractions. QQQ/MS data supported the observed color intensities (Figure 3).

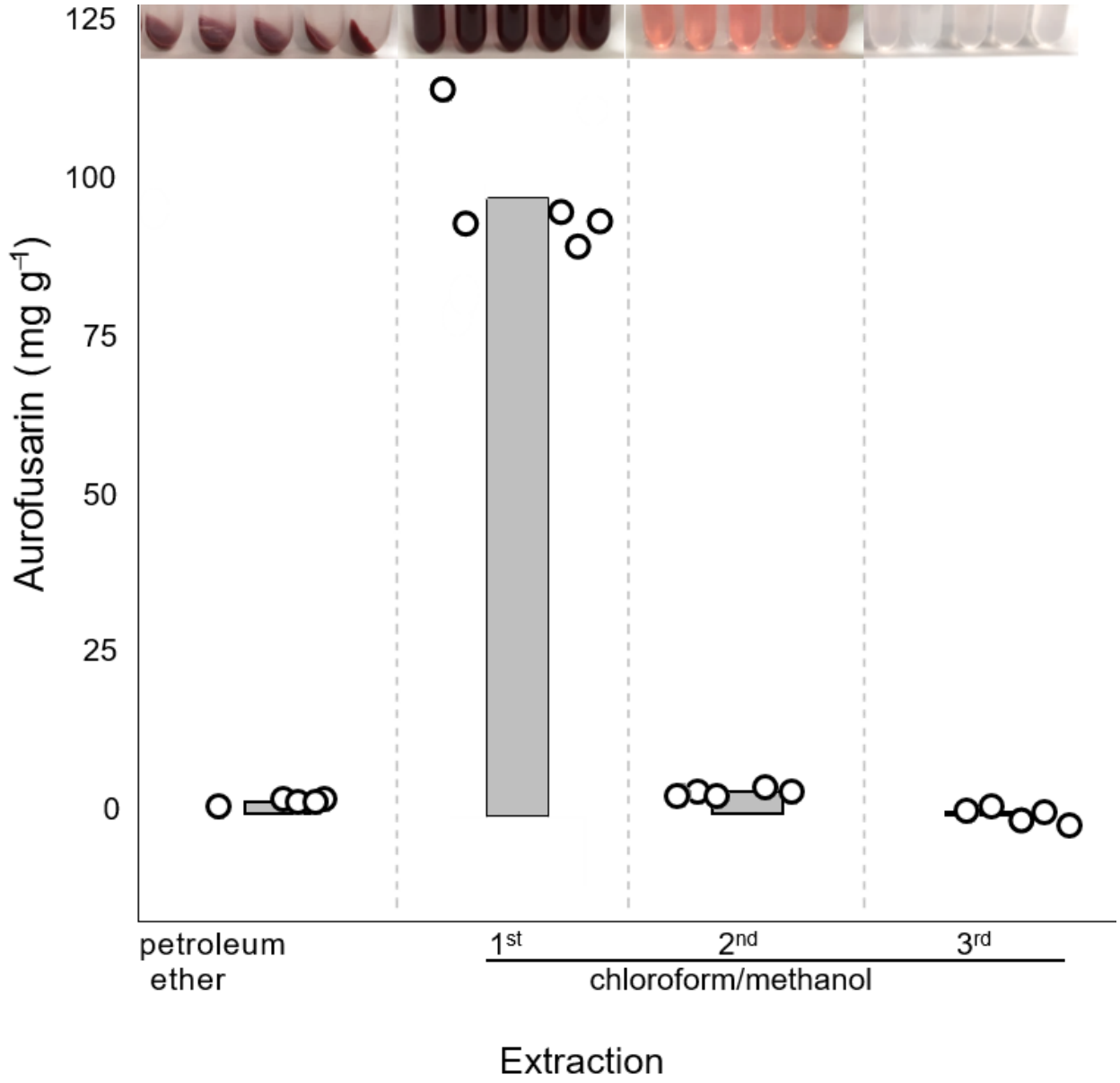


Figure 3. Aurofusarin concentrations in defatting and three consecutive extract fractions. Samples were defatted with petroleum ether, then extracted three consecutive times with chloroform/methanol (8:2, v/v). Aliquots of the extracts were diluted with pure methanol and subjected to LC−QQQ/MS (n = 5).

4.3. Aurofusarin stability

Aurofusarin content in pure methanol, in mixtures of methanol with bidistilled water (1:1, v/v) and ultra-pure water (1:1, v/v) was quantified by LC−QQQ/MS using the MRM mode. Samples were analyzed in time intervals for 30 h at 20°C (Figure 4). In pure methanol, aurofusarin was almost stable. The presence of water in the mixture negatively affected the stability of aurofusarin, both in the case of bidistilled and ultra-pure water. The degradation started directly after sample preparation and showed a continuous exponential decay until the end of the

experiment. A qualitative analysis of the degradation output was performed by LC-TOF/MS after 24 h sample incubation. The total ion chromatogram showed no other product than aurofusarin existed in pure methanol. In contrast, the aurofusarin peak almost disappeared if water was added. Mainly two new decomposition products were generated *m/z* 557.072 and *m/z* 543.055, which corresponded to the sum formulas of $C_{29}H_{16}O_{12}$ and $C_{28}H_{14}O_{12}$, respectively (Figure 5).

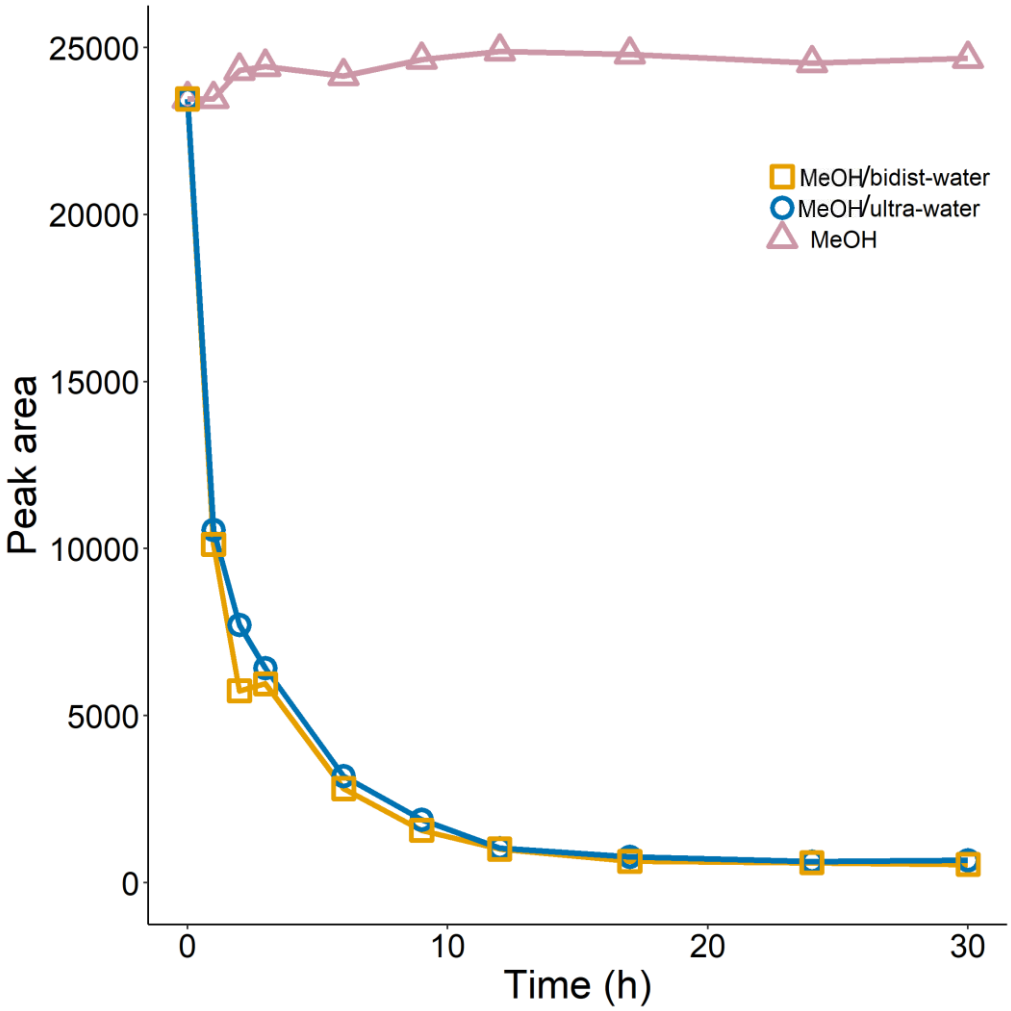


Figure. 4. Degradation of aurofusarin. Aurofusarin stock solution in pure methanol. Aliquots were then diluted in bidistilled water (bidist-water), ultra-pure water (ultra-water), and methanol (MeOH) (v:v). Quantification measurement was performed by LC−QQQ/MS at 20°C with time intervals from 0 to 30 h.

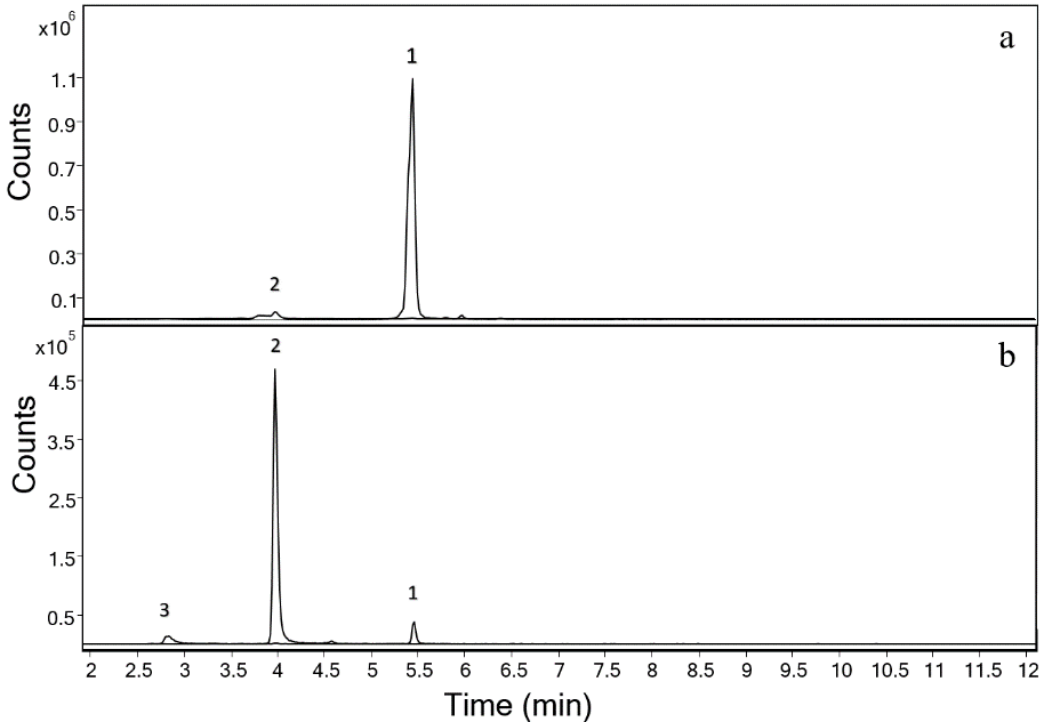


Figure 5. TIC LC−TOF/MS analyses of aurofusarin and its decomposition products. (1) Aurofusarin, $C_{30}H_{18}O_{12}$, *m/z* 571.087; (2) decomposition product $C_{29}H_{16}O_{12}$, *m/z* 557.072; (3) decomposition product $C_{28}H_{14}O_{12}$, *m/z* 543.055; (a) dissolved in pure methanol; (b) dissolved in methanol/water (1:1).

4.4. Assessment of the extraction method and its validation

The efficient extraction method of aurofusarin in chloroform/methanol (8:2, v/v) was further evaluated based on various coefficients (Table 2). The process efficiency indicated that aurofusarin remained more or less stable in the matrix and survived the extraction process. The matrix effect also showed all values close to 100%, suggesting that only negligible interactions with the matrix occurred. Recovery and accuracy also yielded values close to 100 %. The greatest variation between the replicates occurred in set C (spiked fungal extract) and not in set B (spiked mycelium before the extraction), suggesting that the extraction procedure caused no loss of the analyte. A LOQ of 20 ng mL^{-1} was determined (Table 3). The LOD was lower at 7 ng mL^{-1}.

Table 2. Process Efficiency (PE), Matrix Effect (ME) Recovery (RE), Accuracy (%) and Precision (CV%) of the extraction process with chloroform/methanol (8:2, v/v) of aurofusarin-deficient mycelium spiked with 1 mg aurofusarin solution; aurofusarin solution (A), spiked mycelium before the extraction (B), spiked extract (C); n = 5−6.

Precision (CV, %)			Process efficiency (%)	Matrix effect (%)	Recovery (%)	Accuracy (%)
A	B	C				
5	9	15	103	96	107	91

For a detailed description and calculation of coefficients, see 3.8.5

Table 3. LOD and LOQ of aurofusarin in methanolic solution (n = 5).

Slope ± SD	R^2	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
21.9 ± 0.5	0.99	7	20

5. Discussion

5.1. The solubility and extraction of aurofusarin in different solvents

Although some methods to enhance the extraction efficiency of natural pigments have been established (Yang et al., 2013), there is still a need to improve extraction methods for pigments (Lebeau et al., 2017). A recent list summarizes fungal pigments that have been incorporated in the food and textile industry (Narsing Rao et al., 2017; Sen et al., 2019). The discovery of the repellent effect of aurofusarin against the fungivores and its low toxicity to the cell cultures (Xu et al., 2019) may initiate further exploration of aurofusarin in terms of industrial applications. Some chemical and physical features of aurofusarin have been assessed (Gray et al., 1967; Morishita et al., 1968), but the establishment of an efficient extraction method combined with a solubility and stability assessment in various solvents and solvent mixtures lack so far.

Organic solvents, such as methanol, ethyl acetate, acetone, methylene chloride, and chloroform/methanol mixtures were applied to extract several dimeric and mono naphthopyrones from *Aspergillus* spp. (Ehrlich et al., 1984; Li et al., 2016; Song et al., 2004; Xiao et al., 2014). Because of the hydrophobic nature and the dimeric shape, aurofusarin reacts differently in solutions. Although the solubility of aurofusarin is low in many organic solvents, it dissolved well in chloroform, which had been used to extract aurofusarin from several *Fusarium* species in a previous study (Birchall et al., 1966). However, the fact that chloroform is volatilized at room temperature (Estévez & Vilanova, 2014) appeared to negatively affect the extraction process via obtaining a remarkable variation between the samples. Interestingly, adding a small portion of methanol to chloroform had enhanced the extraction efficacy.

It is known that sample defatting can improve extraction efficiency (Johnsson et al., 2000). The insolubility of naphthopyrones in hexane (Ehrlich et al., 1984) and petroleum ether (Ashley et al., 1937; Gray et al., 1967) make these solvents favorable defatting agents. The quantitative analyses revealed that aurofusarin is not soluble in petroleum ether.

Studying the solubility of aurofusarin is important, especially in the context of bioassays. Aurofusarin is known to be insoluble in water (Ashley et al., 1937), and it is only sparingly soluble in polar organic solvents such as ethanol and methanol ($\leq$ 20 mg L^{-1}). Notably, its solubility increased in tandem with the solvent's carbons number. Non-polar solvents have been identified as appropriate to dissolve aurofusarin in former studies (Ashley et al., 1937). In general, nonpolar

solvents with low dielectric constants can dissolve aurofusarin, and the solution turned red. In contrast, the solution of aurofusarin-inpolar solvents appeared pale pink to light red depending on the solvent's properties, keeping in mind the important role of the surrounding temperature and pressure to maintain the solubility constant (Rodrigues et al., 2006).

5.2. Assessment of aurofusarin stability

Dissolution is a kinetic process, and its rate is influenced directly by the solute concentration in a solvent (Jiang et al., 2018). The demonstration of the standard stability during the storage and the analysis period is a prerequisite for validating the chemical analysis (U.S. Food and Drug Administration, 2015). Aurofusarin degraded in aqueous sodium hydroxide, and acetone was generated as a decomposition product (Birchall et al., 1966; Gray et al., 1967). Moreover, it is unstable at room temperature and sensitive to light exposure (Jarolim et al., 2018). During the analytical analysis reducing of the peak areas (Porwal & Upmanyu, 2014) and detecting of new peaks (Westphal et al., 2018) are evidence of the analyte decomposition. Our analyses, however, revealed that aurofusarin is stable in pure methanol. By contrast, it readily degraded if water is involved. Consequently, two decomposition products were detected; their accurate masses are *m/z* = 557.072 and *m/z* = 543.055, matching the chemical sum formulas of $C_{29}H_{16}O_{12}$ and $C_{28}H_{14}O_{12}$. Accordingly, aurofusarin may have lost one and two methylene groups. It was reported that $C_{29}H_{16}O_{12}$ was detected in a Czapek broth, in which *F. graminearum* overexpressed *AurR1* mutant. There is no resembling naphthoquinone natural product that can be found in Reaxys and PubChem databases for this sum formula (Westphal et al., 2018).

5.3. Validation and assessment of the extraction process

Usually, validation procedures commence when method development is accomplished or when a published method is applied for routine analysis (Bratinova et al., 2009). They represent a confirmation that the requirements for acceptable performance of a specific analytical method are fulfilled (Bonfilio et al., 2012). Previously, an ion trap LC−MS MRM method was developed to quantify aurofusarin (Neuhof et al., 2008). Using the same transitions, m/z 571.3 → 556.0 as a quantifier and m/z 571.3 → 511.0 and m/z 571.3 → 541.0 as qualifiers, we developed a method to quantify aurofusarin by QQQ-MS. When the same method parameters are applied, the interlaboratory impact may remain significant and should be considered (Jouyban, 2010, p. 26).

The correct selection of the validation parameters depends on the analytical purpose (Ozkan, 2018). Unanimously approved validation parameters are specificity, linearity, accuracy, precision, range, quantitation limit, and detection limit (U.S. Food and Drug Administration, 2015). Selectivity and specificity provide conclusive parameters for accepting an analytical method (Bratinova et al., 2009). The aurofusarin signal was detected in the presence of co-eluents in the same matrix, which confirms the specificity of the method. The signal was absent in the non-spiked samples confirming the selectivity of the method (Matuszewski et al., 2003). The proportional correlation between responses and corresponding concentrations of the analytical standard was highly linear ($R^2 > 0.997$), which hints at a large linear range (Castillo & Castells, 2001). The LOD and LOQ recommend the start of the applicable concentration range. They were determined by employing the standard deviation of the blank and the calibration curve slope (Wenzl et al., 2016). There are several ways to establish LOD and LOQ. One is visual evaluation, in which the minimum detectable and quantifiable levels determine LOD and LOQ. Another way is the signal-to-noise ratio, 2:1 to assess the LOD and 10:1 for LOQ (Tietje & Brouder, 2010). Despite the fact that LOD and LOQ assessments are ignored in quantification tests (Tietje & Brouder, 2010), they are regarded as important in other tests (Matuszewski et al., 2003). In addition to the previously mentioned criteria, determination of accuracy and precision are conclusive for the method quality assessment. The method accuracy assesses the validated performance of the extraction process and the analytical system. Analysis of aurofusarin solution consecutively proved an acceptable coefficient of variance and provided a guideline for the performance of the analytical system and the detector sensitivity. Differing coefficients of variance in the detector responses indicated a potential but negligible effect of the matrix and/or analyte recovery. Anyway, getting low precision is highly expected when the matrices originate from different sources and when the ion spray interference is taken into consideration (Matuszewski et al., 2003).

A newly established analytical method can be affected by matrix effects (Zhou et al., 2017). Generally, the presence of the co-eluting analytes in the solution can either enhance or reduce the ionization of the target analyte (Ikonomou et al., 1991). In both cases, effects on the analytical performance of the applied method cannot be avoided (George et al., 2018). Matrix effect is an ionization-source-type dependent effect and occurs more prominently in the electrospray ionization (ESI) interface than in other interface types (Matuszewski et al., 2003). Performance of the HPLC system was observed by analyzing aurofusarin in a neat solution (set A) at a concentration resembling the one used for spiking the matrices before (set B) as well as after the extraction (set

C) (Table 2). The matrix effect is ignored at a value of > 100%, but it exists if the value is lower than 100%. For the aurofusarin method, the matrix's suppression effect on the ionization process of aurofusarin is negligible since the value is close to 100% (Zhou et al., 2017). Recovery assessment eliminates the matrix effect and exhibits the true recovery value (Matuszewski et al., 2003). A 60−140 % recovery range is considered acceptable (Bratinova et al., 2009).

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Chapter 4. Bis-naphthopyrone pigments protect filamentous ascomycetes from a wide range of predators

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Author contribution:

Albatol Alsarrag purified the aurofusarin and carried out the HPLC-MS- and HPLC-ELSD analysis.

1. Summary

It is thought that fungi protect themselves from predation by the production of compounds that are toxic to soil-dwelling animals. Here, we show that a nontoxic pigment, the bisnaphthopyrone aurofusarin, protects *Fusarium* fungi from a wide range of animal predators. We find that springtails (primitive hexapods), woodlice (crustaceans), and mealworms (insects) prefer feeding on fungi with disrupted aurofusarin synthesis, and mealworms and springtails are repelled by wheat flour amended with the fungal bis-naphthopyrones aurofusarin, viomellein, or xanthomegnin. Predation stimulates aurofusarin synthesis in several *Fusarium* species and viomellein synthesis in *Aspergillus ochraceus*. Aurofusarin displays low toxicity in mealworms, springtails, isopods, Drosophila, and insect cells, contradicting the common view that fungal defense metabolites are toxic. Our results indicate that bisnaphthopyrones are defense compounds that protect filamentous ascomycetes from predators through a mechanism that does not involve toxicity.

2. Introduction

Soil fungi play a key role in nutrient cycling by degrading recalcitrant plant biomass. Fungal biomass is an attractive source of nutrients for soil invertebrates[1], and predation on fungi disrupts fungal networks[2,3] and modulates the composition[4] and activity[5] of fungal communities, thereby affecting fungal ecosystem services[6]. Because fungi are sessile organisms, their protection from predation consists primarily of chemical defense.

This chemical defence can be mediated by proteins or secondary metabolites. The role of fungal ribosome-inactivating proteins[7], protease inhibitors[8], and lectins[9, 10] in fungal chemical defence has been elucidated at the molecular level. Studies of fungal defence metabolites have a long history, albeit with inconclusive outcomes. In 1977, Daniel Janzen suggested that fungal toxins protect moulded material from consumption by large animals and hinted that the same metabolites may protect infected grain from storage pests[11]. Janzen's ideas led to the hypothesis that mycotoxins protect fungi from predators, and the insecticidal properties of many mycotoxins have since been studied[12, 13, 14]. Apart from their toxicity to insects, circumstantial support for the role of mycotoxins in defence against predators has been drawn from the stimulation of mycotoxin synthesis by arthropod grazing[15] and mechanical injury[16] and from the accumulation of toxic metabolites in fungal reproductive organs[17]. While the ecological function of toxins accumulating in mushrooms (fruiting bodies of basidiomycetes) has been elucidated[18], efforts to substantiate the function of major mycotoxins of filamentous ascomycetes in their defence against predators have remained inconclusive[19, 20]. Mycotoxin gliotoxin facilitates the escape of *Aspergillus flavus* during phagocytosis by a soil amoeba[21]; however, whether gliotoxin protects its producers from animal predators remains unknown. Two polyketides that have not been determined to be mycotoxins have been shown to protect two ascomycetes fungi from animal predation: Asparasone has protected the sclerotia of *Aspergillus flavus* from sap beetles[22], and neurosporin A has protected *Neurospora crassa* from springtail grazing[23]. Nevertheless, there is no indication that these findings can be generalised to related metabolites, other fungal species, or additional predators.

In this work, we investigated the effect of springtail grazing on the transcriptome of the filamentous ascomycete *Fusarium graminearum*. The biosynthesis pathways for several secondary metabolites were induced via grazing. Bis-naphthopyrone aurofusarin was selected for further investigation as similar metabolites are produced by many fungal species. Predation and mechanical damage stimulated aurofusarin synthesis. Mutants of *F. graminearum* with disrupted

aurofusarin synthesis were offered to springtails, isopods, and mealworms, with all predators strongly preferring the mutants over the aurofusarin-producing strains. Food choice experiments with purified aurofusarin, xanthomegnin, and viomellein – which are bis-naphthopyrones produced by *Aspergillus* and *Penicillium* – revealed antifeedant effects of all three metabolites in mealworms and springtails. Toxicity assays with mealworms, springtails, Drosophila larvae, and insect cell cultures revealed a low toxicity of aurofusarin to arthropods. These results suggest that fungal bis-naphthopyrone pigments – which are widespread among ascomycetes – protect fungi from predators by exerting antifeedant effects on a wider range of phylogenetically distant arthropods.

3. Results

3.1. Predation stimulates the synthesis of aurofusarin

Assuming that defense metabolites are synthesized on demand, the transcriptome of the fungus *Fusarium graminearum* that had been exposed to the springtail *Folsomia candida* was studied to reveal which biosynthetic pathways are induced by grazing. RNA was extracted from fungal cultures after grazing, and the mRNA levels of all genes were determined by sequencing (RNAseq). Grazing was found to stimulate the transcription of pathways for the metabolites aurofusarin, fusarin C, and fusaristatin A (Fig. 1 and Supplementary Fig. 1b, c), additional gene clusters that are putatively involved in secondary metabolism (Supplementary Fig. 2), and 7 genes that encode small proteins (Supplementary Fig. 1a). Pathways for the mycotoxins deoxynivalenol and zearalenone – which are toxic to insects[13, 14] – and for necrosis and ethylene-inducing peptide-like proteins – which we hypothesised to be defense agents owing to their similarity to lectins[24] – were not induced by grazing. The RNAseq data are accessible under E-MTAB-6939 at ArrayExpress, EMBL-EBI (www.ebi.ac.uk/arrayexpress), and their analysis for 17,310 genes of *F. graminearum* can be found in Supplementary Data 1.

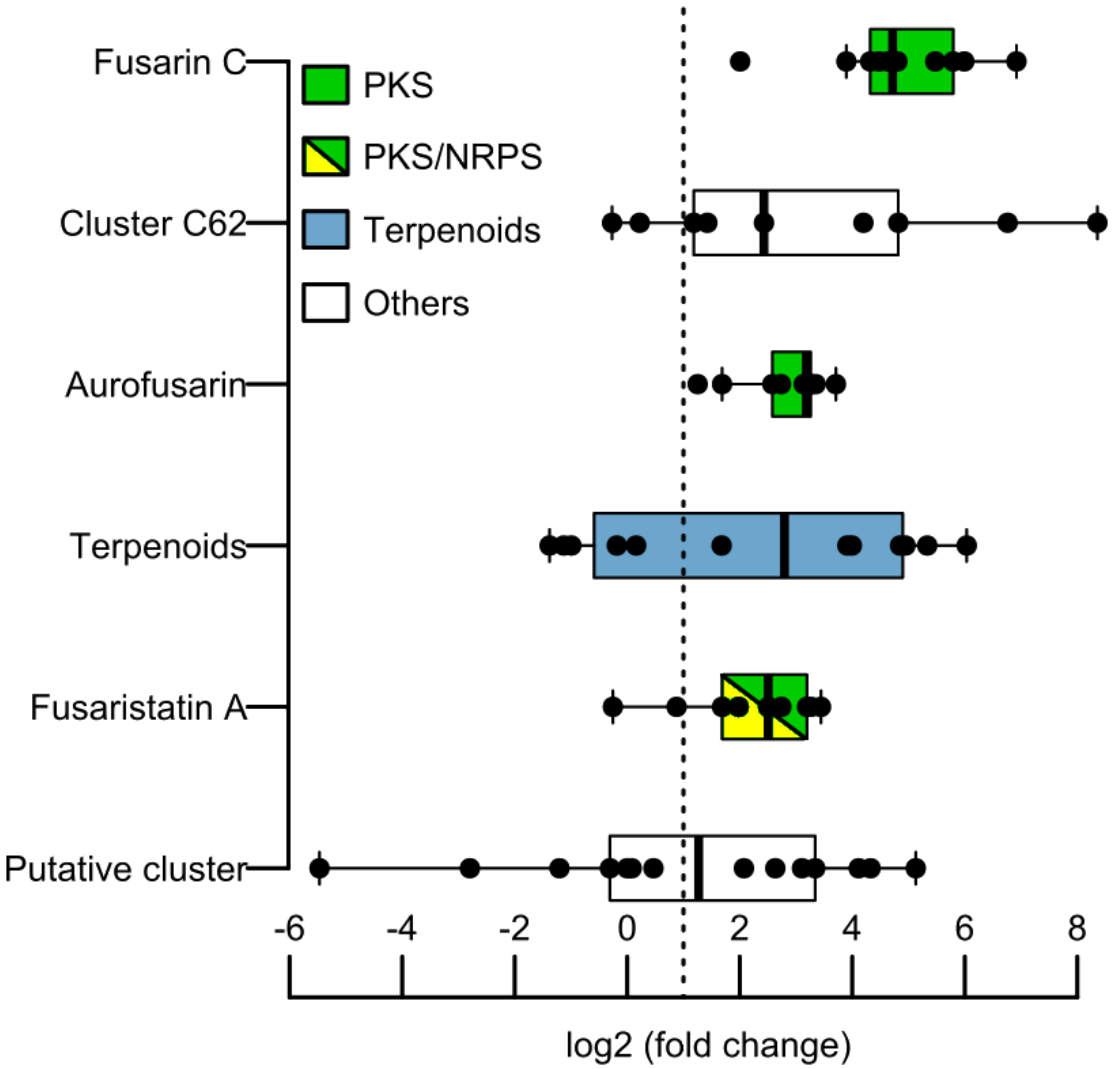

Fig. 1 Secondary metabolite pathways upregulated by grazing in *F. graminearum*. *Fusarium graminearum* IFA66 was exposed to the springtail *Folsomia candida* for 48 h. RNA was extracted, and the levels of individual mRNAs were determined by RNA sequencing (RNAseq). Black points show log2(fold change) values for each gene in grazed versus control cultures. Upregulated gene clusters are defined as having > 50% of all genes and/or having the gene that encodes a signature enzyme be significantly induced (the log2(FPKM) was higher than 1.0 (dotted line), and the q value was lower than 0.01). Accession numbers: fusarin C (FGSG_07798, 07800–07805, and 13222–13224), cluster C62 (FGSG_10606, 10608, 10609, and 10611–10614, 10616, and 10617), aurofusarin (FGSG_02320–02329), terpenoids (FGSG_01737–01749), fusaristatin A (FGSG_08204–08210, 08213, and 08214), and putative cluster (FGSG_10557–10560, 10562–10567, 10569–10571, and 10573). Four biological replicates were used. Box plots show the median and interquartile range. Whiskers indicate the largest and smallest observation or 1.5-fold of the interquartile range, whichever is smaller or larger, respectively (Q1–1.5 × (Q3–Q1) or Q3 + 1.5 × (Q3–Q1)). Source data are provided in a Source Data file.

Aurofusarin was selected for further work because it is produced by many fungal species[25,26] and because metabolites of similar structures are produced by many genera of ascomycetes[27] (see below). Aurofusarin is a red pigment known from maize ears infected with *F. graminearum* (Fig. 2a) and pure cultures of the fungus (Fig. 2b). It belongs to dimeric naphtho-γ-pyrones (Fig. 2f). Springtail grazing stimulated the transcription of all genes of the aurofusarin cluster except one (Fig. 2c). To examine whether aurofusarin synthesis was also induced by other predators, *F. graminearum* and *F. culmorum* were subjected to feeding by the springtail *F. candida* and the nematodes *Aphelenchoides saprophilus* and *Bursaphelenchus mucronatus* for different time periods, and relative mRNA levels for five genes of aurofusarin synthesis[28] were estimated by RT qPCR (Fig. 2g–j). Predation induced the aurofusarin pathway in all fungus/animal combinations. The estimation of the aurofusarin concentration in *F. graminearum* and *F. culmorum* cultures that had been subjected to springtail grazing by high-performance liquid chromatography (HPLC) with light absorption detection (HPLC-DAD) revealed that aurofusarin accumulation was simulated by grazing and that aurofusarin in grazed mycelia amounted to up to 2.5% of the dry weight (Fig. 2d, e). We were not aware of any non-polymeric secondary metabolite that accumulates in fungal mycelia at such a level, and we therefore determined the aurofusarin content in the mycelia of five *Fusarium* species grown in liquid cultures by HPLC with mass spectrometric detection (HPLC-MS/MS) (Supplementary Fig. 3). Aurofusarin levels of 1–7% of dry weight were found in four *Fusarium* species. Because both HPLC-DAD and HPLC-MS rely on aurofusarin standards, which are notoriously unstable (see Methods), extracts of six *F. venenatum* cultures were re-analysed via HPLC with evaporative light-scattering detection (ELSD) for additional verification. ELSD is less accurate than DAD or MS yet does not require aurofusarin standards. The analysis confirmed the high levels of aurofusarin in fungal mycelia.

To determine whether aurofusarin synthesis is stimulated by predation in other *Fusarium* species, cultures of *F. poae*, *F. venenatum*, and *F. avenaceum* on solid media were subjected to grazing by the springtail *F. candida*, and *F. venenatum* and *F. sporotrichioides* were subjected to grazing by the woodlouse Porcellio scaber (Supplementary Fig. 4). Mycelia of *F. venenatum*, *F. sporotrichioides*, and *F. avenaceum* turned red in areas exposed to predation, indicating that the predation had stimulated aurofusarin synthesis.

3.2. Aurofusarin deters a wide range of predators from feeding

A key characteristic of defence metabolites is that they suppress predation. To test whether aurofusarin protected its producers from predation, *F. graminearum* accumulating aurofusarin and genetically engineered strains that were unable to produce aurofusarin were simultaneously offered to predators in food choice experiments (Fig. 3). Predators representing distant arthropod lineages were used: the collembolan *F. candida* (primitive arthropod), the woodlouse *Trichorhina tomentosa* (crustacean), and the mealworm *Tenebrio molitor* (insect). Aurofusarin-producing and nonproducing cultures were placed onto opposite sides of Petri dishes, and the number of animals feeding on each culture was monitored. All predators displayed a strong preference for mutants that did not produce aurofusarin (Fig. 3). Within 1 hour, most mealworms had gathered on the cultures without aurofusarin, where they remained until the end of the experiment. The springtails and woodlice gradually gathered on cultures of non-producers; as shown in Fig. 3, after 8 h most animals were feeding on cultures without aurofusarin.

The disruption of biosynthetic pathways for the mycotoxins deoxynivalenol and zearalenone in *F. graminearum* had no effect on food preference (Supplementary Fig. 5), though both mycotoxins are toxic to insects[13,14]. The reversal of the springtails' food preference for *F. verticillioides* over *F. graminearum* via the disruption of aurofusarin synthesis in *F. graminearum* (Supplementary Fig. 5) indicates that aurofusarin had served as the major—or only—defence metabolite of *F. graminearum* deterring the springtails in this experiment. The disruption of the biosynthetic pathway for aurofusarin synthesis may indirectly affect the synthesis of other metabolites[25,29] which may include unknown attractants. To clarify whether indirect effects of the disruption of the aurofusarin pathway may account for the arthropods' preference for fungi in which aurofusarin does not accumulate, mealworms were offered wheat flour amended with purified aurofusarin and unamended flour (Fig. 4). The larvae's strong preference for flour without aurofusarin revealed that aurofusarin possesses antifeedant activity and efficiently deters predators at a concentration similar to its concentration in fungal mycelia upon grazing (Fig. 2d, e). The exclusion of light in these experiments helped ensure that the animals not recognise aurofusarin by its colour.

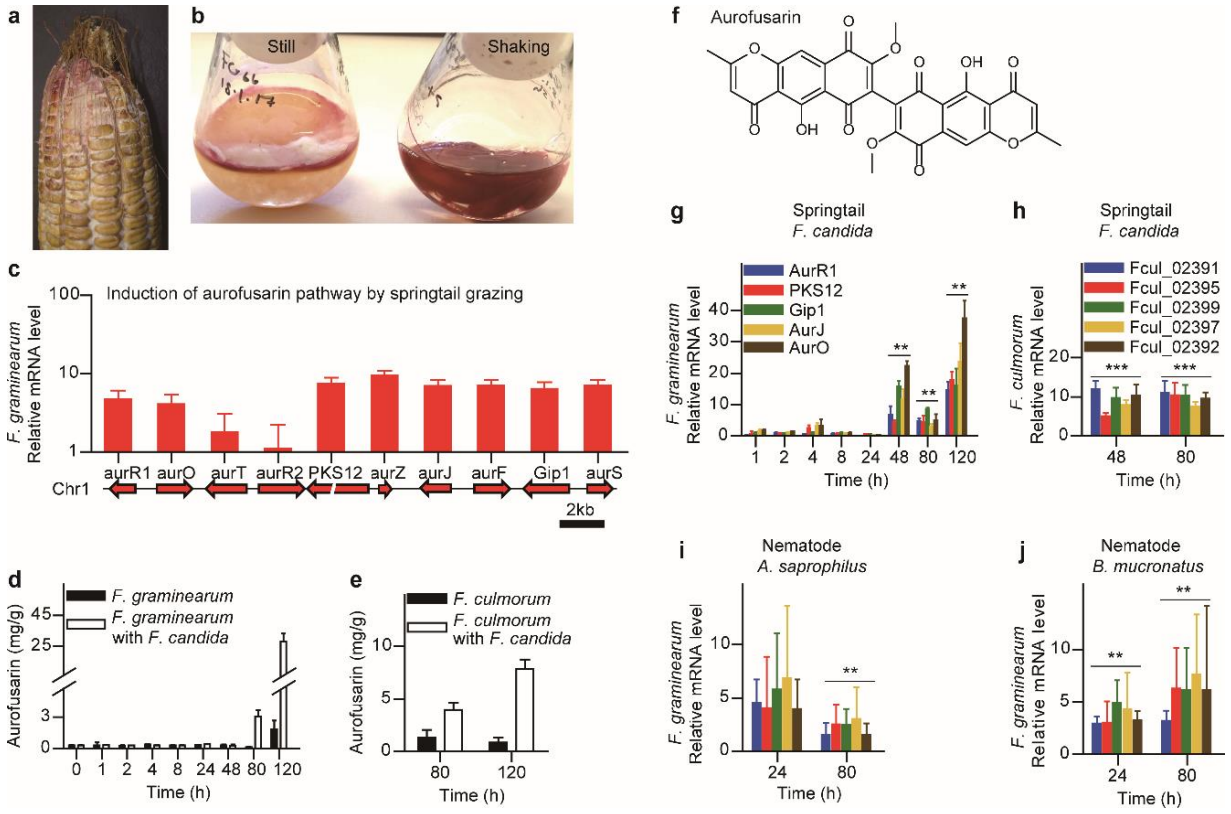

Fig. 2 Aurofusarin synthesis in *Fusarium* spp. is stimulated by predation. a An ear of corn inoculated with *F. graminearum* showing red pigment aurofusarin (courtesy of Dr Belinda J. van Rensburg, ARC South Africa). b *F. graminearum* cultures in potato broth. c Upregulation of genes of aurofusarin biosynthesis in *F. graminearum* after exposure to grazing by the springtail *Folsomia candida* for 48 h (RNAseq; n = 4; see Fig. 1 for details). d, e Aurofusarin accumulation in *F. graminearum* and *F. culmorum* exposed to grazing by the springtail *Folsomia candida* (n = 4). f Structure of aurofusarin. g, h Upregulation of genes of aurofusarin synthesis in *F. graminearum* and *F. culmorum* after grazing by *Folsomia candida* (RT qPCR). i, j Upregulation of genes of aurofusarin synthesis after exposure to the fungivorous nematodes *Aphelenchoides saprophilus* and *Bursaphelenchus mucronatus* (RT qPCR). The gene cluster was labelled as significantly induced when mRNA levels of at least three genes increased at least threefold and the increase was statistically significant (***P* < 0.001, ****P* < 0.0001, two-tailed t test) with both reference genes (glyceraldehyde-3-phosphate dehydrogenase and elongation factor 1a). Error bars show s.e.m. Three to four biological replicates were used in RT qPCR (see Supplementary Data 3). Source data are provided in a Source Data file

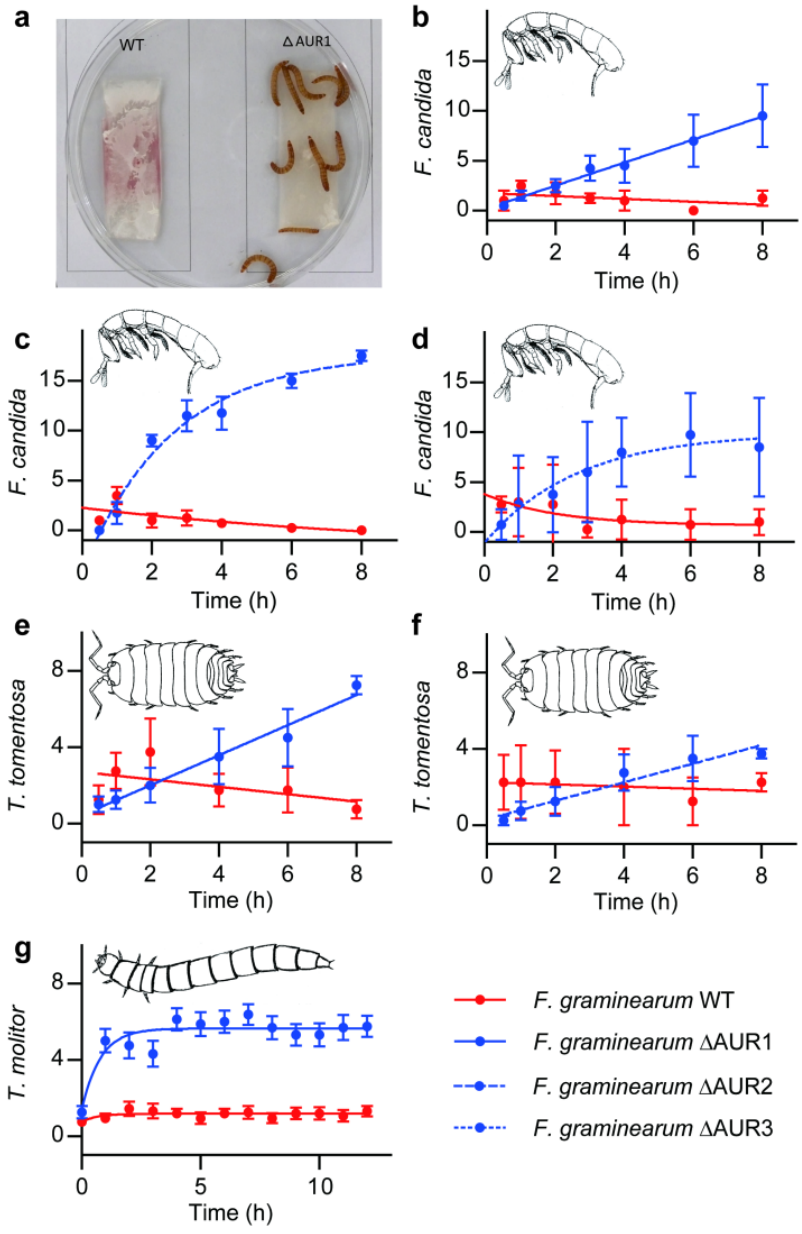

Fig. 3 Predators avoid fungal cultures in which aurofusarin accumulates. a Mealworms in a Petri dish with cultures of *Fusarium graminearum* producing aurofusarin (WT) and a nonproducing mutant (ΔAUR1). b–d The food preferences of the springtail *Folsomia candida* for *F. graminearum* WT and aurofusarin-nonproducing mutants were studied by placing springtails that had been starved for 2 days into the centre of a Petri dish containing fungal cultures and by counting the animals feeding on each culture (20 animals per plate; four replicates). e–f The food preference of the isopod *Trichorhina tomentosa* was tested in the same manner with eight animals per arena and four replicates. g The food preference of the mealworm *Tenebrio molitor* was examined by placing larvae into Petri dishes containing fungal cultures on microscope slides, as shown in a. Sixteen replicates with 10 animals per plate were used. Error bars show 95% CI. Source data are provided in a Source Data file

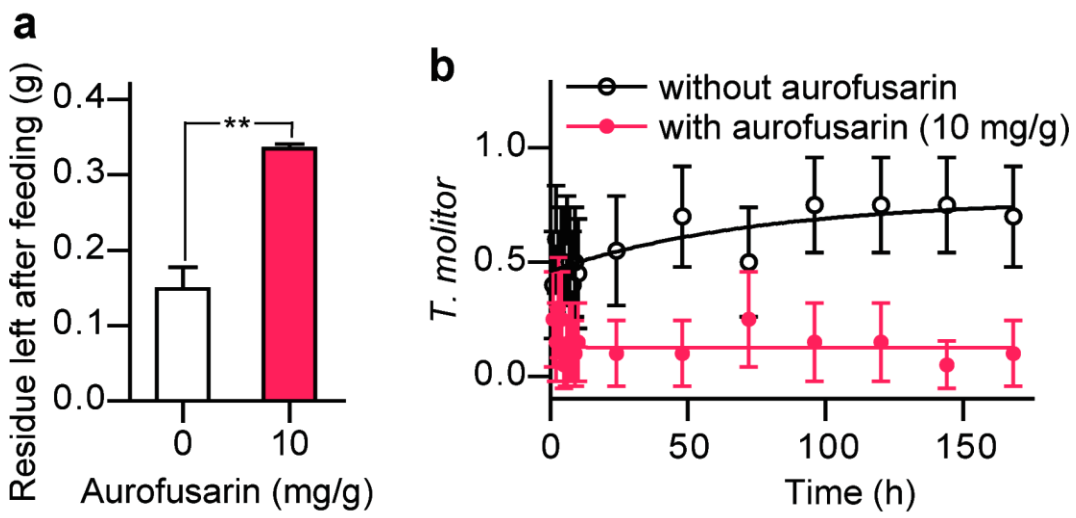

Fig. 4 Aurofusarin in wheat flour repels mealworms. a The weight of the wheat flour and faeces left by five larvae of *T. molitor* after 4 d of feeding on 0.4 g of wheat flour with and without 10 mg g^{-1} of aurofusarin. The significance of the difference was analysed with unpaired two-tailed t test (n = 3, p = 0.0033). b Single mealworms were placed on Petri dishes between two portions of 100 mg of wheat flour, one of which was amended with 10 mg g^{-1} of aurofusarin. The arenas were kept in total darkness and opened only for a second in dim light to record the mealworm location (n = 20, error bars show CI 95%). Source data are provided in a Source Data file

3.3. Aurofusarin is not toxic to arthropods

Why do predators avoid aurofusarin-accumulating fungi? The avoidance of food containing toxins is an adaptation that reduces toxic exposure[7]. Aurofusarin has been reported to be toxic in poultry[30], but metabolites other than aurofusarin might have been responsible for the effects described in this work because the poultry feed used in these trials had not been amended with pure aurofusarin, but rather with a culture of a fungus known to be a potent producer of mycotoxins. To determine whether aurofusarin is toxic to insects, mealworms were fed wheat flour amended with aurofusarin for 10 d, and their weight gain was determined. Aurofusarin at concentrations of up to 1 mg g^{-1} did not affect the mealworms' growth (Fig. 5a). This concentration is two to three orders of magnitude greater than concentrations at which mycotoxins display toxicity in insects[13, 14, 31]. At 10 mg g^{-1}, aurofusarin suppressed the mealworms' growth. At this concentration, aurofusarin substantially reduced feed intake (Fig. 4a), and we therefore assume that the suppression of mealworms' growth on flour with 10 mg g^{-1} of aurofusarin was caused by reduced feeding rather than toxicity.

To test the developmental toxicity of aurofusarin on an arthropod that did not feed on filamentous fungi, we fed larvae of *Drosophila melanogaster* with food amended with aurofusarin at the same level as in the previous experiments as well as at a lower level of 2 mg/g for two days. This feeding was followed by a transfer to a medium without aurofusarin to accomplish the development (Fig. 5b). No differences in the number of adults emerging from pupae in feeding trials with and without aurofusarin were found, which indicated that aurofusarin did not cause developmental toxicity in *D. melanogaster*.

The effect of aurofusarin on the growth and mortality of the springtail *Folsomia candida* and the woodlice *Trichorhina tomentosa* was studied by monitoring the mortality of animals fed on *F. graminearum* and its aurofusarin-nonproducing mutant for 5 weeks, and the size of the animal bodies was estimated at the end of the experiment. The results are shown in Tab. 1. Forced feeding on *F. graminearum* cultures in which aurofusarin had accumulated did not cause any mortality in the springtail *F. candida* or in the isopod *T. tomentosa*. The growth of animals fed on mycelia with aurofusarin was reduced as compared with aurofusarin-nonproducing mutants, but the effects were small, indicating that reduced feed consumption rather than toxicity was the cause. The lack of mortality during five weeks of feeding on aurofusarin-containing mycelia corroborates the lack of toxicity of aurofusarin in springtails and isopods.

Table 1 Growth of predators fed aurofusarin mutants of Fusarium graminearum

Predator	*F. g.*	n	Length (mm)	Rel. length	P-value	Width (mm)	Rel. width	P-value
F. candida	WT	56	0.89 ± 0.13	100%	-	0.20 ± 0.03	100%	-
	ΔAUR1	21	1.08 ± 0.20	121%	< 0.0001	0.23 ± 0.06	123%	< 0.0001
	ΔAUR2	35	1.06 ± 0.23	119%	< 0.0001	0.23 ± 0.06	122%	< 0.0001
	ΔAUR3	35	0.94 ± 0.15	105%	0.12	0.21 ± 0.04	112%	0.0085
T. tomentosa	WT	10	1.47 ± 0.13	100%	-	0.62 ± 0.07	100%	-
	ΔAUR1	12	1.81 ± 0.10	121%	< 0.0001	0.69 ± 0.09	112%	0.048
	ΔAUR2	13	1.76 ± 0.09	117%	< 0.0001	0.70 ± 0.05	114%	0.0029
	ΔAUR3	14	1.82 ± 0.15	122%	< 0.0001	0.73 ± 0.06	119%	< 0.0001

The animals were fed on *F. graminearum* for 5 weeks. *F. candida*, springtail *Folsomia candida*; *T. tomentosa*, isopod *Trichorhina tomentosa*; Rel. length, body length relative to animals fed on WT; Rel. width, body width relative to animals fed on WT. Size at the beginning of trial: *F. candida* length 0.48 ± 0.08 mm, width 0.11 ± 0.02 mm; *T. tomentosa* length 1.20 ± 0.08 mm, width 0.50 ± 0.05 mm. Length and width are shown as mean ± s.d. p values were determined using a two-tailed t test. Source data are provided in a Source Data file

The low toxicity of aurofusarin in mealworms, Drosophila, isopods, and springtails could be accounted for by inefficient absorption, detoxification in the digestive tract, or fast clearance. Toxicity assays with cell cultures circumvent these effects, and we therefore investigated the effect of aurofusarin on a cell culture of the fall armyworm, *Spodoptera frugiperda*, which is an established toxicity model for insects[14]. Aurofusarin also exhibited relatively low toxicity in insect cells (Fig. 5c). The low toxicity of aurofusarin contradicts the hypothesis that fungal defence metabolites are toxic to predators[12, 13, 14, 15, 19, 20].

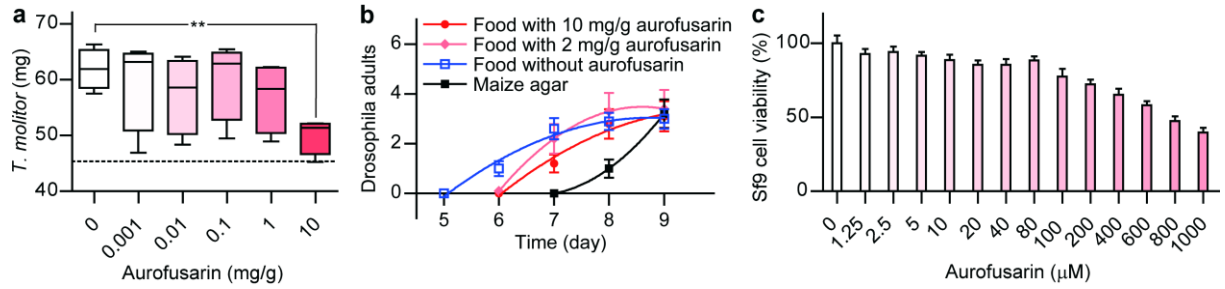

Fig. 5 Toxicity of aurofusarin to arthropods. a The weight of T. molitor after 10 d of feeding on wheat with different concentrations of aurofusarin. The average initial weight is indicated by the dashed line. Means and SD of 4 replicates, each with 10 animals, are shown. The effect of aurofusarin at 10 mg g^{-1} was analysed with two-sided t tests (10 individuals per experiment, n = 4, p = 0.0027). b The developmental toxicity of aurofusarin was tested by feeding larvae of *Drosophila melanogaster* on food with and without aurofusarin for two days, followed by incubation on standard food to accomplish the development (10 larvae per arena, 10 arenas per treatment, error bars show s.e.m.). Maize agar was used to simulate starvation. c The viability of Sf9 cells (fall armyworm *Spodoptera frugiperda*) after 24 h of incubation with aurofusarin (n = 3) shown as means with error bars showing s.e.m. The coloration of data points and bars indicates aurofusarin concentration in flour. Source data are provided in a Source Data file

3.4. Mechanism of the induction of aurofusarin synthesis by grazing

Increased levels of aurofusarin in shaken cultures (Fig. 2b) and in mycelia that had been exposed to a wide range of predators with different feeding modes (Fig. 2d, e, i–j) indicate that mechanical damage alone stimulates the synthesis of aurofusarin in *F. graminearum*. To test this hypothesis, we injured the mycelium of *F. graminearum* using an array of razor blades and monitored the aurofusarin content in the mycelia (Fig. 6). The results confirmed that mechanical damage was sufficient to induce aurofusarin synthesis in *F. graminearum* and showed that the effect was local (remaining confined to damaged parts of mycelia) and that the accumulation of increased levels of aurofusarin continued for at least 120 h after the injury. The mycelia of *F. graminearum* and *F. culmorum* in shaken liquid cultures accumulated more aurofusarin than still cultures, indicating that shaking caused mechanical injury (Supplementary Fig. 3). *F. avenaceum* and *F. poae* accumulated low amounts of aurofusarin in both culture types, but *F. venenatum* produced higher amounts of aurofusarin in still cultures than in shaken cultures, contradicting the results obtained with *F. graminearum* and *F. culmorum*. To clarify the discrepancy, the mycelium of *F. venenatum* that had been growing on an agar medium was injured with a razor blade array (Supplementary Fig. 6). Within 24 h, the injured mycelia turned red, showing that mechanical damage induced aurofusarin synthesis also in *F. venenatum*. As shaking has not stimulated aurofusarin synthesis in *F. venenatum* (Supplementary Fig. 3), it apparently has

not caused damage in this fungus comparable to cutting (Supplementary Fig. 6) or predation (Supplementary Fig. 4b).

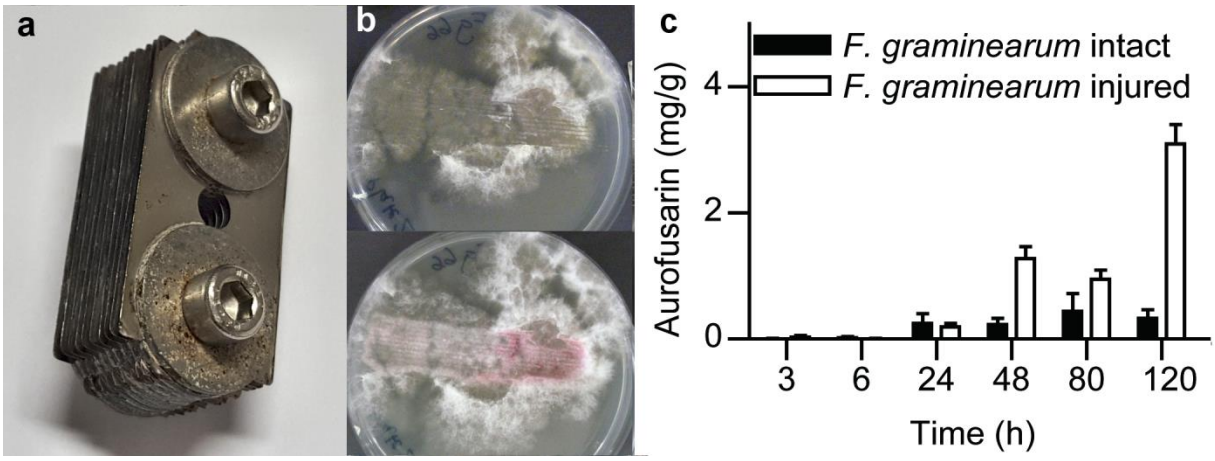

Fig. 6 Mechanical injury induces aurofusarin synthesis. a An array of 10 razor blades assembled at distances of 1 mm. b *Fusarium graminearum* IFA66 on GM7 medium at 22 °C immediately after injury with the razor blade array (upper photo) and 24 h later (lower photo). c *F. graminearum* cultures were grown on rice media for 5 days, injured with the blade array in the same manner as in b, and harvested at the indicated times. The aurofusarin content was determined via HPLC-MS/MS (n = 4, error bars show s.e.m). Source data are provided in a Source Data file

3.5. Further fungal bis-naphthopyrones act as antifeedants

The magnitude of the deterrence effect of aurofusarin and the wide range of predators responsive to the antifeedant indicated that aurofusarin is *F. gramineraum's* major defence metabolite. Dimeric naphthopyrones similar to aurofusarin are produced by many genera of filamentous ascomycetes. Core structures of over 50 such metabolites are shown in Fig. 7. Their biological function is unknown. Viomellein and xanthomegnin—which are produced by many species of *Aspergillus*, *Penicillium*, *Trichophyton*, and other genera—were selected to investigate their inducibility by predation and their antifeedant activity towards arthropods. Induction of the viomellein synthesis by grazing was tested by subjecting cultures of the viomellein producer *Aspergillus ochraceus* to grazing by the springtail *F. candida*. The analysis of extracts of grazed and controlled fungal cultures by HPLC showed that grazing stimulated the synthesis of viomellein in fungal mycelia (Fig. 8a). The deterrent effect of xanthomegnin and viomellein on the springtail *F. candida* was tested by offering baker's yeast spiked with these metabolites to the springtails in food choice experiments. At a spiking level of 10 mg g^{-1}, the springtails strongly avoided yeast

containing either xanthomegnin or viomellein (Fig. 8b, d). After 20 min, hardly any animal was found feeding on food spiked with xanthomegnin or viomellein. The deterrence was less prominent yet still highly significant at a spiking level of 2 mg g^{-1} (Fig. 8c, e). The results showed that the fungal bis-naphthopyrones viomellein and xanthomegnin are antifeedants that exert effects similar to aurofusarin on the springtail *F. candida*.

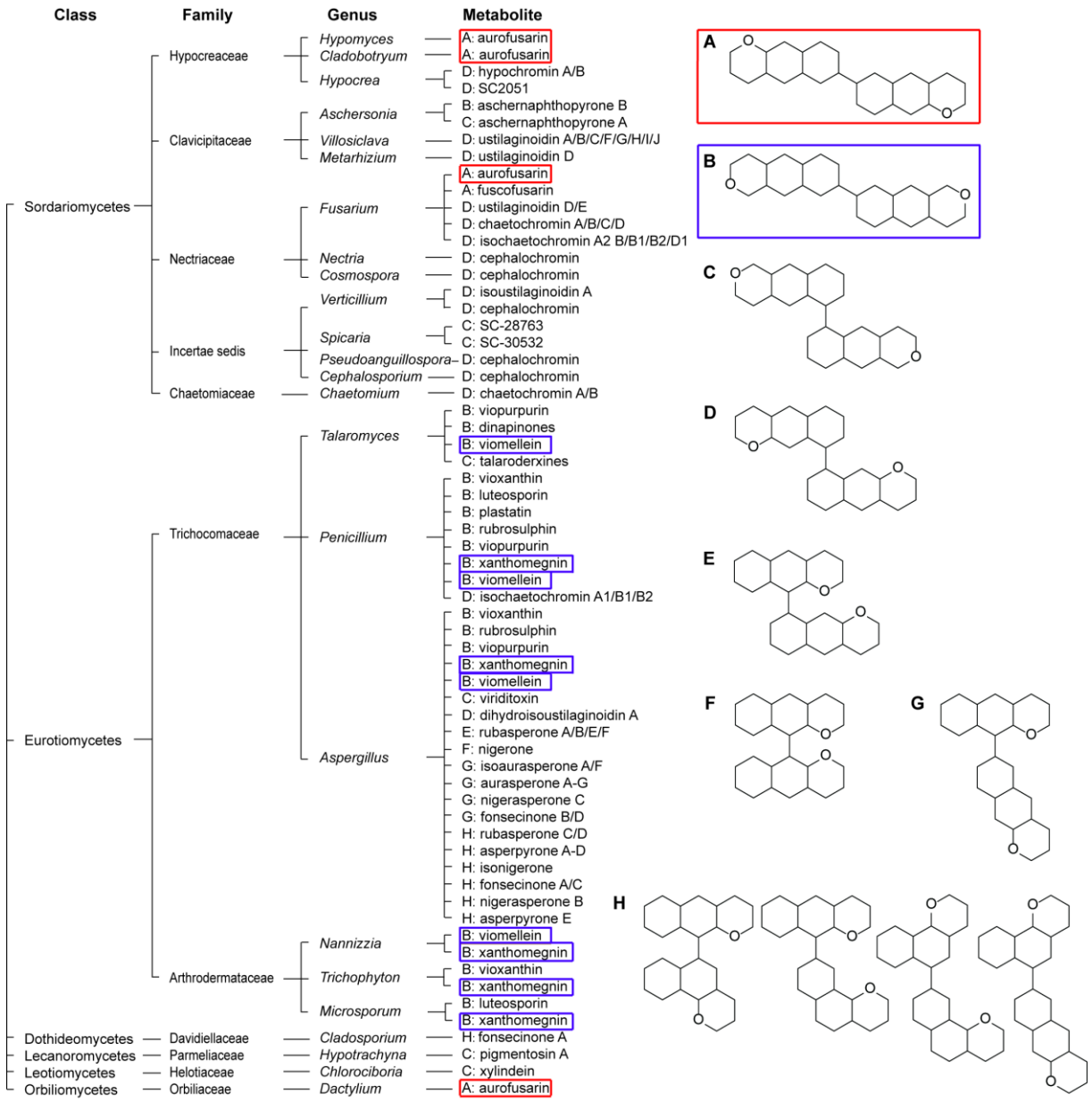

Fig. 7 Production of dimeric naphthopyrones by ascomycetous fungi. The taxonomic affiliation of selected fungal genera that produce dimeric naphthopyrones[27] is shown on the left with schematic structures of bis-naphthopyrones on the right. The Structure Classes A to G contain dimeric naphtho-α-pyrones and naphtho-γ-pyrones consisting of linear heptaketides; the classes differ by the position of the link between monomers and by the presence of either α- or γ-pyrones. The metabolites of Class H contain angular heptaketides, and all metabolites of this class listed here contain γ-pyrones. The metabolites used in this study have been labelled

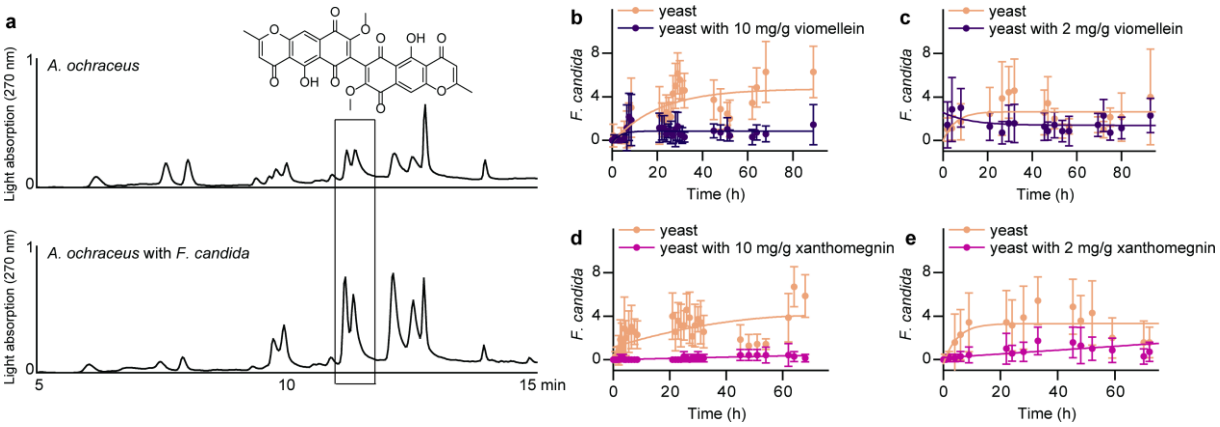

Fig. 8 Antifeedant activity of viomellein and xanthomegnin. a *Aspergillus ochraceus* was grown on rice kernels for 5 d. The springtail *Folsomia candida* was added to fungal cultures and allowed to feed for 6 d. The cultures were extracted with chloroform/methanol (80:20) and analysed via HPLC with light absorption detection. Viomellein appeared as two peaks originating from stereoisomers (atropisomers) and separated owing to restricted intramolecular rotation around the biaryl axis. b–e The food preference of springtails *Folsomia candida* for yeast amended with viomellein or xanthomegnin. The animals were placed into the middle of arenas containing dry yeast on one side and dry yeast amended with viomellein or xanthomegnin on the other side. The number of springtails visiting each diet was recorded in a time series. Seven replicates were used, each with 20 animals. No statistical test was used for the data obtained for 10 mg g^{-1} of bis-naphthopyrones b, d. For the data obtained with 2 mg g^{-1} of bis-naphthopyrones c, e, cumulative values for all time points beginning with 20 h were compared using two-tailed t tests. The preference for yeast without naphthopyrones was highly significant for both metabolites (viomellein: $n = 14$, $p = 0.0054$; xanthomegnin: $n = 10$, $p < 0.0001$). Error bars show 95% CI. Source data are provided in a Source Data file

4. Discussion

Secondary metabolite synthesis in fungi is highly diverse, and most secondary metabolite pathways are species-specific[32,33]. Identical or similar structures are rarely found among secondary metabolites produced by more than two fungal genera. In contrast to other secondary metabolites, dimeric naphthopyrones have been found in all genera of filamentous ascomycetes investigated thus far, suggesting that they fulfil a common and widespread biological function. Our study indicates that this function is defence against animal predators.

Fungal strains defective in secondary metabolism owing to dysfunctional global regulator velvet complex[20,34], which controls secondary metabolism and development[35], and strains with constitutively stimulated secondary metabolism[36] were used in food choice experiments. Predators preferred strains impaired in secondary metabolite synthesis and avoided strains with constitutively stimulated secondary metabolite synthesis, but the pleiotropic character of these mutations prevented identification of metabolites responsible for the effects. Mycotoxin sterigmatocystin was most often implicated in defence, but pathwayspecific mutants failed to confirm its role[20]. Our

results indicate that the metabolites responsible for the loss of protection against predation in fungal strains with globally suppressed secondary metabolism were dimeric naphthopyrones.

If bis-naphthopyrones are defence metabolites ubiquitous among ascomycetes, why was the induction of their synthesis by predation not observed earlier? Transcriptomic studies in fungi have included various sorts of treatments, but surprisingly the effect of predation on fungal transcriptome has not been investigated. The effect of predation on fungal metabolome was addressed in a single study, in which *Aspergillus nidulans* was exposed to grazing by the springtail *F. candida*[15]. The metabolites found to be stimulated by predation did not include naphthopyrones. *A. nidulans* produces naphthopyrone YWA1, which is similar to the aurofusarin precursor rubrofusarin[28] and is dimerised into green pigment[37] in the same way that rubrofusarin is dimerised into aurofusarin[28]. Why was the synthesis of the pigment not stimulated by grazing? The likely reason was that as in most studies of interactions between *A. nidulans* and arthropods conducted thus far, in this study, an *A. nidulans* strain carrying mutation veA1 was used, which is defective in secondary metabolite production and defence responses[38]. VeA is part of the velvet complex, which is needed for aurofusarin synthesis in *F. graminearum*[39]. The metabolites induced by grazing in *A. nidulans* veA1 may belong to a second-tier level of defence, though our results indicate that in F. graminearum, bisnaphthopyrones are mainly—if not only—defence metabolites that target predators.

One *Fusarium* species that does not produce dimeric naphthopyrones is *F. proliferatum*. The absence of antifeedant metabolites in this species is in line with the observation that wheat kernels colonised with *F. proliferatum* attracted rather than repelled the mealworm *Tenebrio molitor*[40]. *F. proliferatum* survives a passage through the digestive system of *T. molitor*, and its propagules continue to be disseminated by faeces of the beetle long after ingestion[41], suggesting that the loss of bisnaphthopyrone synthesis may have been selected in this species during its adaptation to dissemination by insects.

Aurofusarin synthesis in *Fusarium* species was induced by predators with different feeding modes and by cutting mycelia with razor blades, indicating that mechanical damage was sufficient to trigger chemical defence against predation. The potential for the activation of defence responses by shaking or stirring fungal cultures in buffled flasks and fermenters should be considered in physiological studies on fungi in liquid media. The induction of bis-naphthopyrones synthesis in fungi by wounding contrasts with the chemical defence of land plants against herbivory, which requires specific signals in addition to wounding[42]. Defence response induction in plants by

wounding alone would lead to frequent false alarms because plant shoots are often injured by being hit by solid objects in the wind and owing to passing animals, whereas fungal hyphae are protected by the solid substrates inside of which they grow. The second difference between the chemical defence of plants and fungi against predation is that plant defence against herbivory spreads systemically and even reaches neighbouring plants via volatile signals[43], whereas fungal defence remains confined to the area affected by the damage (Fig. 6, Supplementary Figs. 4 and 6).

The presence of defence metabolites of the same structural class that presumably possess the same mode of action in many fungal species is likely to exert a strong selection pressure on the predators. If insects are notorious for their rapid development of resistance to insecticides[44] and mushroom-feeding insects tolerate mushroom toxins[18], why are bis-naphthopyrones still active against a wide range of fungivores that have likely been exposed to these metabolites for hundreds of millions of years? Aurofusarin content in *F. graminearum* that has been exposed to predation is very high (Figs. 2d, e). The maintenance of the high production of aurofusarin that may infer substantial fitness costs —as evidenced by markedly increased growth rates of mutants with disrupted aurofusarin synthesis[25,29]—must have been subjected to a strong selection pressure. We hypothesise that high levels of aurofusarin in fungal mycelia prevented predators from adaptation by saturating molecular targets of aurofusarin with binding affinities reduced by mutations, or by overwhelming enzymatic degradation. Mycotoxins never accumulate in comparably high concentrations, which would probably cause selfpoisoning that even protection mechanisms of mycotoxin producers[45] could not prevent. The synthesis of an antifeedant of low toxicity in large amounts exemplifies a new concept in fungal chemical defence, with aurofusarin, viomellein, and xanthomegnin serving as the first examples. The ubiquity of bisnaphthopyrone pigments in ascomycetes indicates that this defence mechanism is widespread. The defence function of aurofusarin extends and modifies Janzen's 40-year-old hypothesis that fungi protect their substrates from animals via poisonous chemicals[11]. Rather than structurally diverse toxins produced in low amounts, structurally similar antifeedants accumulating at high amounts protect fungi from soil-dwelling predators. The intriguing question for future research is how a single metabolite class deters a wide range of predators. The presence of gustatory receptors triggered by bis-naphthopyrones in phylogenetically distant arthropods, including crustaceans, springtails, and insects, indicates that natural ligands of these receptors are compounds common in food substrates of all arthropods, such as proteins or polysaccharides.

5. Methods

5.1. Animals

The larvae of the beetle *Tenebrio molitor* (Insecta: Coleoptera) and the isopod *Trichorhina tomentosa* (Crustacea: Oniscidea) were purchased from Zoo & Co. Zoo-Busch and b.t.b.e. Insektenzucht GmbH (Schnürpflingen, Germany). The culture of *Folsomia candida* (strain: Berlin) was obtained from the Institute of Zoology, University of Goettingen, Germany, and was kept on Petri dishes filled with a layer of gypsum plaster with charcoal (9: 1). The culture of *Porcellio scaber* was initiated using animals collected in Göttingen, Germany, in spring 2017. The culture of *D. melanogaster* was initiated with animals caught in 2006 in Kiel, Germany[46]. Cultures of the nematodes *Aphelenchoides saprophilus* and *Bursaphelenchus mucronatus* were obtained from Prof. Liliane Ruess, Humboldt University of Berlin, Germany, and Prof. Jiafu Hu, Zhejiang Agriculture and Forestry University, China, respectively.

5.2. Fungal strains

The *Fusarium graminearum* strain IFA66[47] (DON chemotype) was obtained from Dr Marc Lemmens (BOKU, Tull, Austria) via Prof. Thomas Miedaner (University of Hohenheim, Germany). *F. graminearum* 1003 and its aurofusarin-deficient mutants were generated by disrupting the polyketide synthase gene[48] and were labelled △AUR1, △AUR2, and △AUR3. The zearalenone-deficient mutant △ZEN was generated from the same parent by disrupting the PKS gene involved in zearalenone synthesis. The deoxynivalenol-deficient mutant △DON was generated from the strain *F. graminearum* 3211 by disrupting Tri5 gene[49]. *F. culmorum* 3.37[40] was a gift from Prof. Heinz-Wilhelm Dehne (University of Bonn, Germany). *A. ochraceus* 6692 was obtained from Prof. Rolf Geisen, Max Rubner-Institut, Karlsruhe, Germany. *F. avenaceum* BBA92013 was obtained from Prof. Tapani Yli-Mattila, Turku, Finland. *F. tricinctum* RD30, *F. venenatum* RD15, and *F. venenatum* RD90 were isolated from weed samples in Germany and provided by Dr Raana Dastjerdi, University of Goettingen, Germany. *F. poae* DSMZ62376 was obtained from DSMZ (Braunschweig, Germany), and *F. verticillioides* M-8114 was obtained from the *Fusarium* Research Center (University Park, PA, USA).

F. sporotrichioides IPP0249 was obtained from Prof. A. von Tiedemann (University of Goettingen, Göttingen, Germany).

5.3. Fungal media and cultures

The potato broth medium (PDB) was prepared by boiling 200 g of potatoes with the peel in 1 L of tap water for 20 min and autoclaving the filtrate with 20 g of glucose. GM7 medium was prepared as described before[50]. Liquid cultures of *Fusarium* spp. for the analysis of aurofusarin content were grown in 30 ml of PDB in 100-ml Erlenmeyer flasks at 23°C. Rice medium for the analysis of aurofusarin accumulation in *Fusarium* spp. cultures after injury with a razor blade array was prepared by autoclaving a mixture of 1.5 ml of tap water with 0.5 g of rice powder (Alnatura GmbH, Bickenbach, Germany) in glass Petri dishes with a diameter of 5 cm. Rice medium for the investigation of the effect of predation on pigment accumulation was prepared by autoclaving 20 g of rice powder and 20 g of agar in 1 L of tap water and was poured into plastic Petri dishes with a diameter of 9 cm.

5.4. Purification of aurofusarin

Aurofusarin was extracted from *F. graminearum* IFA66 that had been grown in PDB for 2 weeks at 25 °C with shaking at 200 rpm. Fungal mycelium was freeze–dried, ground, and extracted with 50 ml of chloroform/methanol (80:20) per gram of mycelium. The extract was cleared by centrifugation and the solvent removed in vacuum. Aurofusarin was purified by ethanol precipitation from phenol49 at 50 °C followed by crystallisation from glacial acetic acid[50]. The purity of crystallised aurofusarin was verified by HPLC-ELSD (see "Aurofusarin analysis by HPLC-MS/MS and HPLC-ELSD").

5.5. Exposure of *F. graminearum* and *F. culmorum* to predation

For transcriptome analysis, 5,000 fungal spores in 5 µl of water were inoculated onto three rice kernels that had been autoclaved with 200 µl of demineralised water (for arthropods) or onto 40 mg of rice flour that had been autoclaved with 150 µl of demineralised water (for nematodes) in 15-ml Falcon tubes and incubated at 15°C in the dark. After 7 d, 20 mg (around 200 individuals) of the springtail *F. candida* were starved for two days, 2,000–3,000 individuals of the nematode

Aphelenchoides saprophilus or 1,000–2,000 individuals of the nematode *Bursaphelenchus mucronatus* in 100 µl of water were placed into the mycelia, and the incubation was continued. Controls were incubated under the same conditions without animals. Each group consisted of four replicates.

5.6. Transcriptome analysis by RNAseq

F. graminearum IFA66 was exposed to predation by *F. candida*, as described above. After 48 h, four cultures with predators and four control cultures were harvested, the animals were removed, and the total RNA was extracted using the RNAsnap method[53], which was modified as follows: Fungal cultures were suspended in 400 µl of RNA extraction solution [95% deionised formamide, 18 mM EDTA, 0.025% SDS, and 1% 2-mercaptoethanol] and disrupted via shaking with zirconia beads (2.0 mm in diameter, Carl Roth, Karlsruhe, Germany) in the reciprocal mill MM 200 (Retsch, Haan, Germany) for 2 min at maximum power, followed by incubation at 95°C for 7 min. Cell debris was removed by centrifugation at 16,000x *g* for 5 min at room temperature. The supernatant was transferred into an RNA precipitation mixture consisting of 800 µl of isobutanol, 400 µl of 5 M guanidine thiocyanate, and 5 µl of linear polyacrylamide used as a co-precipitant (Co-Precipitant Pink, Bioline, London, UK). The mixture was centrifuged at 16,000x *g* for 5 min at room temperature, and the pellet was washed with 75% ethanol, dried, and dissolved in RNAase-free water.

Strand-specific cDNA libraries were prepared using Illumina's TruSeq stranded mRNA kit (75 bp paired-end) and sequenced on Illumina NextSeq 500V2. Data were analysed using the public server of Galaxy[54] (https://usegalaxy.org/). Before the analysis, the reads were trimmed to remove low-quality sequences. The reads were mapped to the reference genome (*F. graminearum* PH-1) using Hisat2 v2.0.5.1 with the default options for single-end data[55]. Cufflinks v2.2.1[56] was used to determine the abundance of transcripts in FPKM (Fragments Per Kilobase of exon per million fragments mapped): The maximum intron length was set to 1,000 nt, and the last annotated genome (ASM24013v3) was used as a reference. Cuffdiff v2.2.1.3[56] was used to determine the changes in gene expression compared with the control using an FDR (false discovery rate) of 0.05. Only genes with a $\log_2$ FPKM (fold change) higher than 1.0 and a q value lower than 0.01 were considered to have been significantly induced.

To identify upregulated secondary metabolite gene clusters, all upregulated genes were checked in the National Center for Biotechnology Information (NCBI) and in the European Bioinformatics Institute (EMBL–EBI) (UniProt) databases for signatures of polyketide synthetases (PKS), nonribosomal peptide synthetases, and terpenoid synthetases. Once more than 50% of the genes in a cluster and/or the signature enzyme of a cluster had been significantly induced (log2 FPKM (fold change) higher than 1.0 and q value lower than 0.01), the literature related to the cluster was consulted to reveal the associated secondary metabolites. In this manner, gene clusters for the biosynthesis of aurofusarin[55], C62[56,57], fusarin C[56,58,59], fusaristatin A[56,57], and terpenoids[56] were identified. The results were corroborated with the help of AntiSMASH 3.0[60]. The upregulated genes that had been located immediately before and after the signature enzymes were considered part of the cluster. Putative clusters without known signature enzymes were identified using AntiSMASH 3.0[60] and corroborated manually

5.7. Transcription analysis of aurofusarin pathway by RT qPCR

After exposure to *F. candida* grazing for 0, 1, 2, 4, 8, 24, 48, 80, and 120 h (*F. graminearum*) or 80 and 120 h (*F. culmorum*) and after nematode feeding for 24 and 80 h (*F. graminearum*), fungal mycelia were harvested, frozen in liquid nitrogen, and ground. RNA was extracted using the guanidinium thiocyanate-phenol-chloroform method[61], precipitated with 4 M LiCl for 3 h on ice, and reverse transcribed with RevertAid Reverse Transcriptase (Thermo Fisher Scientific, California, USA) and random primers according to the manufacturer's instructions using 400 ng of RNA in 20-µl reactions. The cDNA obtained was used as a template for PCR, which contained ThermoPol Reaction Buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 0.1% Triton-X-100, pH 8.8 at 25°C) with 2.5–4.5 mM $MgCl_2$, 200 µM dNTP, 0.3 µM forward and reverse primers (Supplementary Data File 2), SYBR Green I (Invitrogen, Karlsruhe, Germany), 1 mg ml^{-1} bovine serum albumin, 0.03 U $µl^{-1}$ Taq polymerase (New England Biolabs, UK), and 1 µl cDNA as a template. PCR conditions were as follows: 95°C for 2 min, 35 cycles of 94°C for 20–30 s, 59°C for 30–40 s and 68°C for 30 s, with a final extension of 68°C for 15 min followed by a melting curve analysis beginning at 95°C with a decrement of 0.5°C–55°C. GAPDH-(glyceraldehyde 3-phosphate dehydrogenase) and EFIA (elongation factor 1-alpha) genes were used as an internal reference, and the primers are shown in Supplementary Data File 2. Three to eight biological replicates were analysed (Suppl. File 3). The amplification efficiency for each gene

obtained with the help of serial dilutions was used to calculate relative transcript levels (fold change)[62]. The significance of differences between cultures subjected to predators and controls was determined, as described in "Statistics and reproducibility".

5.8. Analysis of food preference

The preference of the springtail *F. candida* and the isopod *Trichorhina tomentosa* for fungal strains was studied on Petri dishes (92 mm in diameter) filled with a mixture of gypsum plaster and charcoal (9:1). Fungal cultures were grown on potato agar prepared from potato broth (see above) solidified with 15 g of agar L^{-1} and kept at 25°C for 7–8 d in the dark. Agar plagues were cut from the edge of fungal colonies using a sterile cord borer (12 mm in diameter) and placed onto discs of matching size cut from Parafilm and placed on the opposite sides of the Petri dishes. Twenty *F. candida* individuals or 8 *T. tomentosa* individuals that had been starved for two days were placed into the center of the Petri dishes, and the plates were incubated at 15°C in the dark. The number of collembolans and isopods on each mycelium was recorded.

Food choice experiments with the mealworm *T. molitor* were carried out with fungal mycelia as well as with wheat flour amended with aurofusarin. In an experiment with fungal mycelia, *F. graminearum* strains were cultured on glass slides covered with PDA. After 7–8 d at 25°C, the slides were placed on the opposite sides of Petri dishes (150 mm in diameter), and eight *T. molitor* larvae were placed in the middle of the plates. The number of animals visiting each fungal culture was recorded by taking photos at time intervals for 12 h, and animals inside 13 x 6-cm rectangles that had been drawn around each slide were counted. The experiments with wheat flour were carried out with groups of animals and with single animals. Wheat flour (summer wheat variety: Taifun) was amended with aurofusarin that had been dissolved in chloroform to reach a final concentration of 10 mg g^{-1}. After shaking at room temperature for 1 h, the chloroform was removed in a vacuum and the flour was left in an open Petri dish overnight in a fume hood. Flour samples used as a control were treated with pure chloroform in the same manner. Portions of 0.4 g of flour with and without aurofusarin were placed on the opposite sides of Petri dishes. Five larvae of *T. molitor* were added to each of three Petri dishes and allowed to feed for 4 d at room temperature and in ambient light. The animals were subsequently removed, and the weight of the remaining wheat flour with and without aurofusarin (mixed with faecal pellets left by the animals) was determined. The experiments with single animals were carried out with portions of 100 mg of

flour in Petri dishes covered with light-tight plant pot saucer trays. Single three-month-old mealworms (23.9 ± 1.8 mm) were placed into the center of 20 Petri dishes with one portion of flour with aurofusarin and another portion without aurofusarin. The light-blocking trays were opened for two seconds in dim light at fixed time intervals to record the mealworm location (n=20).

Food choice experiments addressing the effect of xanthomegnin and viomellein on *F. candida* were carried out with dry yeast (Dr. Oetker Dry Yeast, Dr. August Oetker Nahrungsmittel KG, Bielefeld, Germany). The yeast was amended with aurofusarin dissolved in chloroform to achieve final concentrations of 2 mg/g and 10 mg/g, chloroform was removed in vacuum and the yeast was left in open Petri dish overnight in a fume hood. Yeast for the controls was treated with pure chloroform. 20 collembolans starved for two days were added to Petri dishes filled with charcoal/plaster (1:9) and that contained 3 mg of dry yeast on one side and 3 mg of dry yeast amended with viomellein or xanthomegnin on the other side and the number of springtails visiting each diet was recorded in a time series.

5.9. Toxicity of aurofusarin to mealworm *Tenebrio molitor*

Wheat flour was amended with aurofusarin dissolved in chloroform, and the solvent was evaporated, leading to aurofusarin concentrations of 0, 1, 10, 100, 1,000, and 10,000 µg g^{-1}. Groups of ten larvae of *T. molitor* were weighed individually and placed in Petri dishes containing 1 g of wheat flour amended with different amounts of aurofusarin. After 10 d at 18°C in the dark, the weight of the animals was determined again. Each treatment consisted of four replicates, and two independent experiments were carried out.

5.10. Toxicity of aurofusarin to insect cells in tissue culture

The *Spodoptera frugiperda* 9 (Sf9) cell line was maintained in a Sf-900 II medium (Thermo Fisher Scientific China, Shanghai, China) and grown at 28°C. The cells were seeded into 96-well cell culture plates with 8×10^3 cells in 100 µl medium per well, incubated for 24 h and treated with 100 µl medium containing aurofusarin dissolved in dimethyl sulfoxide (DMSO) for 24 h with the final DMSO concentration not exceeding 0.1%. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (0.5 mg ml^{-1}) was added to each well, and incubation was continued for 4 h. Formazan precipitate was dissolved in 150 µl of DMSO, and the absorbance of

the solution at 490 nm was determined. Medium containing 0.1% DMSO was used as a negative control. The test was performed in triplicate.

5.11. Developmental toxicity of aurofusarin in Drosophila

Flies were reared at room temperature in BugDorm cages (MegaView Science, Taichung, Taiwan). Eggs were collected in Petri dishes left in the cages and incubated at 22°C in the dark for hatching. Larvae were transferred into 2 ml-Eppendorf tubes containing 160 mg of medium, which were closed with sponge stoppers and incubated in a humid chamber in the dark at 25°C. The full medium consisted of 62.5 g of baker's yeast, 62.5 g of cornmeal, 62.5 g of sucrose, and 12.5 g of agar per litre; the cornmeal medium consisted of 188 g of cornmeal and 12.5 g agar per litre. The medium amended with aurofusarin was prepared by adding a chloroform solution of aurofusarin to a mixture of yeast, cornmeal, and sucrose, followed by removing the chloroform in a vacuum and adding the required amount of 1.25% agar at 60°C. After incubation on the medium with aurofusarin for 2 days (with the full medium and the cornmeal medium used as controls), all larvae were transferred to a fresh medium to accomplish the development, and the flies that emerged from pupae were counted daily.

5.12. Inducibility of aurofusarin synthesis by mechanical injury

To visualise aurofusarin induction by injury, fungal spores were spread onto GM7 medium incubated at 22°C. The developed mycelia were injured with an array of 10 razor blades spaced at 1 mm apart. 24 h later, the aurofusarin accumulation was monitored as red pigment. For HPLC analysis of the aurofusarin accumulation after injury, 10,000 spores of *F. graminearum* IFA66 in 10 µl of water were inoculated into the middle of rice medium in Petri dishes and incubated at 23°C in the dark for 5 d. Mycelium on the plate surface was injured by 5 cuts with an array of 10 razor blades spaced at 1 mm apart. The cultures were kept at 23°C, harvested at a time series, and extracted for aurofusarin analysis.

5.13. Exposure of *Aspergillus ochraceus* to predation

For treatment with the springtails, *A. ochraceus* 6692 was grown on three autoclaved rice kernels, as described for *F. graminearum* under "Exposure of *Fusarium graminearum* and

F. culmorum...". After 5 d at room temperature in the dark, the springtail *F. candida* was added (20 mg, ca. 200 individuals) and allowed to feed for 6 d. For treatments with mealworms, *A. ochraceus* was grown in a medium made by autoclaving 1 g of maize flour with 4 ml of tap water in 100-ml Erlenmeyer flasks. After 5 d at room temperature in the dark, 8 larvae of *T. molitor* were added to each flask and allowed to feed for 6 d. The animals were removed, the cultures were freeze-dried, and viomellein was extracted with chloroform/methanol (80:20) using a 2-ml solvent for rice cultures and a 15-ml solvent for maize cultures. The extracts were cleared by centrifugation and the solvent was removed in a vacuum. The residue was dissolved in DMSO.

5.14. Determination of aurofusarin and viomellein content by HPLC-DAD

Aurofusarin was extracted from freeze-dried cultures using chloroform/methanol (80:20). The extracts were cleared by centrifugation, the solvent was removed in a vacuum, and the residue was dissolved in DMSO. The aurofusarin content was determined by HPLC with a diode-array detector (DAD, Varian Prostar) using a polar-modified C18 column (Polaris Ether, 100 x 2.0 mm; Varian, Darmstadt, Germany) that was kept at 40°C, which was eluted with a gradient of Solvents A (water with 0.05% acetic acid and 5% acetonitrile) and B (methanol with 0.05% acetic acid): 0.1 min 60% B, 11.9 min 60–98% B, 2 min 98% B, 1 min 98–60% B, 8 min 60% B. Light absorption was monitored at 243 nm. The aurofusarin standard turned out to be extremely unstable in protic solvents and under light, as has been observed by other researchers[64]. Stock solutions in DMSO were therefore kept at -80°C. We found that it was crucial to prepare calibration standards in pure methanol instead of using the mobile phase to prevent degradation during the run. Viomellein was extracted in the same manner as aurofusarin. The residue after solvent removal was dissolved in DMSO and analysed by HPLC on a C18 column (Kinetex 2.6 µm, 50 x 2.1 mm, Phenomenex, Aschaffenburg, Germany) that was kept at 40°C, which was eluted with a gradient of solvents A (water with 3% acetic acid and 5% acetonitrile) and B (methanol with 3% acetic acid) as follows: 2 min 40% B, 12 min 40–98% B, 2 min 98–98%, and 1 min 98–40% B, 8 min 40% B. Viomellein was detected by light absorption at 270 nm. Aurofusarin and viomellein standards were purchased from Bioviotica (Göttingen, Germany).

5.15. Aurofusarin analysis by HPLC-MS/MS and HPLC-ELSD

Because the concentrations of aurofusarin in fungal mycelia that were determined by HPLC-DAD turned out to be unprecedentedly high, two other methods were used for confirmation. The first method was HPLC-MS/MS with separation on a polar-modified C18 column (Polaris Ether, 100 x 2.0 mm; Varian, Darmstadt, Germany) that was kept at 40°C, which was eluted with a gradient of Solvents A (water with 0.1% formic acid) and B (methanol) as follows: 0.2 min 40% B, 6.8 min 40–98% B, 2 min 98% B, 1 min 98–40% B, 5 min 40% B. The eluent was ionised by electrospray in a positive mode with a capillary voltage of 4,000 V. The triple quadrupole 6460 (Agilent, Darmstadt, Germany) that was used as a detector was operated in a selected reaction monitoring mode with the transition m/z 571.3->556.0. Aurofusarin standards were prepared shortly before analysis by diluting a stock solution in DMSO with pure methanol (see previous paragraph). As an additional verification method independent of the aurofusarin standard, HPLC coupled with an evaporative light scattering detector (ELSD) was used. The analyte was chromatographically separated (as described above), and the eluent was directed into the ELSD 1260 detector (Agilent, Darmstadt, Germany) operated at a nebuliser temperature of 40°C, an evaporator temperature of 42°C, and an evaporator gas flow of 1.6 standard litres per minute. Tetracycline hydrochloride (Sigma-Aldrich, Munich, Germany) was used as a standard.

5.16. Statistics and reproducibility

The investigators were not blinded. In the RNAseq experiment, four biological replicates were used because we were interested in the gene clusters that were most strongly induced. In the food choice experiments, the number of animals per arena was limited to 8–20 (depending on the species) due to the requirement that the animals not consume all of the food before the end of the experiment. Statistical tests were deemed unnecessary in certain food choice experiments (Fig. 3, Ext. Data Fig. 6) and in the stimulation of aurofusarin synthesis by grazing (Fig. 2d,e) due to the high magnitude of the effects.

Bar graphs show means ±SEM, and line graphs show means ±95% CI. Box plots show medians and lower and quartiles (Q1 and Q3), with whiskers showing the largest (smallest) observation or 1.5-fold of the interquartile range, whichever is smaller (larger) [Q1-1.5*(Q3-Q1), Q3+1.5*(Q3-Q1)]. The significance of the differences between the means for the weight gain of mealworms and for the consumption of wheat flour with/without aurofusarin was tested via un-

paired t-tests. The significance of differences in the transcription of the aurofusarin gene cluster was determined as follows: Ct values for target genes and two internal reference genes (glyceraldehyde-3-phosphate dehydrogenase and elongation factor 1a) were adjusted for amplification efficiencies, which were obtained from dilution series[63]. ΔCt was calculated for predated cultures and controls, and the two groups were compared by unpaired two-tailed t-tests. The induction of gene expression was regarded as significant when relative mRNA levels of at least three genes increased at least 3-fold and the increase was statistically significant with both reference genes (*0.05<P<0.01, **0.01<P<0.0001, ***P<0.0001). Sample size and p-values are shown in Supplementary Data File 3.

6. References

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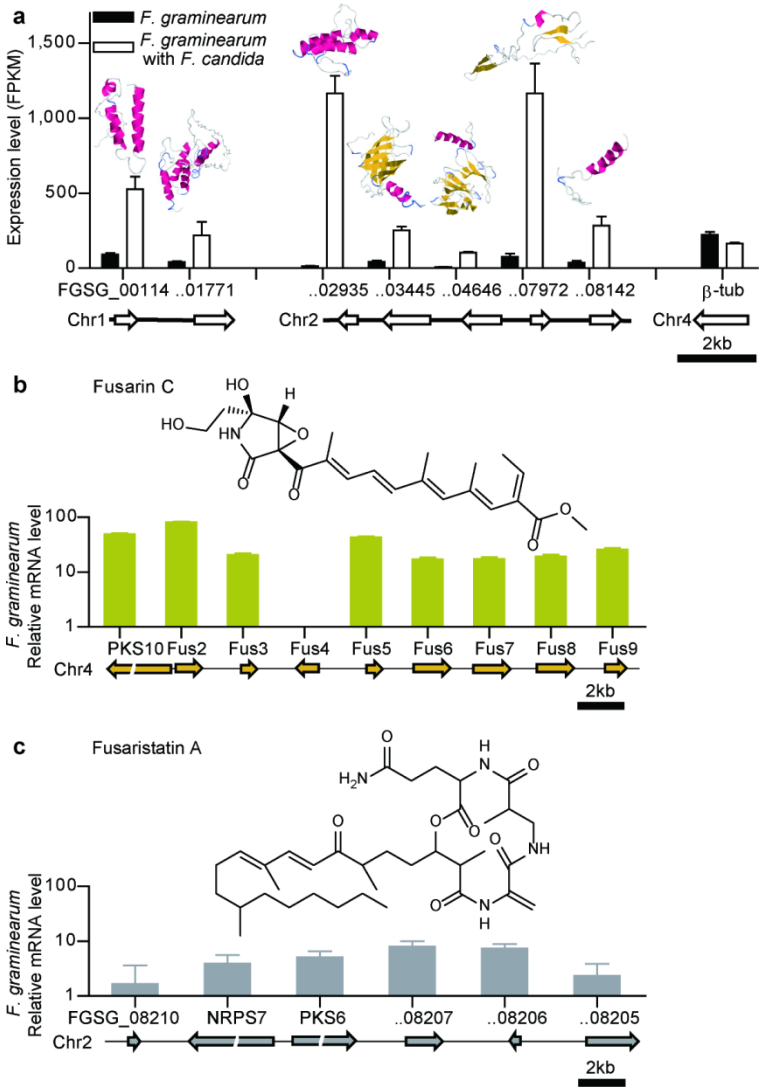
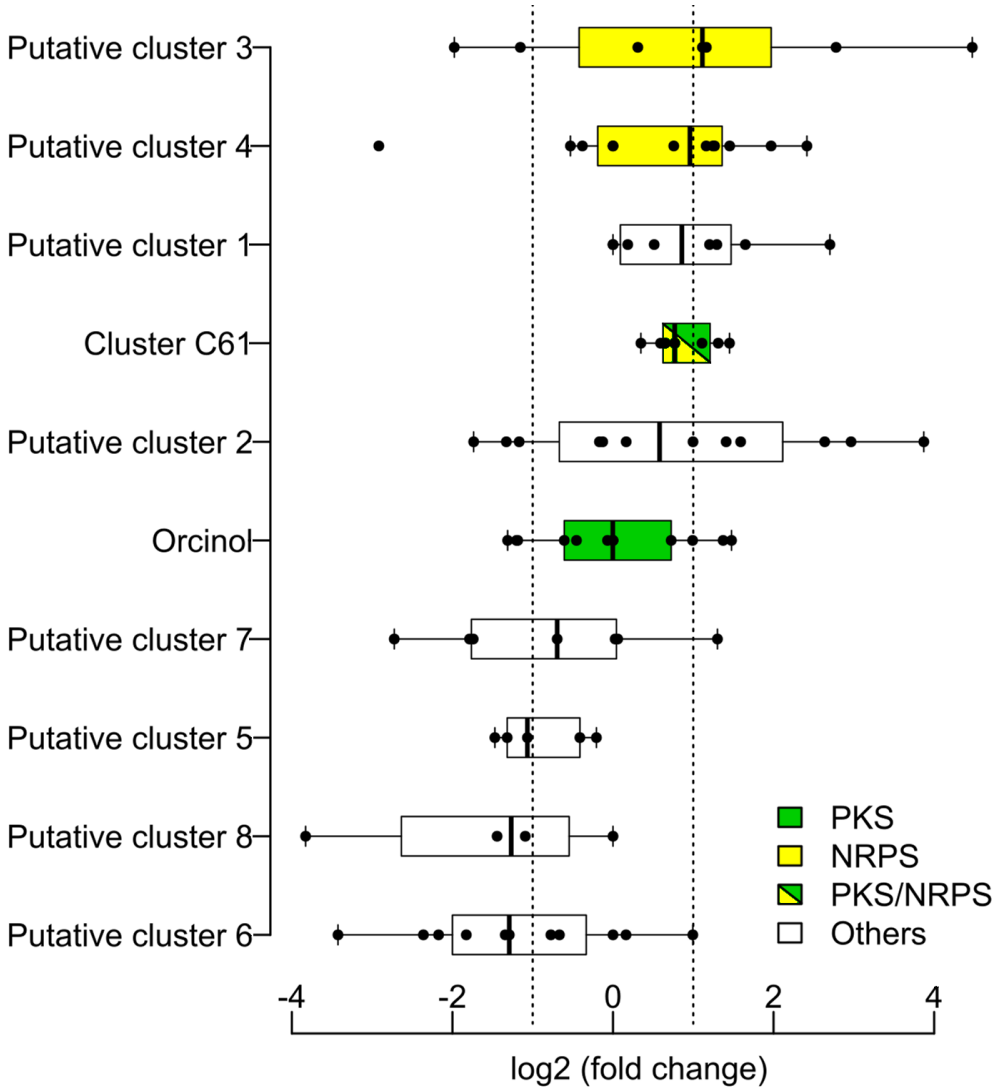
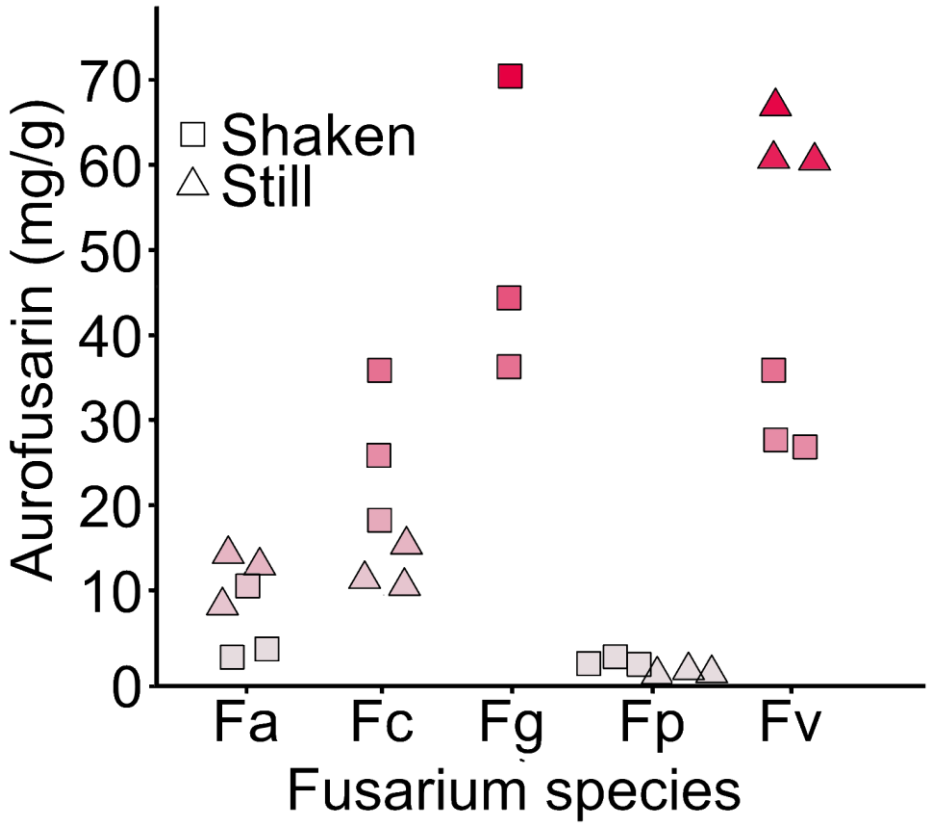
7. SUPPLEMENTARY INFORMATION



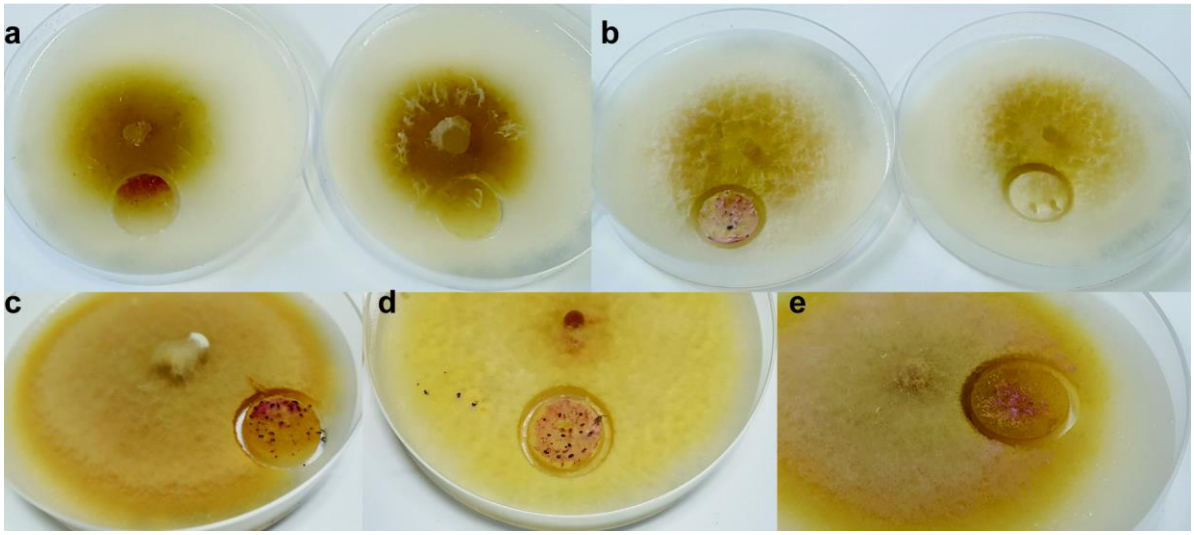
Supplementary Fig. 1 Springtails grazing induces biosynthetic pathways for cysteine-rich proteins, fusarin C and fusaristatin A in *F. graminearum*. **a** Fragments per kilobase of mapped reads (FPKM) generated by Cufflinks for 7 most strongly up-regulated genes encoding small secreted cysteine-rich proteins in *F. graminearum* when predated by the springtail *F. candida* for 48 h according to RNAseq data; beta-tubulin gene is shown as a reference. **b,c** Up-regulation of fusarin C and fusaristatin A pathways in *F. graminearum* after exposure to the springtail *F. candida* for 48 h. The RNAseq analysis was carried out with four biological replicates; the whiskers show SEM. Source data are provided in a Source Data file.



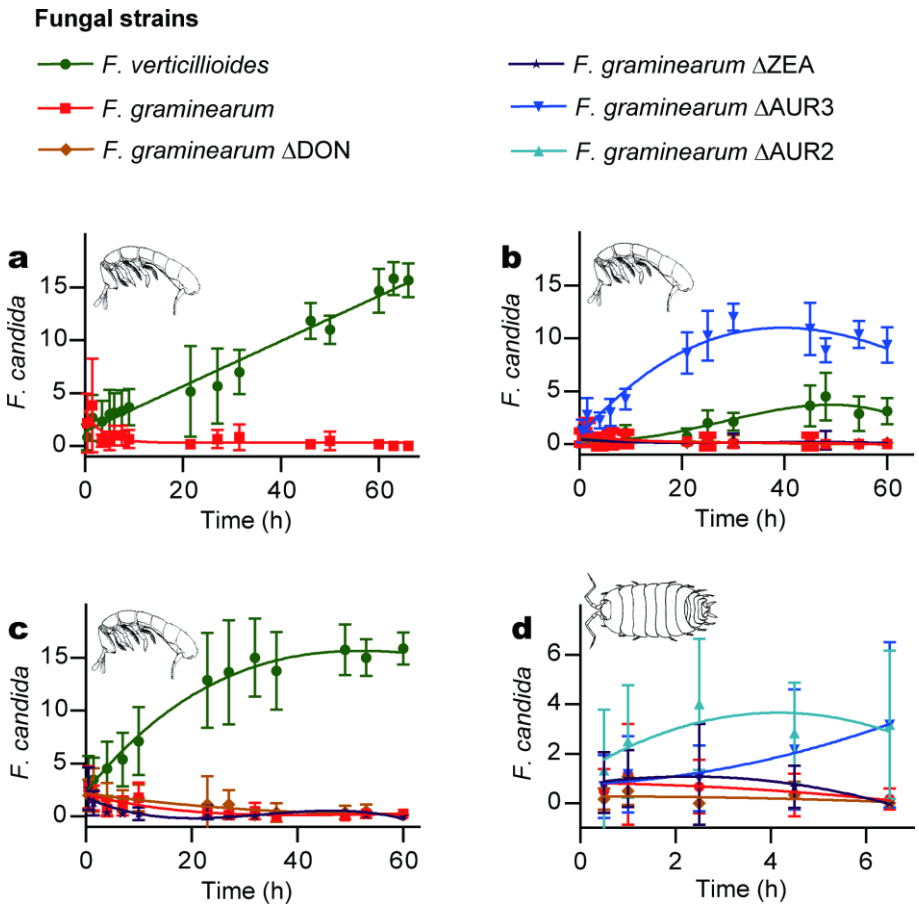
Supplementary Fig. 2 Effect of springtail grazing on the transcription of further gene clusters for secondary metabolites in *Fusarium graminearum*. *F. graminearum* was exposed to the springtail *F. candida* for 48 h and mRNA levels were determined by RNAseq. Accession numbers: putative cluster 3 (FGSG_03428 to FGSG_03434), putative cluster 4 (FGSG_07820 to FGSG_07831) putative cluster 1 (FGSG_01672, FGSG_01673, FGSG_01675 to FGSG_01677, FGSG_01679 to FGSG_01681), cluster C61 (FGSG_10542, FGSG_10543, FGSG_10545 to FGSG_10547, FGSG_10549, FGSG_13782), putative cluster 2 (FGSG_11984 to FGSG_11989, FGSG_01685 to FGSG_01690), orcinol (FGSG_03956 to FGSG_03959, FGSG_03962 to FGSG_03971), putative cluster 7 (FGSG_09060 to FGSG_09066), putative cluster 5 (FGSG_04173 to FGSG_04177), putative cluster 8 (FGSG_10494 to FGSG_10496, FGSG_10498), putative cluster 6 (FGSG_04740 to FGSG_04750). The RNAseq analysis was carried out on four biological replicates. Box plots show the median for each cluster and lower and upper quartiles (Q1 and Q3). Up-regulated clusters were defined as having >50% of the genes and/or the gene encoding the signatures enzyme induced [log2 FPKM (fold change) higher than 1.0 (dotted line) and a q value lower than 0.01]. Whiskers show the largest (smallest) observation or 1.5-fold of the interquartile range, whichever is smaller (larger) [Q1-1.5*(Q3-Q1) and Q3+1.5*(Q3-Q1)].



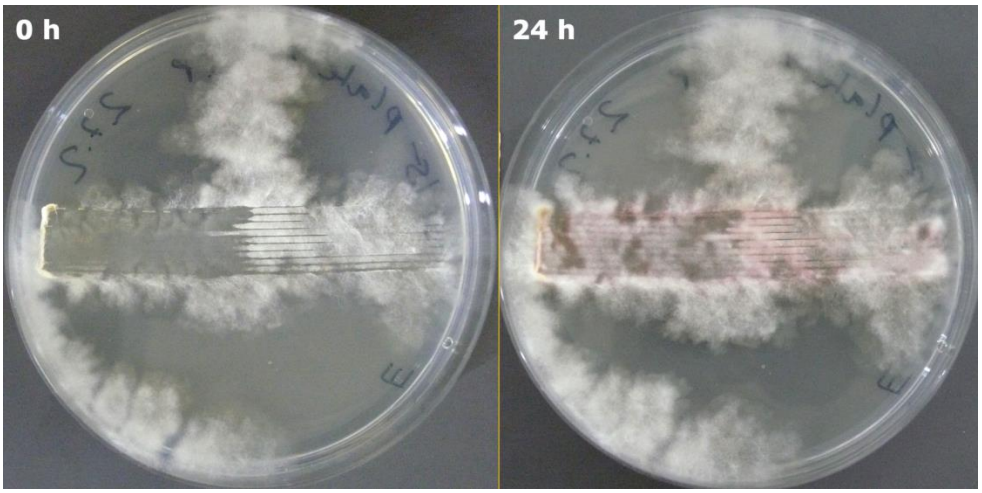
Supplementary Fig. 3 The concentration of aurofusarin in mycelia of *Fusarium avenaceum* BBA92013 (Fa), *F. culmorum* 3.37 (Fc), *F. graminearum* IFA66 (Fg), *F. poae* DSMZ62376 (Fp), and *F. venenatum* RD90 (Fv) were grown in still and shaken (140 rpm) cultures in PDB for 14 d at 23°C, mycelia were harvested by filtration, freeze-dried, extracted with chloroform-methanol (80:20) and aurofusarin content was determined by HPLC-MS/MS. The coloration of symbols indicates aurofusarin concentration in mycelia. Source data are provided in a Source Data file.



Supplementary Fig. 4 Stimulation of the synthesis of red pigment in *Fusarium* spp. by grazing. **a,** Left: *F. poae* DSMZ 62376 culture on rice agar with an arena exposed to *F. candida* grazing for 2 d; right: control with the same arena without animals. **b,** *F. venenatum* RD15 culture with an arena exposure to *F. candida* grazing for 2 d; right: control with the same arena without animals. **c,** *F. sporotrichoides* IPP 0249 culture with an arena exposed to the isopod *Porcellio scaber* for 2 d. **d,** *F. venenatum* RD 90 culture with an arena exposure to the isopod *Porcellio scaber* for 2 d. **e,** *F. avenaceum* BBA92013 culture with an arena exposure to *F. candida* for 2 d. The animals were confined to grazing arenas of 15 mm diameter with plastic cylinders inserted into the agar; the cylinders and animals were removed before the photos were taken.



Supplementary Fig. 5 Effect of aurofusarin and mycotoxins deoxynivalenol and zearalenone on the food preference in arthropods. **a** Preference of the springtail *F. candida* for *Fusarium verticillioides* over *F. graminearum*. The springtails starved for two days were placed into the centre of a Petri dish with the fungal cultures on the opposite sides of the dish and the number of animals feeding on each culture was monitored (20 animals per plate; 6 replicates). **b** Disruption of aurofusarin synthesis in *F. graminearum* (ΔAUR2, ΔAUR3) reversed the preference of *F. candida* for *Fusarium* species; disruption of zearalenone (ΔZEN) and deoxynivalenol (ΔDON) synthesis did not affect the food preference. The fungal cultures were placed along inner edges of Petri dishes in equal distances (20 animals per plate; 8 replicates). **c** *F. candida* preferred *F. verticillioides* over *F. graminearum* wild type and *F. graminearum* strains with disrupted synthesis of zearalenone and deoxynivalenol (20 animals per plate; 8 replicates). **d** Isopod *T. tomentosa* preferred *F. graminearum* strains with disrupted synthesis of aurofusarin over wild type strain and strains with disrupted synthesis of zearalenone and deoxynivalenol (8 animals per plate; 5 replicates). Error bars show 95% CI. Source data are provided in a Source Data file.



Supplementary Fig. 6 Effect of mechanical damage on *Fusarium venenatum*. Culture of *F. venenatum* RD15 growing on GM7 medium at was injured with a razor blade array as in Fig. 6 and photos were taken immediately after the injury (left) and 24 h late (right).

Chapter 5. Investigation of the secondary metabolites in *Microdochium nivale* and *M. majus*

Albatol Alsarrag, Mohammad Alhussein, Tuvshinjargal Budragchaa, Petr Karlovsky

1. Abstract

The filamentous fungi *Microdochium nivale* and *M. majus* are known as pink snow mold and renowned as important pathogens on grasses and winter cereals that cause dramatic losses in grain yield and severe damage to lawns every year. However, over the years, studies have provided conflicting information about the toxicity of *M. nivale*. Some claimed its toxicity, whereas others demonstrated that *M. nivale* is a non-toxic species.

In the present study, the toxicity of metabolites, which several strains of *M. nivale and M. majus* produce, were explored using mealworms, springtails, brine shrimps, duckweeds, and radish seed bioassays. The results revealed that strains related to *M. nivale* caused stronger negative impacts on the tested organisms than *M. majus*. Among the screened *M. nivale* strains, secondary metabolites from the most efficient strain were extracted and subjected to a bioassays-guided fractionation that resulted in the isolation of two compounds. The first compound inhibited the germination of radish seeds (phytotoxic), and the second compound displayed toxicity to brine shrimps (cytotoxic), each yielding $LD_{50} \leq 13$ μg ml^{-1}. The accurate masses of the toxic compounds were assigned by LC–TOF/MS. Further, the structure elucidation revealed that the phytotoxic compound is valienol (streptol)**,** which is commonly produced by Actinobacteria, and to our knowledge, this is the first time to isolate streptol from fungi.

Keywords *Microdochium nivale*; *Microdochium majus*; bioassays-guided fractionation; cytotoxic; phytotoxic

2. Introduction

The ascomycete *Microdochium nivale* (Fr.) Samuels & Hallett, teleomorph *Monographella nivalis* (Schaffnit) E. Müller, also known as pink snow mold, is an important pathogen on grasses and winter cereals (Dwyer et al., 2017; Matsumoto & Hsiang, 2016, p. 82; Tronsmo et al., 2001). The taxonomy changed several times to its current state. The name *Lanosa nivalis* had been introduced by Fries (1825) for the disease-causing snow mold on grasses. In 1924, Hans Sydow defined the genus *Microdochium* by describing *M. phragmitis* isolated from reed (Noble & Montgomerie, 1956). The fungus was known as *Fusarium nivale* (*Calonectriae graminicolae*). Then the taxon *F. nivale* was changed by Gams & Müller to *Gerlachia nivalis* (Ces. Ex Sacc.) because of the annellate conidiogenous cells and the amphisphaeriaceous teleomorph. In 1983 Samuels & Hallett classifed this anamorph as *Microdochium nivale* (Fr.) because of the high correlation and shared features between *Microdochium* and the *F. nivale* were noted by; they regarded *Gerlachia* as a synonym to *Microdochium* (Gams & Müller, 1980; Samuels & Hallett, 1983). Based on conidia morphology, two varieties of *F. nivale* were differentiated, *F. nivale* var. *nivale* and *F. nivale* var. *majus*. They were distinguishable by conidia size and the number of conidial septa (Gerlach & Nirenberg, 1982; Wollenweber, 1931). However, many researchers did not accept this classification of *F. nivale*, arguing that the conidial morphology was insufficient proof. In 1995, Lees and his team meticulously isolated single spore cultures of several *M. nivale* isolates from wheat across the United Kingdom. They utilized random amplified polymorphic DNA (RAPD) and conidial morphology to confirm the validity of the previous classification of *F. nivale* (Lees et al., 1995). Subsequently, based on the elongation factor 1 alpha (*EF-1α*) DNA sequences, the two sub-groups of *M. nivale* were separated into two species, *M. nivale* and *M. majus* (Glynn et al., 2005). Later studies confirmed this separation (Hofgaard et al., 2006; Jewell & Hsiang, 2013). Recently, new species and combinations of *Microdochium* were described and respectively proposed depending on morphological analysis and DNA sequence data that confirmed *Monographella* as a sexual morph and *Microdochium* as an asexual genus. Based on these new insights, the authors reclassified it into a new family *Microdochiaceae* within Xylariales. The anamorph represents this family to which conidiogenous cells are polyblastic, sympodial, or annellidic, and the conidia are hyaline without appendages (Hernández-Restrepo et al., 2016).

Among the four types of snow mold complex diseases, the pink snow mold that occurs after the infection by *M. nivale* and *M. majus* is considered the most ubiquitous and harmful disease on

winter crops (Ponomareva et al., 2021; Tronsmo et al., 2001), causing a dramatic yield loss every year (Humphreys et al., 1997) harming turfgrasses extensively (Abdelhalim et al., 2016; Hofgaard et al., 2006). In addition, *M. nivale*, together with some *Fusarium* species, has been recognized as the most efficient causal agent of *Fusarium* ear blight (FEB) (Parry et al., 1995), causing a loss in the yield of several grain crops (Bennett, 1933).

Generally, *M. majus* and *M. nivale* are pathogens on wheat and rye; the latter species is more aggressive and displays host specificity to oat (Simpson et al., 2000). *M. nivale* is distributed worldwide and prefers moist and cooler climates, causing two different diseases, depending on the environmental conditions; pink snow mold and scab. The first disease is initiated by a soil-borne inoculum and develops under snow in unfrozen soil. Scab establishes itself by airborne ascospores, and the infection increases in winters with high humidity (Inglis & Cook, 1981). *M. nivale* produces conidia and ascospores but no chlamydospores (Naito & Koshimizu, 1974; Tronsmo et al., 2001; Von Arx, 1984). The infection of plant tissue occurs via haustoria (Dubas et al., 2011). Snow cover stimulates infections (Ergon et al., 2003). The disease becomes very severe after extended periods of snow covering, causing brownish patches known as *Fusarium* patches (Tronsmo et al., 2001).

Disease severity and the host preference of *M. nivale* are temperature-dependent. In vitro, the *Lolium perenne* infestation remained symptomless, and the germination of the spores was slow at 0–1°C but enhanced at 18–20°C (Prończuk & Messyasz, 1991). Numerous studies exist on morphological characterization (Diamond et al., 1998; Gagkaeva et al., 2020; Hofgaard et al., 2006; Lees et al., 1995; Marin-Felix et al., 2019; Simpson et al., 2000), pathogenicity and phylogenies of *M. nivale*. However, studies on the secondary metabolites producing by *M. nivale* were not pursued as the fungus was considered to be non-toxic (Chełkowski et al., 1991; Gagkaeva et al., 2020; Logrieco et al., 1991; Nielsen et al., 2013). The few studies on fungal secondary metabolites identified *Fusarium nivale* as a producer of nivalenol, fusarenon-X (Ueno et al., 1973), and vomitoxin (Vesonder et al., 1981).

The conflicting reports about the toxicity of *M. nivale* and its common history with *Fusarium* encouraged us to perform this work, aiming to I) Investigate the biological activity of several strains of *M. majus* and *M. nivale*. II) Explore the common *Fusarium* mycotoxins in the extract of *Microduchium* strains. III) Isolate and identify the potential biologically active secondary metabolites in a more extensive way than hitherto accomplished.

3. Materials and methods

An overview of the experimental design is shown in Figure 1.

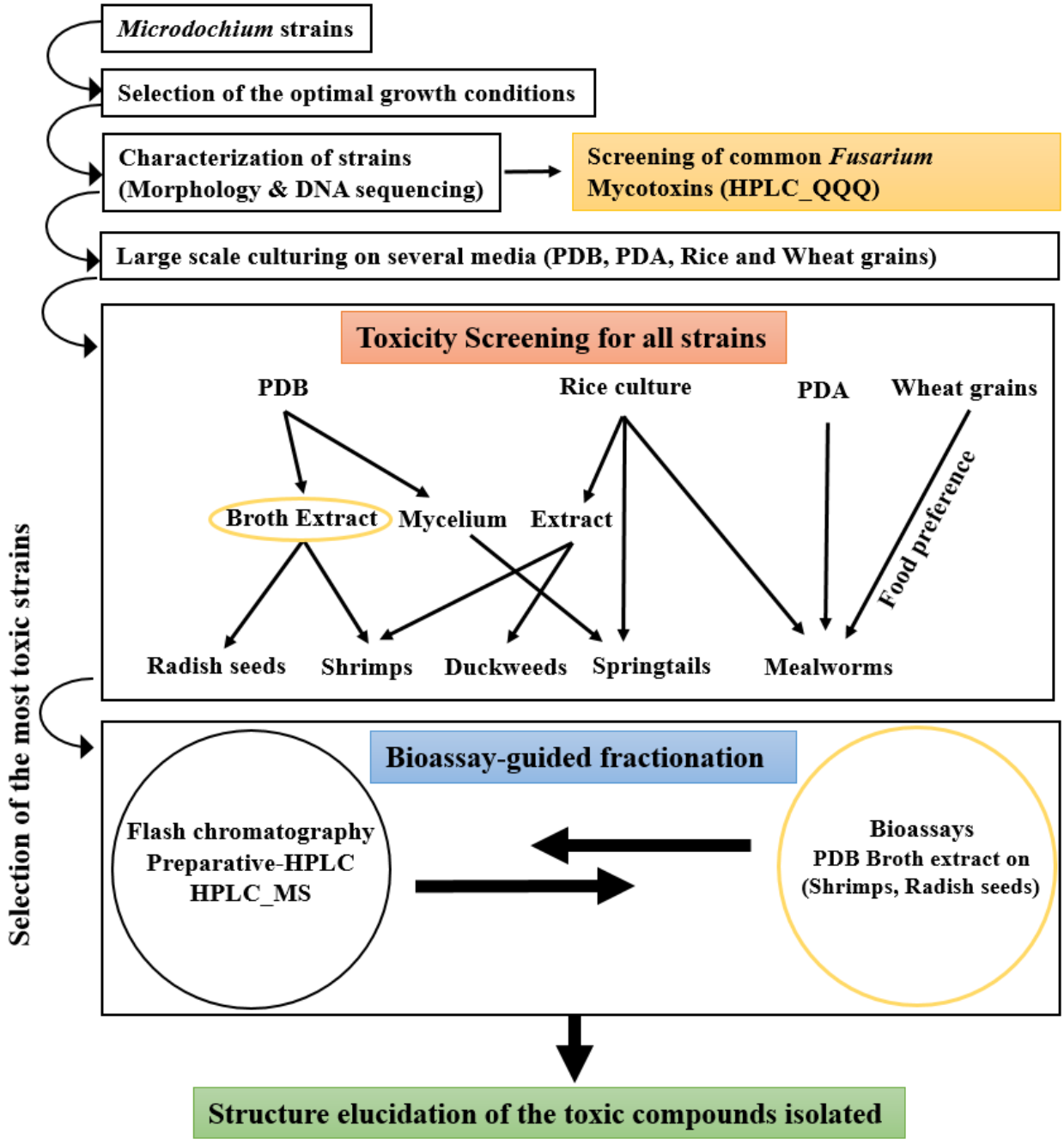


Figure 1. Scheme of the experimental design.

3.1. Fungal strains

Five strains of each *Microdochium nivale* and *Microdochium majus* were provided by Dr. Pavel Matusinsky, Agrotest Fyto, Kroměříž, Czech Republic (Table 1). In addition, *F. graminearum* strain IFA66 (DON chemotype) was obtained from Dr. Marc Lemmens (BOKU, Tulln, Austria) via Professor Thomas Miedaner (University of Hohenheim, Germany). *Rhizopus oryzae* CJM28.327 was isolated from fresh tempeh by Dr. Riyan Anggriawan (Indonesia, 2015).

Table 1. *M. nivale* and *M. majus* fungal strains

Species	Isolate	Year	Host cultivar wheat/ barley
M. nivale	Mn1	2013	Baletka
M. nivale	Mn206	2013	Baletka
M. nivale	Mn33	2013	Avenue
M. nivale	Mn8	2013	Federer
M. nivale	Mn94	2013	Fredericus
M. majus	Mm179	2013	Bakfis
M. majus	Mm185	2013	Evina
M. majus	Mm216	2013	Chevalier
M. majus	Mm50	2013	Sakura
M. majus	Mm72	2016	Potenzial

3.2. Media and cultures

Several cultures (fungi-medium) were prepared to serve various purposes: Rice culture was offered to springtails and mealworms, and the culture extract (See 3.3) was tested on brine shrimps and duckweeds; Potato dextrose agar (PDA) was offered to mealworms; Fungal mycelium from liquid cultures in potato-dextrose broth (PDB) was offered to springtails and the broth extract (See 3.3) to brine shrimps and radish seeds; Infected and intact wheat grains were utilized to perform the food preference on mealworms.

Rice cultures were prepared by soaking 25 g polished rice in 35 mL tap water using 100 mL Erlenmeyer flasks. Five grams of wheat grains were soaked in a 50 mL falcon tube including 5 mL

tap water. PDA was prepared by cooking 200 g unpeeled potatoes, then 20 g glucose and 20 g agar were added to filtered soup, which was completed with demineralized water to 1 L before autoclaving. PDB was prepared following the same procedure of preparing PDA without the addition of agar. The four media types were autoclaved at 121°C for 20 min. Nine cm PDA plates were prepared. PDA plugs (5 mm diam.) taken from stock cultures were used to perform the inoculation. Plates were incubated in the dark at 22°C for two weeks. PDB was shaken at 130 rpm. *Fusarium graminearum* and *Rhizopus oryzae* were used as mycotoxin producers and non-producers control. Wheat grains inoculated with *M. nivale* and *M. majus* strains. The cultures were incubated for two weeks at 23°C in the dark. Non-infected grains faced the same procedures and were offered to the worms simultaneously as a negative control. Fungal mycelium cultured in PDB was filtered for later use. The liquid medium itself was extracted with ethyl acetate (for details, see chapter 3.3).

3.3. Extraction of the secondary metabolites from fungal strains

The fungal strains were grown on rice grains (4 g) were extracted with 40 mL acetonitrile: water (84:16, v/v), and the extract of intact rice grains serve as a negative control. Filtered PDB was extracted twice with ethyl acetate (v/v) at room temperature for two hours. First, the extracts were concentrated to dryness under vacuum, and then the extracts were weighed.

3.4. Optimizing the growth temperature

Microdochium strains were cultured on PDA in the dark at 8, 10, 12, 16, 20, 24, and 28°C separately and prepared in sets of three biological replicates per temperature regime. Colony diameter (mm) was recorded daily to determine the optimal temperature for growth. The measurements started one day after the inoculation and were continued for four days consecutively.

3.5. DNA extraction and qPCR

Microdochium strains were cultured on PDA for one week at 23°C in the dark. Fungal mycelium was harvested (30 mg per strain), lyophilized, ground, and extracted using a cetyltrimethylammonium bromide (CTAB)-based protocol (Brandfass & Karlovsky, 2008). The extracted DNA was assessed on agarose gels (0.8% (w/v) in 1 × Tris-acetate-EDTA buffer) stained

with ethidium bromide. Gel electrophoresis was applied for 60 min at 4.6 V/cm. The pellets were washed, dissolved in ethanol, dried under vacuum, and again dissolved in 50 µL TE-buffer (10 mM Tris buffer, 1 mM EDTA, pH 8.0). A dilution step with bi-distilled water was accomplished before the qPCR. PCR was performed using primers ITS1 (5'- TCCGTAGGTGAACCTTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and Taq® polymerase (New England Bioline, London, UK). The components of the reaction mixture were: Taq Polymerase with ThermoPol Buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton-X-100, pH 8.8 at 25°C); 0.15 mM of each dNTP; 2.5 mM $MgCl_2$ (Bioline, Lückenwalde, Germany); 0.3 µL of each primer; and SYBR Green I (Invitrogen, Karlsruhe, Germany). The initial denaturation started at 94°C for 1 min, followed by 30 cycles with a denaturation step at 94°C for 20 s, then an annealing step at 58-64°C for 1 min, and finally an elongation step at 72°C for 45 s. The purified PCR products were subjected to sequencing (Macrogen Europe, Amsterdam, Netherlands).

3.6. Bioassays and Samples preparation

3.6.1. Springtail *Folsomia candida*

The springtails *F. candida* strain Berlin were supplied by the Institute of Zoology (University of Göttingen, Germany). The animals were cultured on dry yeast on a gypsum plaster in 9 cm Petri dishes. Springtails were allowed to graze on the fresh *Microdochium* mycelia cultured on rice grains for nine days and PDB for two weeks separately. On rice grains, the animals were cultured for ten days in a set of four replicates per treatment and eight springtails per replicate, and at the experiment end, the animals' mortality was recorded. Rice grains overgrown with *F. graminearum* and uninoculated rice grains represented positive and negative controls. In the other experiment, springtails were incubated in 55 mm Petri dishes furnished with wet filter paper and allowed to feed for two weeks at 22°C in the dark on *Microdochium* mycelium that was filtered from PDB. This set was prepared in five replicates with twenty animals per replicate. Mycelia of *F. graminearum* and *R. oryzae* represented positive and negative controls. Animal productivity (their ability to lay eggs), activity (their ability to move), and the number of dead animals were recorded at the experiment end.

3.6.2. Mealworm *Tenebrio molitor*

Mealworms were purchased from Zoo & Co. Zoo-Busch GmbH (Göttingen, Germany). They were starved for two days before starting the experiment. Fitness experiments were performed by offering fresh fungal mycelia cultured on rice and PDA separately. Rice grains with *Michrodochium* strains and *F. graminearum* for nine days and the uninoculated rice were used to feed the starved mealworms. Four replicates per treatment and ten mealworms per replicate were prepared in 100 mL Erlenmeyer flasks. The total weight of 10 mealworms (one replicate) was observed initially and after three weeks after feeding at 22°C in the dark. On PDA, the length and weight of the starved mealworms were measured at the beginning of the trial and after three weeks of feeding on PDA blocks overgrown with fungal mycelium. Twenty replicates were prepared, one animal per replicate.

3.6.3. Brine shrimp *Artemia salina*

Artemia eggs (Artemio ® Mix, JBL, Neuhofen, Germany), 26 g, were hatched in 1 L demineralized water after 24 hours exposure to a classic light at 55 W and 28°C and aeration. The hatched small brine shrimps were attracted by illumination and transferred into 96 well microtiter plates. Each well contained fifty µL of the *Artemia* suspension including 20–40 animals, and this volum completed to 200 µL with ethyl acetate extract of pure and inoculated PDB (after removing the fungal mycelium) at several concentrations. First, the extracts were dissolved in dimethylsulfoxide then diluted in shrimp's water (suspension-excluded shrimps) to obtain a two-fold dilution series starting at 31 to 1000 µg mL^{-1}. Ten replicates were prepared per concentration. Brine shrimps were maintained in shrimp's water as a control, with corresponding percentage of dimethylsulfoxide. The toxicity of the purified fraction was tested at a range of 10–100 µg mL^{-1}. The final dimethylsulfoxide conc. was ≤ 2% in all treatments.

3.6.4. Radish seeds *Raphanus sativus*

Radish seeds (Saxa3, Bruno Nebelung GmbH, Everswinkel, Germany) were purchased from a local store. First, the toxicity of the ethyl acetate fraction of filtered PDB was explored by preparing a dilution series from 62 to 500 µg mL^{-1} in sterile tap water with 2 % dimethylsulfoxid. The chromatography fractions were prepared in a similar way as previously described to obtain a dilution series from 25 to 200 µg mL^{-1}. Water with ≤ 2% dimethylsulfoxide served as a negative

control. Four seeds were placed on two layers of sterile filter paper (Ederol, Nr. 75) in 3.5 cm diam. Petri dishes. Subsequently, 550 µL of the extract solution was added. The plates were incubated in the dark at 25°C. The germination rate was recorded after three days of incubation. The toxicity of the purified fraction was tested from 7 to 30 µg mL^{-1}.

3.6.5. Duckweed *Lemna minor*

Duckweed plants were purchased from Aqua Nord Market (Norden, Germany). The plants were sterilized by soaking in 0.5 % sodium hypochlorite for 5 min, then the plants were directly washed with sterile tap water. Dead and strangely-looking plants were eliminated. The others were transferred to 20 cm diam. glass Petri dishes filled with modified Swedish standard *Lemna* growth medium that was prepared as described in OECD Guideline (2006). The modification was to exclude 3-(*N*-morpholino)-propane sulfonic acid (MOPS) buffer. The plants were covered with a glass lid and sub-cultured weekly for three weeks at 25°C and a 16:8 h light regime. One plant with 2-3 fronds was transferred into glass tube (1 cm diam., 3 cm height) and was considered a replicate, and four replicates were prepared for each treatment. Extracts of rice cultures (non-inoculated is a control) were dissolved in sterile tap water with 2% dimethylsulfoxide to obtain a final concentration of 0.02, 0.2, and 2.0 mg mL^{-1}. The plants were covered with a glass lid and incubated for one week at 25°C and a 16:8 h light regime. Photos were taken at the beginning and the end of the experiment using a stereomicroscope equipped with a digital camera. The leaves area (mm) was determined by ImageJ software (Rasband, 1997), and the frond number was recorded.

3.6.6. Food preference

A single *T. molitor* worm was placed in a petri dish (9 cm diam.) and provided with 3–4 intact and infected grains on two opposite sides (n= 20). A glass slice was set in the middle of the Petri dishes to prevent the grains displacing by the worms. The worms were kept in darkness except for the moment of location scoring. Time-series (0.5–96 h) data were generated.

3.7. Purification and structure elucidation

3.7.1. Flash chromatography

The fungal mycelium was filtered after two weeks of growth in PDB. The remaining PDB medium was extracted twice with ethyl acetate. The extract was concentrated under a vacuum to dryness. The dried extract was dissolved in methanol:water (1:1, v/v) and fractionated using flash chromatography (Sepacore® Flash system X10/X50, comprised of a binary pump Module C-601/C-605, a UV C-635 detector; a fraction collector C-660 and run by SepacoreControl software; Büchi, Flawil, Switzerland). The detection wavelength was 270 nm. The separation was achieved by a reverse-phase column (Chromoband® Flash cartridge RS C_{18}, 40–63 µm, 240 x 30 mm, MACHEREY-NAGEL, Düren, Germany). The flow rate was 30 mL/min. The mobile phase A was water with 0.25 % acetic acid (v/v), and B was methanol with 0.25 % acetic acid (v/v). The elution gradient started at 10% methanol for 2 min; 10–100% methanol, 78 min; 100% methanol, 15 min. The toxicity of all the collected fractions was assayed using radish seeds and brine shrimps.

3.7.2. Preparative HPLC

Preparative high-pressure liquid chromatography (HPLC) was applied to purify the active fractions from flash-chromatography. The separation was performed on reversed-phase silica (Nucleodur C18 HTec, 5 µm, 250 x 10mm, Macherey Nagel, Düren, Germany) at a flow rate of 3 mL min^{-1}. The pump was a binary pump PU-2086 plus (JASCO Inc., Gross-Umstadt, Germany). The eluent mixtures A (0.25 % aqueous acetic acid) and B (methanol with 0.25 % acetic acid) were used. The gradient for the cytotoxic fraction was as follows: 0–5 min, 10 % B; 5–25 min, 10–50 % B; 25–80 min, 50-98 % B; 80–90 min, 98 % B; 90–105 min, 98–100 % B; 105–120 min, 10% B. The gradient for the phytotoxic fraction was as follows: 0–5 min, 5% B; 5–65 min, 5–60 % B; 65–80 min, 60-98 % B; 80–90 min, 98 % B; 90-105 min, 98-5 % B; 105-115 min, 5% B. A UV/VIS detector (Jasco UV-970, JASCO Inc., Gross-Umstadt, Germany) was set to 270 nm as detection wavelength. A fraction collector (CHF122SC, Advantec MFS Inc., Japan) was used to collect the fractions. The system was run by JASCOChromPass, chromatography data system, version 1.8.6.1.

3.7.3. Mycotoxins analysis

An HPLC-system was linked via an ESI interface (positive mode, capillary voltage 4000 V) to an Agilent QQQ/MS 6460 detector (Agilent Technologies, Waldbronn, Germany) which was adjusted in the multiple reaction monitoring (MRM) mode. Zorbax Eclipse Plus C18 column, 50 x 2.1 mm with 1.8 µm particle size (Agilent Technologies, Waldbronn, Germany), the flow rate 0.4 mL min^{-1} that was thermostatted at 40°C. Solvent A was water with 0.1% formic acid (v/v), B was methanol with 0.1% formic acid in (v/v). The solvent gradient was applied as follows: 0.2 min 5% B, 6 min 35% B, 4 min 98% B, 2 min 98%, 0.5 min 5%B. Ionization was implemented by electrospray in a positive mode and a capillary voltage of 4000 V. The acquired data were processed using Mass Hunter Workstation, Version B.08.02 (Agilent Technologies, Waldbronn, Germany). The extracts of all *Microdochium* strains were analyzed as described by Beule et al. (2019) to ensure that our *Microdochium* strains are non-producers of the common *Fusarium* mycotoxins. Particularly, the following mycotoxins were explored: Nivalenol, deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3-ADON), 15-acetyl-deoxynivalenol (15-ADON), diacetoxyscirpenol (DAS), T-2 toxin, HT-2 toxin, fusaric acid, enniatins (A, A1, B and B1), zearalenone, beauvericin, NX2, NX4, fusarin X, and aurofusarin.

3.7.4. Analysis by LC–TOF/MS and LC–ELSD

LC–TOF/MS analysis was performed using Agilent 1290 Infinity II HPLC system (Agilent Technologies, Waldbronn, Germany) coupled with the Agilent 6545 QTOF-MS detector (Agilent Technologies, Waldbronn, Germany). The cytotoxic fraction was separated using a Zorbax Eclipse Plus C18 column, 50 x 2.1 mm with 1.8 µm particle size (Agilent Technologies, Waldbronn, Germany). The column was thermostatted at 40 °C, and the flow rate was 0.4 mL min^{-1} at an injection volume of 10 µL. Solvent A was water with 0.1% formic acid (v/v), and solvent B was methanol with 0.1% formic acid (v/v). The gradient was applied as follows: 0–0.2 min, 5% B; 0.2–7 min, 5-98% B; 7–7.5 min, 98% B; 7.5–8 min, 98-5 % B; 8–12 min, 5% B. The phytotoxic fraction was separated using a Hilic EC 150/2 Nucleoshell, 2.7 µm (Macherey-Nagel, Düren, Germany). The column was thermostatted at 40 °C, and the flow rate was 0.25 mL min^{-1} at an injection volume of 10 µL. Solvent A was water with 0.1% formic acid (v/v), and solvent D was acetonitrile with 0.1% formic acid (v/v). The gradient was applied as follows: 0–0.2 min, 95% D; 0.2–20 min, 95–30% D; 20–24 min, 30% D; 24–24.5 min, 30-95% D; 24.5–31 min, 95% D. The

eluent was ionized using a dual Agilent Jet Stream Electrospray Ionization source (Dual AJS ESI). MS data were acquired in full scan mode (MS1) and positive ionization. The mass range was from 100 to 1,700 *m/z,* and the acquisition rate was 2 spectra/s. The source conditions were as follows: the sheath gas temperature was 350°C with a flow rate of 11 L/min, capillary voltage 3,500 V, Nozzle voltage 1,000 V, nebulizer pressure of 35 psig, drying gas temperature 320°C with a flow rate of 8 L/min, fragmentor voltage 175 V, skimmer voltage 65 V. and Octopole RF Peak 750 Vpp. The MS/MS spectra were acquired in targeted MS/MS mode at a collision energy of 10 V. The Acquisition data were processed using the software Mass Hunter Workstation, Qualitative Analysis Navigator Version B.08.00 (Agilent, Darmstadt, Germany). The purity of the isolated compounds was confirmed by HPLC coupled to an evaporative light scattering detector (ELSD). The separation was performed as described above, and the analytes were detected by an ELSD 1260 detector (Agilent, Darmstadt, Germany) operated at a nebulizer temperature of 40°C, an evaporator temperature of 42°C, and an evaporator gas flow of 1.6 L min^{-1}.

3.7.5. Structure elucidation of the bioactive compounds

The chemical structure of the purified bioactive compounds was determined by Dr. Tuvshinjargal Budragchaa (Leibniz-Institut für Pflanzenbiochemie, Halle, Germany), employing ^{13}C, ^{1}H, 2D NMR, and the optical rotation measurement. All NMR spectra were recorded on a 500 MHz Bruker AVANCE NEO NMR spectrometer. The optical specific rotation was measured using a JASCO P-2000 digital polarimeter.

3.8. Statistics

Dose-response curves on brine shrimps and radish seeds were estimated using the 'LL.2'-function in the 'drc' R package (Ritz et al., 2015); the lower limit is fixed at 0, and the upper limit is fixed at 1. Pairwise (Wilcox.test) mean comparison between two groups (considering rice or Ro as references) were determined using the 'stat_compare_means'-function, in the 'ggpubr' (Kassambara, 2020). Data visualization was performed by the 'ggplot2' R package (Wickham, 2009) in the R v 4.0.2 (R Core Team, 2020).

4. Results

4.1. Phenotypic assessment of the isolates

The morphological appearance of ten *Microdochium* isolates was observed on different media. The growth pattern of *M. majus* strains on agar culture was morphologically congeneric. The mycelium is superficial, white, fluffy, and concentrated around the center of the plate. In comparison, *M. nivale* cultures were characterized by the immersed mycelium and the hyaline and smooth hyphae. Some strains produced superficially light orange conidial spores at advanced growth stages, forming sporodochia (Figure 2).

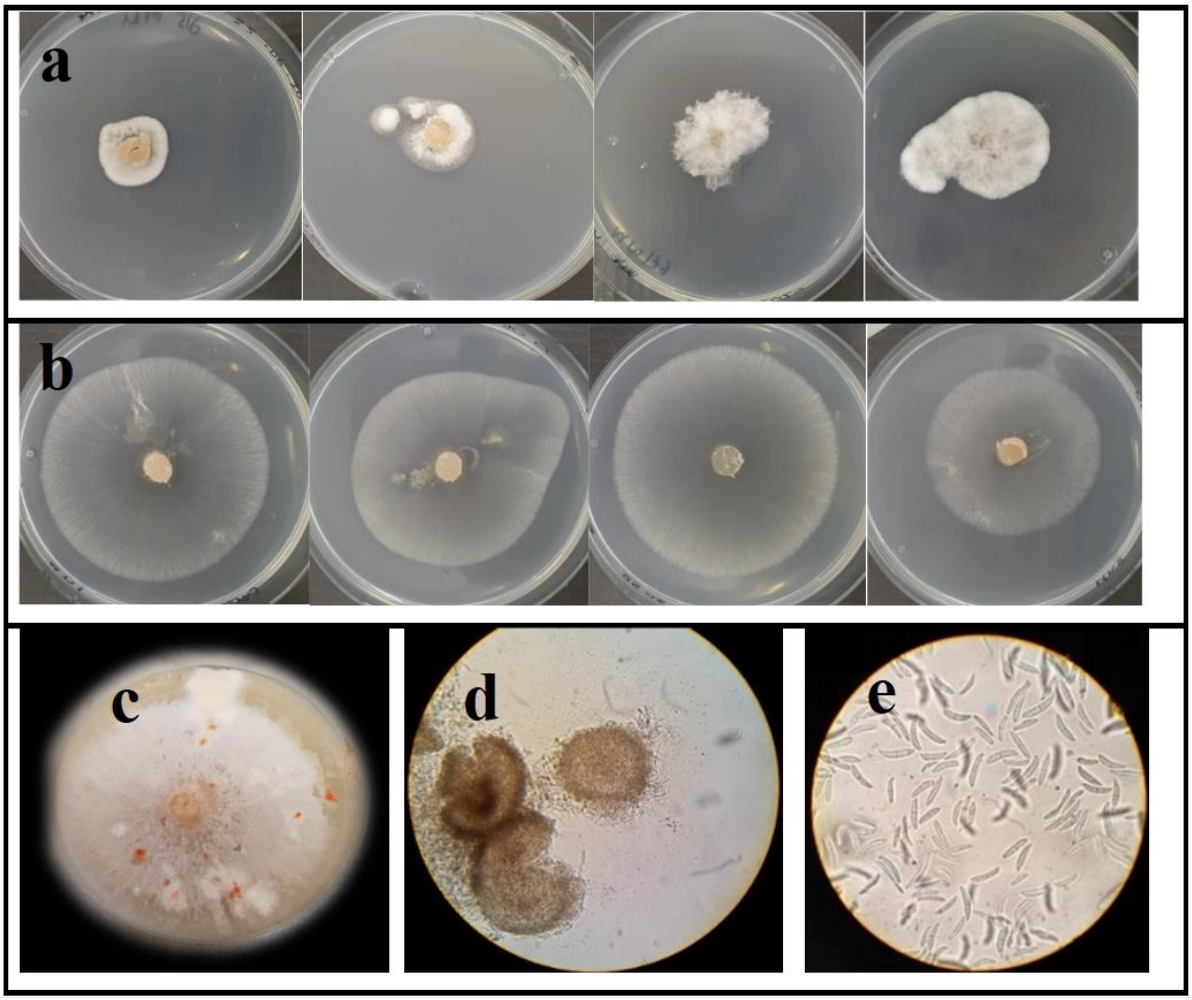


Figure 2. *Microdochium* strains on PDA. The strains were cultured at 4°C for one week. a) *M. majus* strains. b) *M. nivale* strains. c) light orange superficially formed sporodochia on *M. nivale* cultured for one month. d) sporodochia at 40x magnification. e) Conidia at 100x magnification

Cultures of *M. majus* and *M .nivale* on PDB homemade were indistinguishable from each other. The mycelium was colorless and grew continuously during the incubation period. (Supplementary Figure 1a,b). Rice grains overgrown with *M. majus* were pinkish, and the top of cultures was covered with fluffy white mycelium. Molded rice grains with *M .nivale* appeared light brownish, and the top of cultures covered with fluffy white mycelium (Supplementary Figure 1c,d). In all cultures, no sporodochia or conidia were produced. The molecular analysis confirmed the subordination of the fungal strains to *M. majus* and *M. nivale* (Supplementary Table 1).

4.2. Influence of the temperature on the fungal growth

M. majus and *M. nivale* responded equally to the temperature changes. Figure 3 illustrates one strain per species. The rest of the strains are shown in Supplementary Figure 2. Optimum growth temperatures were between 16 and 24°C. At 8°C the fungal growth was slowed down and at 28°C was inhibited.

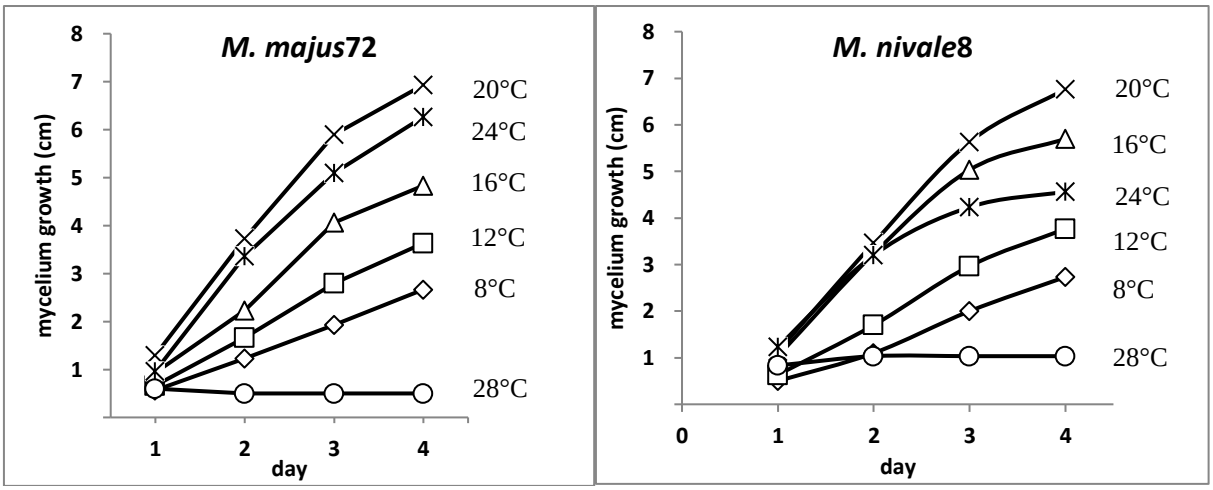


Figure 3. Fungal growth rates at different temperatures *M. majus* (Mm72) and *M. nivale* (Mn8) were cultured on PDB at 8, 12, 16, 20, 24, and 28°C. The measurements took place with time intervals from 1 to 4 days (n= 3)

4.3. Bioassays

4.3.1. Toxicity to the *Folsomia candida*

The toxicity of *Microdochium* mycelia (filtered out of PDB) was explored (Figure 4). Generally, some *M. nivale* strains but not all caused strongly increased lethality of springtails compared to *M. majus*. *M. nivale* Mn8 and Mn206 significantly reduced springtail survival and Mn8 even to the same extent as *F. graminearum*. No impact was observed by mycelium extracts from *M. majus* strains and the rest of the tested *M. nivale* strains. The animals' activity and fecundity confirmed the previous results (Table 1). Springtails fed on Mn8, Mn206, and *F. graminearum* were inactive and, in addition, Mn8, and *F. graminearum* did not lay any eggs. Among the *M. majus* strains, Mm72 and Mm179 did not lay eggs despite being unaffected in terms of lethality.

The fitness of springtails fed on rice grains overgrown with *M. nivale* and *M. majus* strains for ten days at 22°C in the dark did not differ. However, two *M. nivale* strains (Mn8 and Mn33) caused higher death rates to springtails than the *M. majus* strains. In contrast, the rest of *M. nivale* strains and *M. majus* displayed a death ratio closer to natural death, which occurred when the springtails were fed on uninoculated rice cultures (Supplementary Figure 3).

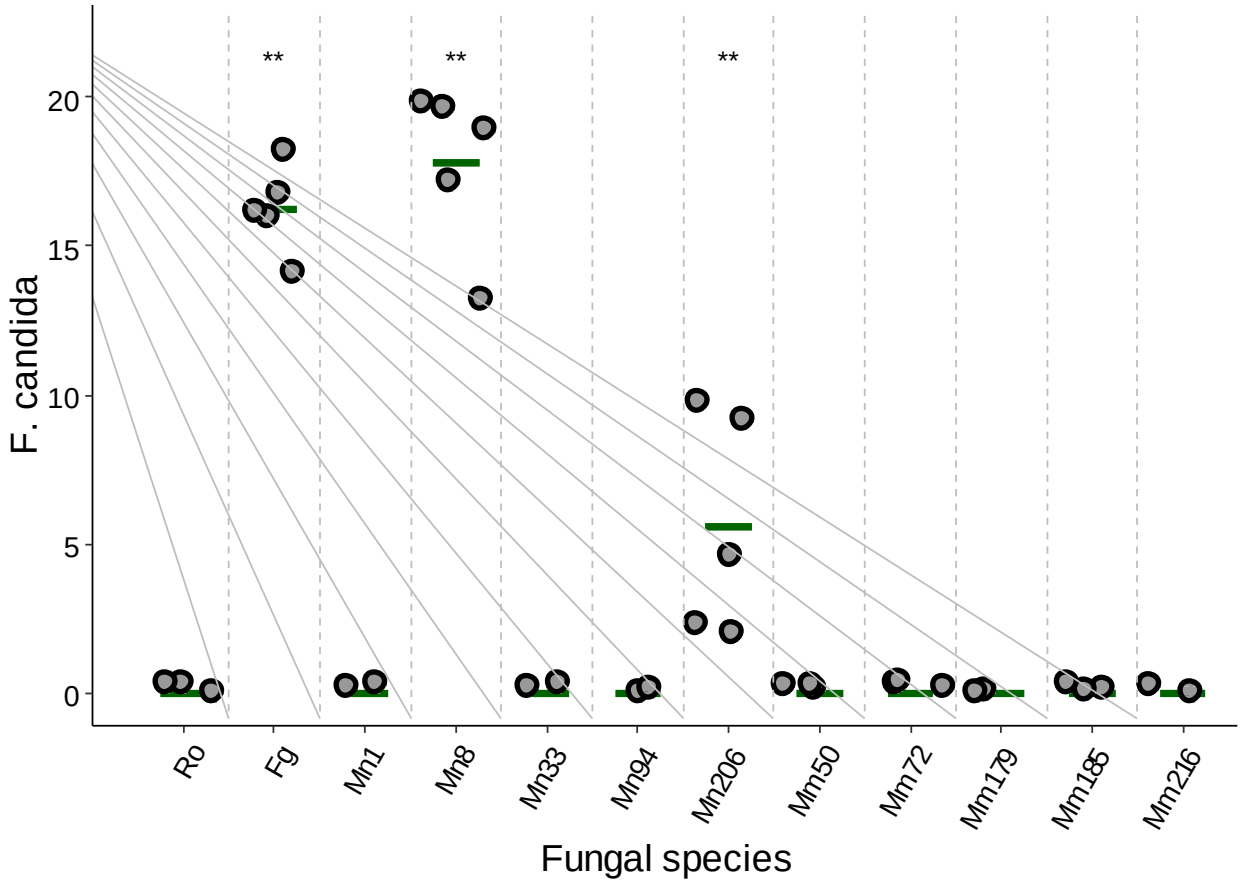


Figure 4. Toxicity of *M. nivale* (Mn) and *M. majus* (Mm) strains to springtails *F. candida*. *Microdochium* (mycelia filtered out of PDB) was offered to springtails at 22°C in the dark for two weeks. The number of dead animals was recorded (n= 5, twenty animals per replicate). *F. graminearum* (Fg) and *R. oryzae* (Ro) served as a positive and negative control. Pairwise comparison of fungal strains against Ro as a reference in multiple comparisons. $*P \leq 0.05$; $**P \leq 0.01$ (Wilcoxon test). The horizontal lines represent means

Table 1. The activity and the productivity of springtail *F. candida* fed on *M. nivale* (Mn), *M. majus* (Mm) strains, *F. graminearum* (Fg), and *R. oryzae* (Ro) for two weeks at 22°C in the dark (n= 5)

	Mn					Mm					Fg	Ro
	1	8	33	94	206	50	72	179	185	216		
Activity	yes	no	yes	yes	no	yes	yes	yes	yes	yes	no	yes
Eggs	yes	no	yes	yes	yes	yes	no	no	yes	yes	no	yes

Yes. The animals are able to move and lay eggs
No. The animals are stable and sterile

4.3.2. Toxicity to *Tenebrio molitor*

The toxicity of *M. nivale* to mealworms was assessed by gaining less weight than the mealworms cultured on uninoculated rice grains. Offering the mealworms with *M. nivale* infected rice grains reduced their weight gain compared to those that were fed on uninoculated rice. The only exception was Mn94. Interestingly, offering the mealworms with *M. nivale* infected rice grains reduced their weight gain dramatically compared to the worms cultured on rice grains infected with *M. majus* (Figure 5). Moreover, offering the mealworms with *M. majus* infected rice grains improved the fitness of the mealworms and their weight gain exceeded that of the control.

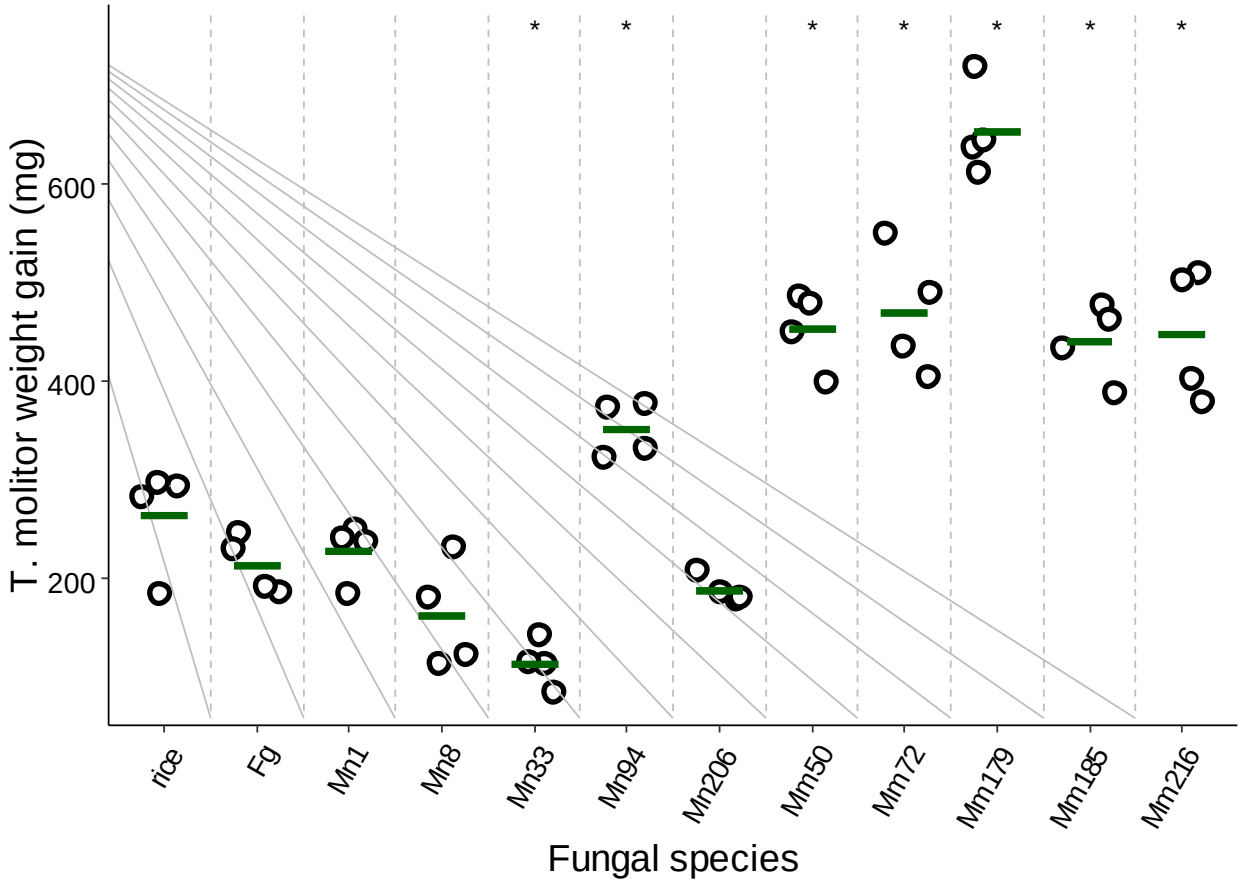


Figure 5. Weight gain by mealworm *T. molitor* feeding on rice grain infected with *M. nivale* (Mn) and *M. majus* (Mm). Rice kernels overgrown with *Microdochium* strains were supplied to the mealworms. After three weeks of feeding at 22°C in the dark, weight gain of the animals was recorded (n= 4, ten animals per replicate). Rice grains infected with *F. graminearum* (Fg) and intact rice grains served as positive and negative controls. Pairwise comparison of fungal strains against rice as a reference in multiple comparisons. Pairwise Wilcoxon, $*P \leq 0.05$. The horizontal lines represent means

A similar experiment was conducted by offering fungal mycelium on agar plugs under similar experimental conditions. The toxicity of *Microdochium* strains to mealworms was assessed

by weight loss (Supplementary Figure 4) and decreased body length (Supplementary Figure 5) compared to the control. Remarkably, mealworms cultured with *M. nivale* gained significantly less weight and length than mealworms cultured on *M. majus*. Examining the impact of the fungal strains individually and comparing them to the reference *R. oryzae* revealed that all *M.nivale* strains and the positive control *F. graminearum* reduced the weight and significantly the length of the worms than *R. oryzae*. Noteworthy, Mn8 was the most effective. In contrast, all *M.majus* strains enhanced the weight and the length of the worms more than *R. oryzae*. Mm185 was the exception.

4.3.3. Effects *Microdochium* isolates on food preference of *Tenebrio molitor*

The behavior of a single mealworm to choose between non-infected and infected grains was recorded for four days consecutively (Figure 6). The observation revealed that most worms gathered on the non-infected grains during the first five hours in all treatments. Later, worms provided with *M. majus* demonstrated a consistent preference for the infected grains until the measurement ended. In comparison, worms that were offered *M. nivale* displayed a wobbling behavior during some time intervals. Anyway, the number of worms feeding on infected grains increased at the measurement end compared to the non-infected controls.

Interestingly, Mn8 infected grains were mostly avoided during four days of incubation. However, in some instances, worms preferred consuming the infected grains with Mm179 rather than the intact grains during the whole measurement period. The opposite case was observed on worms cultivated with Mm185 that preferred feeding on uninfected rather than infected grains.

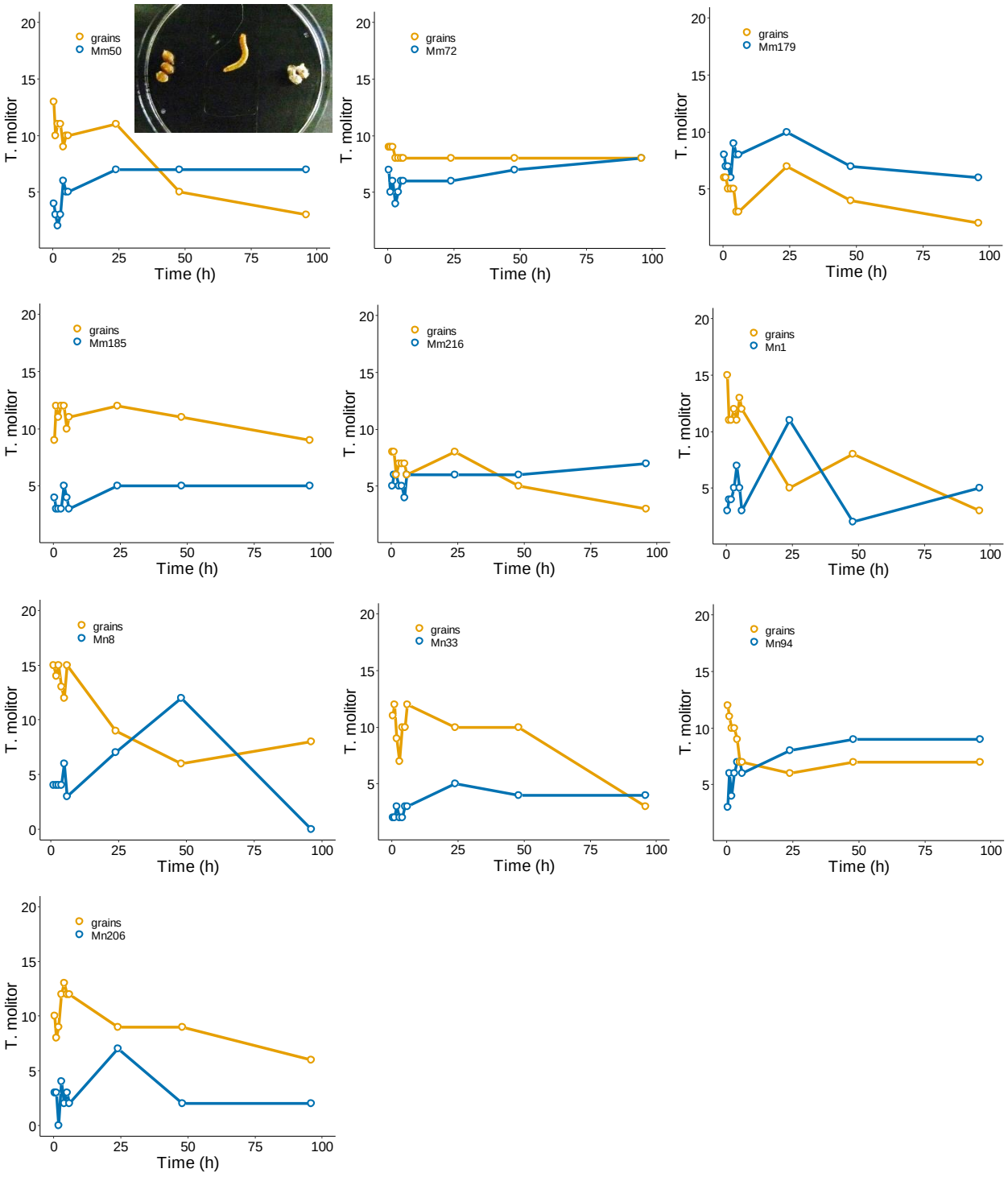


Figure 6. Food choice by mealworms. A single mealworm was placed on Petri dishes between two portions of wheat grains (3-4 grains), one of which was inoculated with *M. nivale* strains Mn1, Mn8, Mn33, Mn94, and Mn206 and *M. majus* strains Mm50, Mm72, Mm179, Mm185, and Mm216 separately, the other remained inoculated. The experiment was carried out at room temperature in the dark. The location of mealworms was recorded within time intervals from 0.5– 96 h (n=20)

4.3.4. Toxicity to Duckweed

Figure 7 illustrates the effects of the extracts of rice cultures inoculated with Mn8, Mm72 and *F. graminearum* on duckweed development. Conc. of 0.2 mg mL^{-1} were tested and the results showed that Mn8 almost suppressed the development of the plant fronds, which appeared colorless whereas, the fronds remained green and growing in the other treatments. The growth rate of duckweeds treated with *F. graminearum* and Mm72 extracts was slower than the plants treated with rice grain extract (Figure 7).

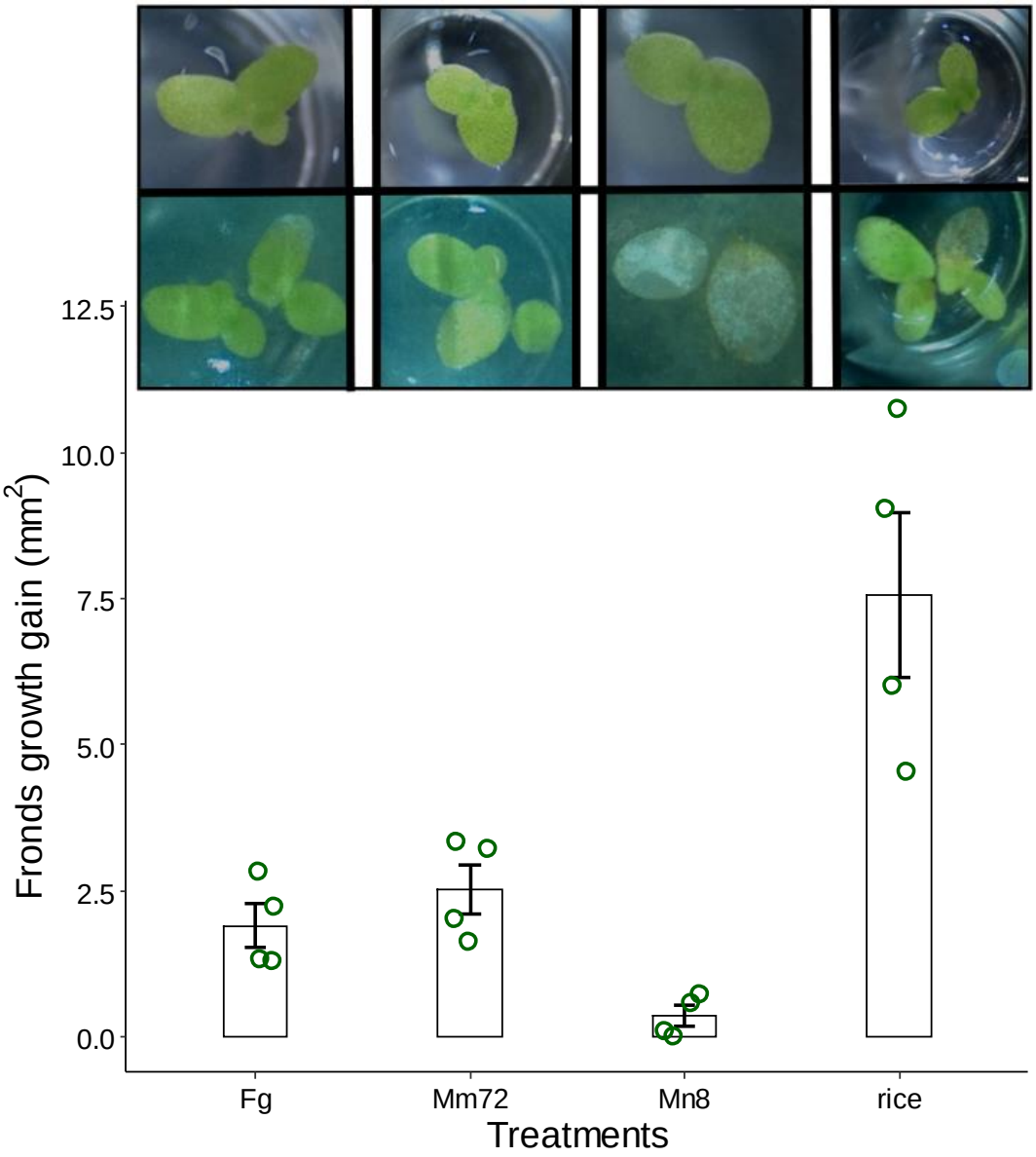


Figure 7. Effect of fungal extracts from rice cultures on duckweed fronds development, *M. nivale* strain (Mn8), *M. majus* (Mm72), and *F. graminearum* rice cultures at 25°C and a 16:8 h light regime for one week (n=4)

4.3.5. Toxicity of *Microdochium* metabolites against brine shrimps and radish seeds

The toxicity of the ethyl acetate extracts of the PDB from fungal cultures was assayed for toxicity against brine shrimps and radish seeds. In brine shrimps, scoring took place after 24 h exposure to the fungal extract by recording the number of dead animals. Figure 8 shows dose-response curves from Mm50 and Mn8; Supplementary Figure 6 shows all strains. Table 2 lists the LD_{50} values of all tested strains from the two *Microdochium* species. The LD_{50} was found for Mn8 (23 µg mL^{-1}). This concentration was much lower than the lethal concentrations determined for the other *M. nivale* strains, Mn1, Mn33, Mn94, Mn206, Mm50, Mn72, Mn179, Mn185, and Mn216 (Table 2). The fitness of brine shrimps in PDB extract showed no difference between the treatments and the control.

In the radish seed assays, the germinated seeds were counted 72 h after exposure to the fungal extract. LD_{50} values are listed in Table 2. Mn8 inhibited the seed germination in all tested concentrations, Mn33, Mn94, and Mn206 yielded LD_{50}'s in the test concentration range; all others failed to do so, including all *M. majus* strains.

Table 2. Lethal doses (LD_{50}) required to affect 50% of the brine shrimps and radish seeds with the fiducial limits

Isolates	**Brine shrimps**	**Radish seeds**
	LD_{50} (µg mL^{-1})	**LD_{50} (µg mL^{-1})**
Mn1	93 (86-99)	a
Mn8	23 (19-27)	b
Mn33	114 (105-122)	55 (66-45)
Mn94	83 (78-90)	36 (45-26)
Mn206	49 (46-53)	23 (35-10)
Mm50	186 (171-202)	a
Mm72	74 (69-77)	a
Mm179	95 (88-102)	a
Mm185	160 (147-173)	a
Mm216	92 (83-101)	a

Brine shrimps and radish seeds were cultured with ethyl acetate fractions of fungi-filtered PDB at several concentrations. Lethal doses (LD_{50}) associated with *M. nivale* (Mn) and *M. majus* (Mm) were recorded
a Lethal doses are higher than the highest tested concentration
b Lethal doses are lower than the lowest tested concentration

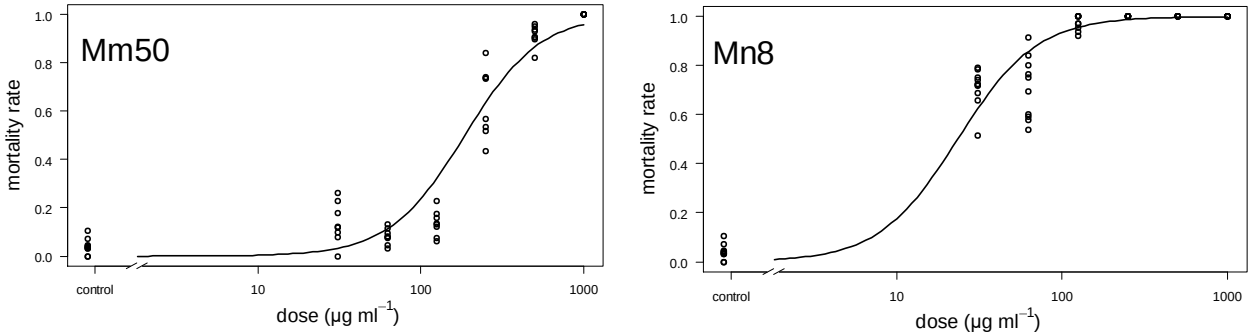


Figure 8. Dose-response curve. The mortality of brine shrimps responds to various concentrations of ethyl acetate fractions of PDB excluded *M. majus* (Mn50) and *M. nivale* (Mn8). The curve fitting of a two-parameter log-logistic model of binomially distributed data with a lower limit is fixed at 0, and the upper limit is fixed at 1. Black dots represent replicates at different concentrations (n= 10)

4.4. Screening of common *Fusarium* mycotoxins.

LC-QQQ/MS analyses revealed that *Microdochium* strains are non-producers of these mycotoxins: Nivalenol, deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3-ADON), 15-acetyl-deoxynivalenol (15-ADON), diacetoxyscirpenol (DAS), T-2 toxin, HT-2 toxin, fusaric acid, enniatins (A, A1, B and B1), zearalenone, beauvericin, NX2, NX4, fusarin X, and aurofusarin.

4.5. Bioassay-guided fractionation and structure elucidation of active compounds

Fitness of brine shrimps and radish seeds treated with ethyl acetate fractions (of the filtered potato broth media cultured with *M. nivale* strain Mn8 then had been fractionated by flash chromatography) showed that out of eight fractions, one fraction exhibited a notable toxic effect against the radish seeds, and another fraction was toxic to brine shrimps (Supplementary Figure 7). Lethal doses of the phytotoxic and cytotoxic fractions required to affect 50% of brine shrimps and radish seeds were 16 µg mL^{-1} each.

4.6. Structure elucidation of the purified compounds.

Figure 9 summarizes bioassay-guided fractionation. Preparative high-pressure liquid chromatography was applied for further purification of the toxic fractions that were isolated by flash chromatography, against brine shrimps and radish seeds. Two metabolites were isolated, a phytotoxic and a cytotoxic metabolite, each with an $LD_{50} \leq 13$ µg mL^{-1} (Supplementary Figure 8).

The purity of the isolated compounds was checked by LC−ELSD, and their accurate masses were determined by LC−TOF/MS in the positive and negative modes. The suggested sum formula for the phytotoxic and the cytotoxic compounds was $C_7H_{12}O_5$ and $C_{11}H_{18}O_2$ according to their predicted accurate masses of *m/z* = 176.068 (the calculated monoisotopic in negative mode M^-H^- is 175.061) and *m/z* = 182.130 (the calculated monoisotopic in positive mode M^+Na^+ is 285.119), respectively. ^{13}C, ^1H, 2D NMR and the optical rotation data showed that the phytotoxic compound is a C7-cyclitol, called streptol (IUPAC name : (1*S*,2*S*,3*S*,4*R*)-5-(hydroxymethyl)cyclohex-5-ene-1,2,3,4-tetrol)). The analysis of the cytotoxic compound is still in progress, and its MS/MS spectra is provided in (Supplementary Figure 9).

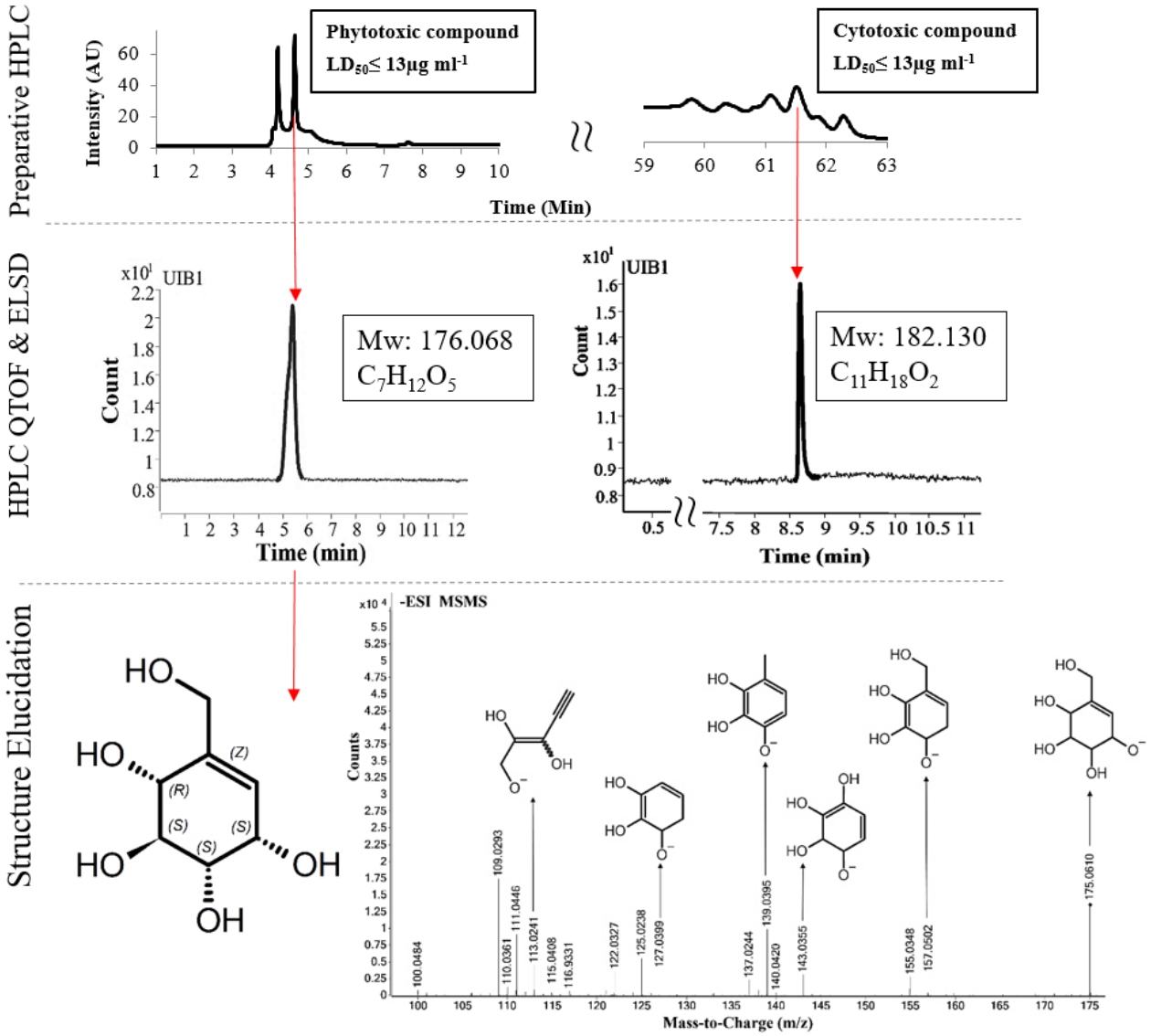


Figure 9. Bioassay-guided fractionation and identification of the bioactive compounds. Two promising compounds were isolated using flash chromatography and preparative HPLC based on their toxicity on brine shrimps and radish seeds inhibition bioassays. The purified compounds were subjected to LC−TOF/MS. The accurate mass of the cytotoxic compound is 182.130 matching the suggested sum formula $C_{11}H_{18}O_2$. The accurate mass of the phytotoxic compound is 176.068, matching the suggested sum formula $C_7H_{12}O_5$. The phytotoxic compound was identified as streptol using ^{13}C, ^{1}H, 2D NMR and optical rotation measurements

5. Discussion

5.1. Identification of the isolates

Colonies of *M. majus* appeared congeneric on freshly prepared PDA, and sporulation occurred in orang sporodochia formed on the agar surface. Under the microscope, mono-septated conidial spores were detected (Gerlach & Nirenberg, 1982). Colonies of *M. nivale* are distinguishable from many *Fusarium* species by the absence of a reddish pigment (Colhoun & Park, 1964). On rice grains, *M. majus* mycelia appeared light pinkish, whereas it is light brownish in *M. nivale*, and in both species the fluffy mycelium covered the top of rice culture. The formation of perithecia depends on the ambient conditions (Lees et al., 1995). Under the current experimental conditions, no perithecia were detected.

5.2. Influence of the temperature on the fungal growth rate

Temperature is a crucial factor in the disease severity of *M.nivale* (Dwyer et al., 2017; Humphreys et al., 1997; Simpson et al., 2000). *M. nivale* grew perfectly at 20−21°C, and the fungal growth was reduced at 0−1°C and 32−33°C (Bennett, 1933). The present work confirmed this finding. In laboratory, *M. nivale* and *M. majus* grew optimally at 16–24°C. The growth was reduced at 8°C and ceased at 28°C.

5.3. Toxicity assessment of *M. nivale*

M. nivale was known as *Fusarium nivale* until 1983, when the fungus has been reclassified into the genus *Microdochium* because of the high phenotypic correlation and the resemblance to species in this genus (Samuels & Hallett, 1983). For many years, *M. nivale* was reported to be a mycotoxin producer for nivalenol and its derivative fusarenon X (Tatsuno et al., 1968). However, reinvestigation of the putatively toxigenic strains that are related to the genus *Fusarium* species has identified *M. nivale* (*F. nivale*) to be non-toxic species, except for one strain, NRRL 3289, which produced a low level of deoxynivalenol (DON). Contrary to earlier reports, screening the same strain a few years later revealed an inability of *M. nivale* to produce DON. The authors of later studies speculated that the reason for these controversial results could be related to re-culturing the

strains for many years without preserving samples as lyophilized stocks (Logrieco et al., 1991; Vesonder et al., 1981).

Assaying extracts of twelve *M. nivale* strains revealed a phytotoxic effect on wheat seedlings. In particular, three of the tested strains displayed strong toxicity to wheat seedlings (Mańka & Chelkowski, 1985). However, all recent studies unanimously reported that *M. nivale* is a non-toxigenic species (Chełkowski et al., 1991; Gagkaeva et al., 2020).

Bioassays against brine shrimp, *A. salina*, mealworm, *T. molitor*, springtail, *F. candida*, radish seed, *R. sativus*, and duckweed, *L. minor* guided the research to exclude the non-toxic species *M. majus* strains from further investigation and purified the bioactive compounds from the most efficient isolate of *M. nivale* strain, which reflects or even exceeds *F. graminearum*. In terms of methods to incorporate the fungal extracts into the diet that is consumed by the test organisms, fungal mycelium from liquid cultures proved more reliable than PDA or rice grain cultures. The springtails and mealworms could accept rice grain and agar as food sources too.

Extracts from fungal strains classified to the same species often show variable bioactivity (Logrieco et al., 1991; Nesbitt et al., 1962). Many factors can affect the biosynthesis and secretion of fungal secondary metabolites even through strains of the same fungal species. For instance, several isolates of *M. nivale* displayed different disease severity because of genetic variation (Abdelhalem et al., 2020). Even under identical cultural circumstances, aurofusarin production can vary between strains (Chapter 2).

Bioassays-guided fractionation is an efficient method for the identification of bioactive compounds (Weller, 2012). Two compounds were isolated successfully, one of which was phytotoxic, the other of which was cytotoxic. The phytotoxic compound could be identified as a C7-cyclitol, called streptol. Streptol was first purified in 1987 from a culture of *Streptomyces* sp. displaying a phytotoxic effect against lettuce seedlings (Isogai et al., 1987). Moreover, streptol had been isolated from the actinobacterium *Dactylosporangium aurantiacum* also on the basis of phytotoxic effects. (Kizuka et al., 2002). It was suggested that streptol is a degradation product of the α-glucosidase inhibitor acarbose isolated from the fermented liquid culture of an actinobacterium, *Actinoplanes* spp. (Mahmud et al., 1999). Recently it is shown that streptol is derived from the biosynthetic pathway of acarbose (Zhao et al., 2020). To our knowledge, this is the first time that streptol was detected as a fungal metabolite.

6. References

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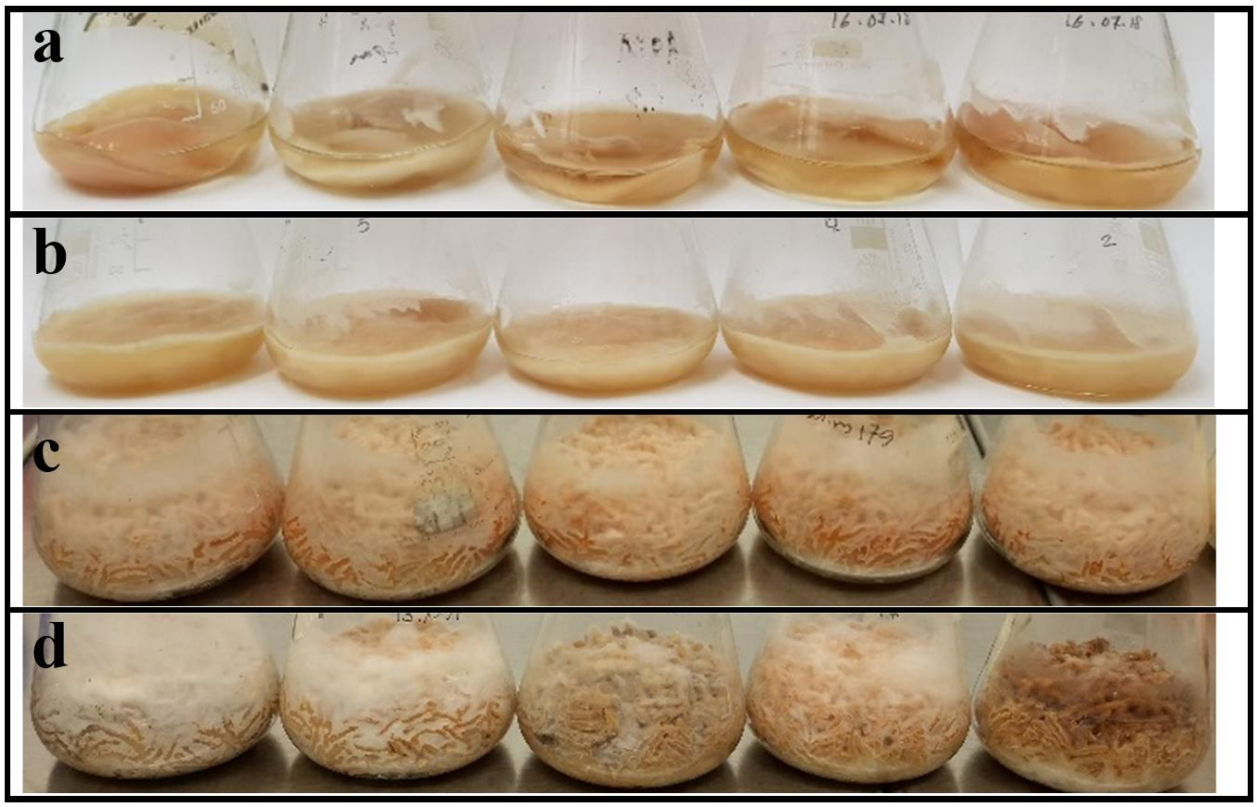
7. Supplementary information



Supplementary Figure 1. Colonies of *Microdochium* strains on PDB and rice grain cultures. They were incubated at 22°C; a) *M. majus* on PDB; b) *M. nivale* on PDB; c) *M. majus* on rice grain cultures d) *M. nivale* on rice grain cultures.

Supplementary Table 1. Accession numbers for reference strains of *Microdochium* spp. used for verifying the identities of the fungal strains used in this study. Nucleotide sequences were obtained from NCBI genbank based on BLAST ®.

Strain used in this study	Species	Identity (%)	Accession
Mn1	*M.nivale*	96	KT692594.1
Mn8	*M.nivale*	99	MN313307.1
Mn33	*M.nivale*	100	MW720810.1
Mn94	*M.nivale*	99	MG098306.1
Mn206	*M.nivale*	100	MN313307.1
Mm50	*M. majus*	99	KP859001.1
Mm72	*M. majus*	100	MZ447941.1
Mm179	*M. majus*	99	MZ447941.1
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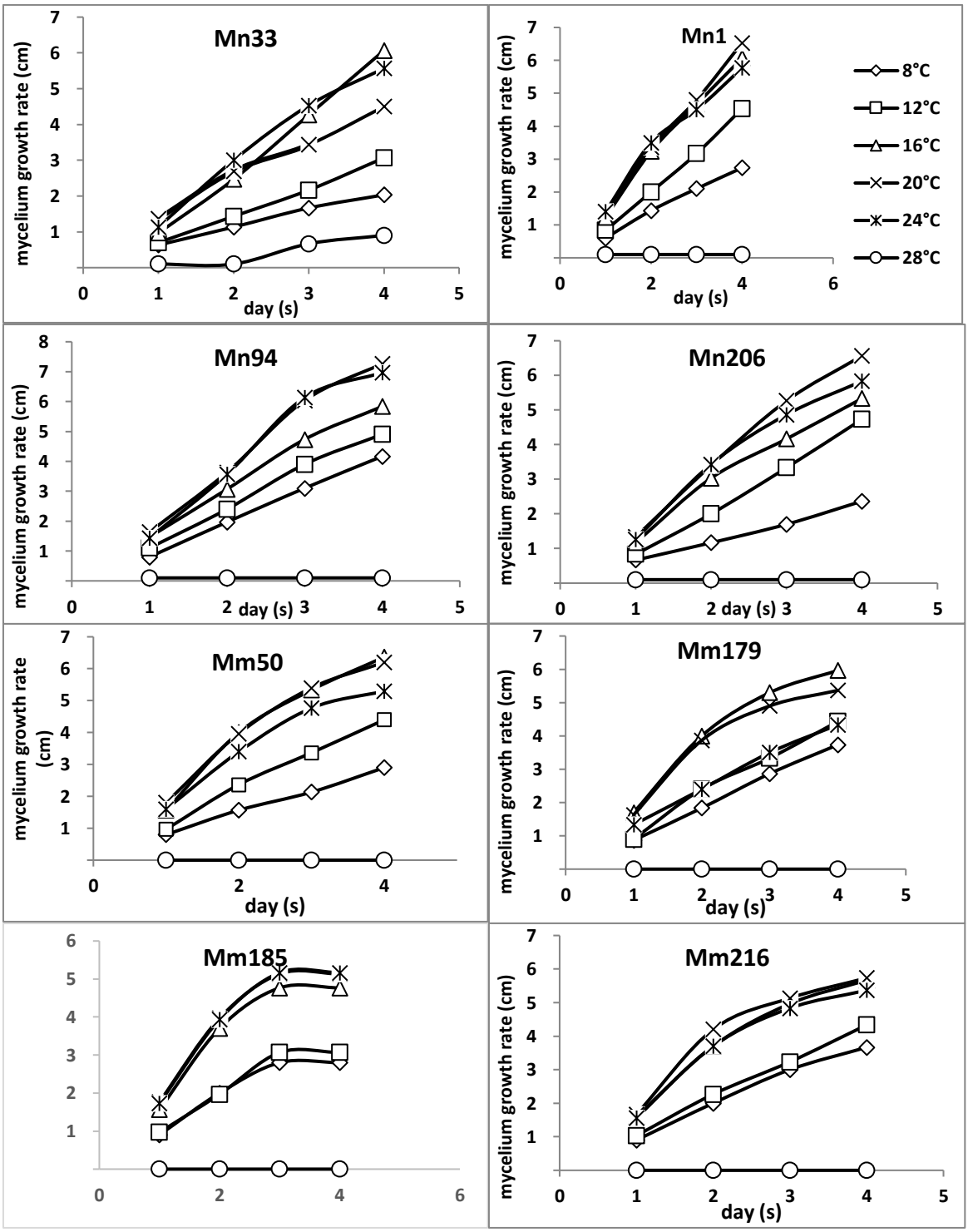
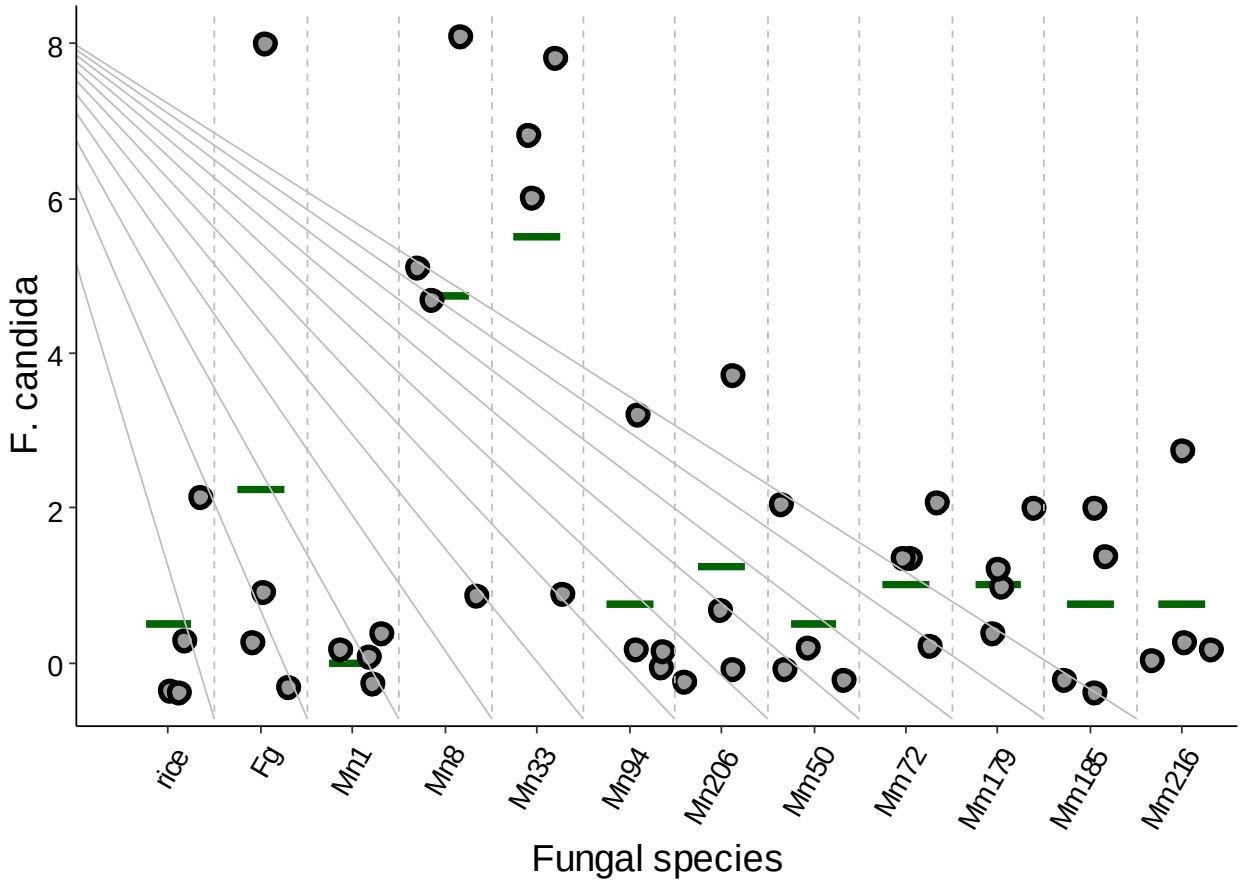
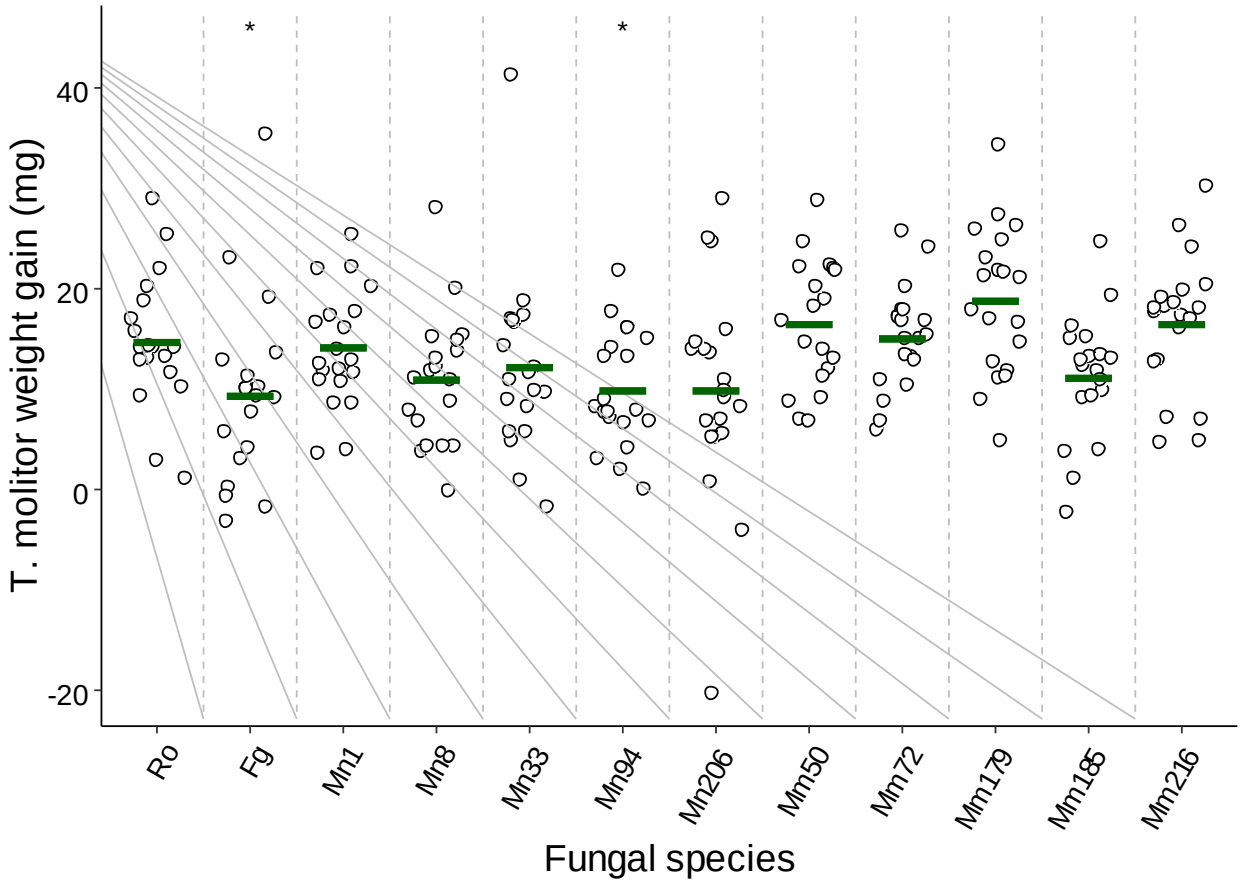
Mm216	*M. majus*	100	MZ447941.1



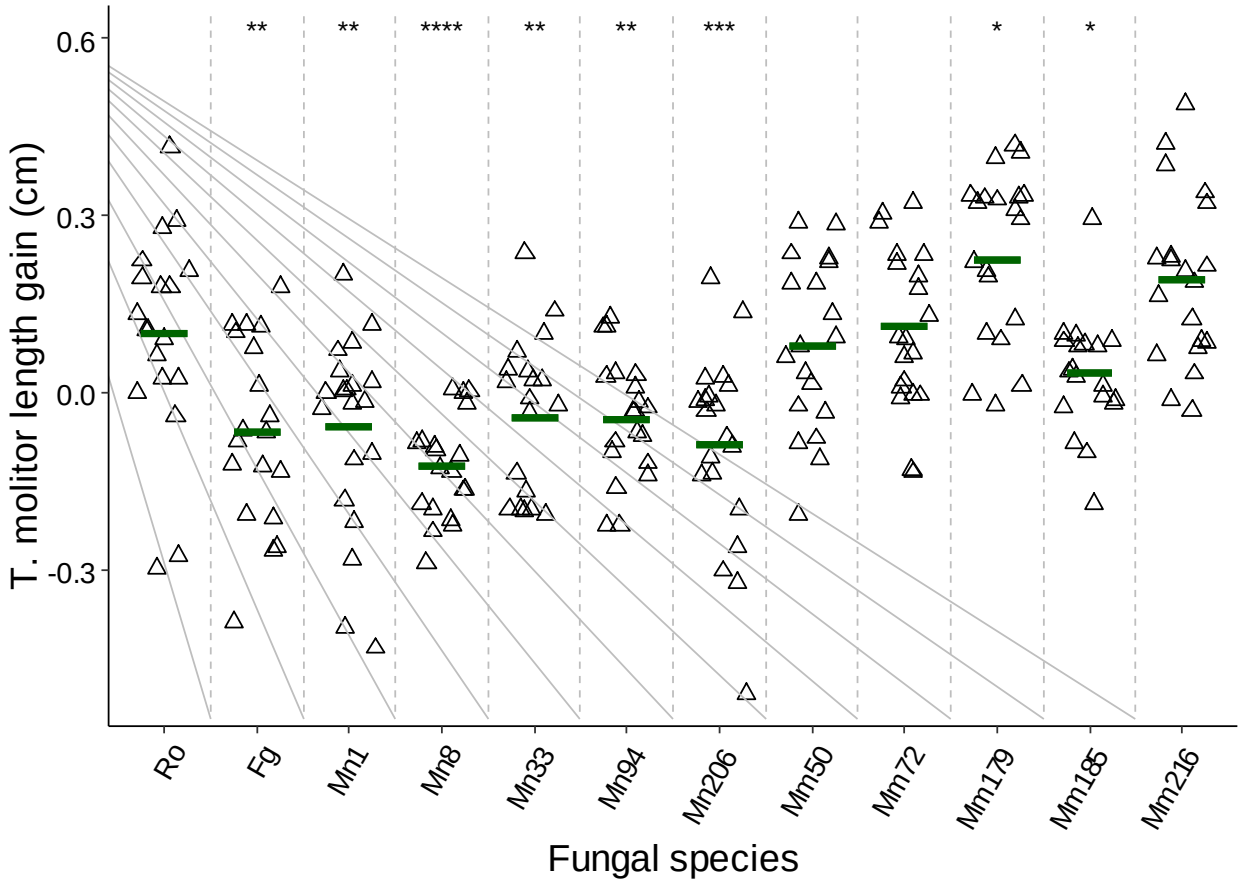
Supplementary Figure 2. Fungal growth rate at different temperatures. Strains of *M. majus* (Mm) and *M. nivale* (Mn) were cultured on PDB at 8, 12, 16, 20, 24, and 28°C. Measurements took place with time intervals from 1 to 4 days (n= 3).



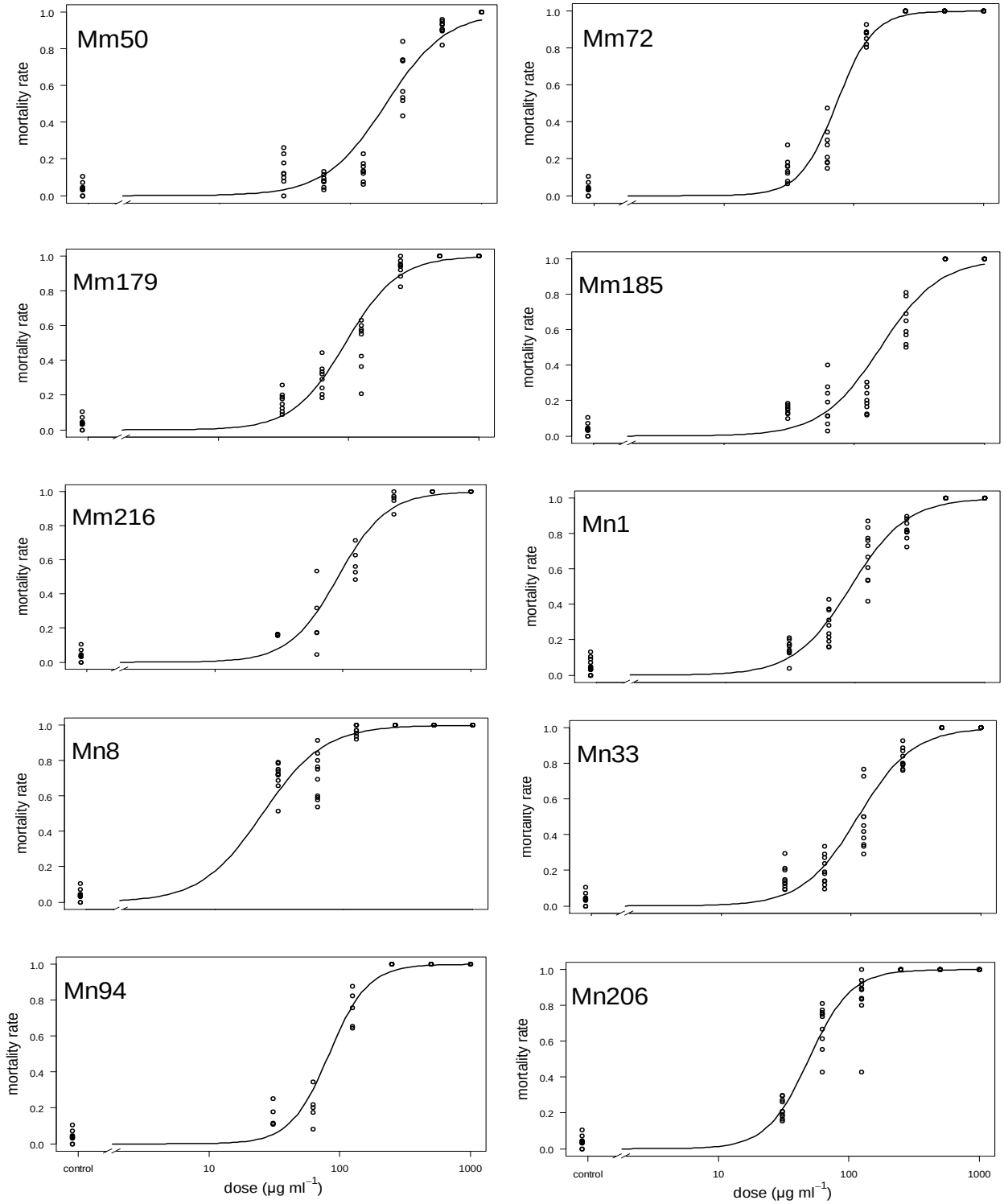
Supplementary Figure 3. Toxicity of *M. nivale* (Mn) and *M. majus* (Mm) strain to the springtails *F. candida*. The animals were cultured with rice grain inoculated with *Microdochium* strains at 22°C for ten days in the dark. On the last day, the number of dead animals was recorded (n= 4, eight animals per replicate). Rice inoculated with *F. graminearum* (Fg) and non-inoculated rice grains served as positive and negative controls. Wilcoxon.test. pairwise comparison with non-inoculated rice grain. The horizontal lines represent the mean.



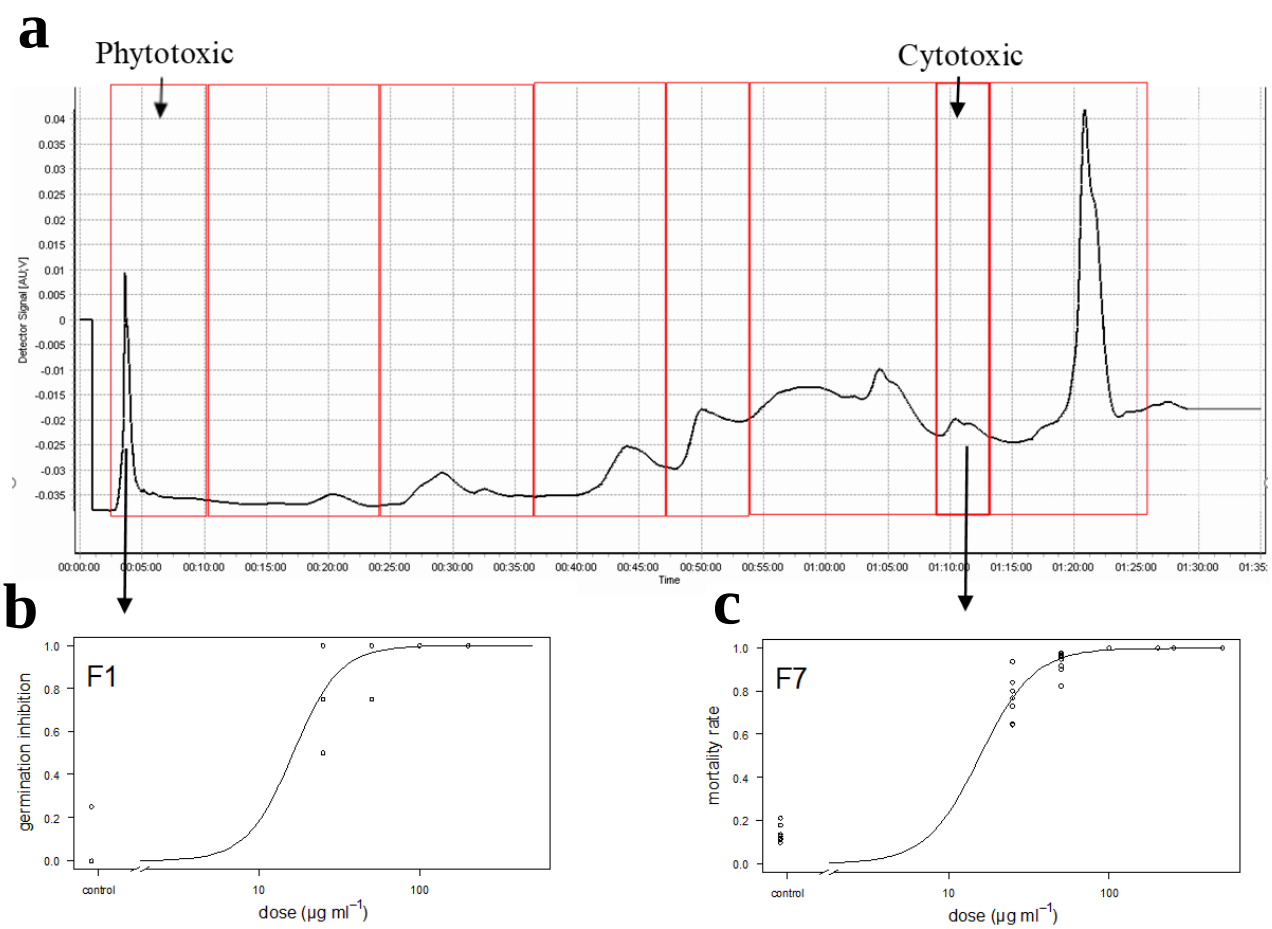
Supplementary Figure 4. Toxicity of *M. nivale* (Mn) and *M. majus* (Mm) to mealworm *T. molitor*. The animals fed for three weeks in the dark at 22°C on *Microdochium* strains cultured on PDA. Gain weight of the animals was recorded individually (n= 20, one worm per replicate). *F. graminearum* (Fg) and *R. oryzae* (Ro) served as positive and negative controls, respectively. Wilcoxon.test. pairwise comparison of fungal strains against Ro as a reference in multiple comparisons. $P > 0.05^{ns}$; $P \leq 0.05$*; $P \leq 0.01$**; $P \leq 0.001$***; $P \leq 0.0001$****. Dots represent data points, and the horizontal lines represent means.



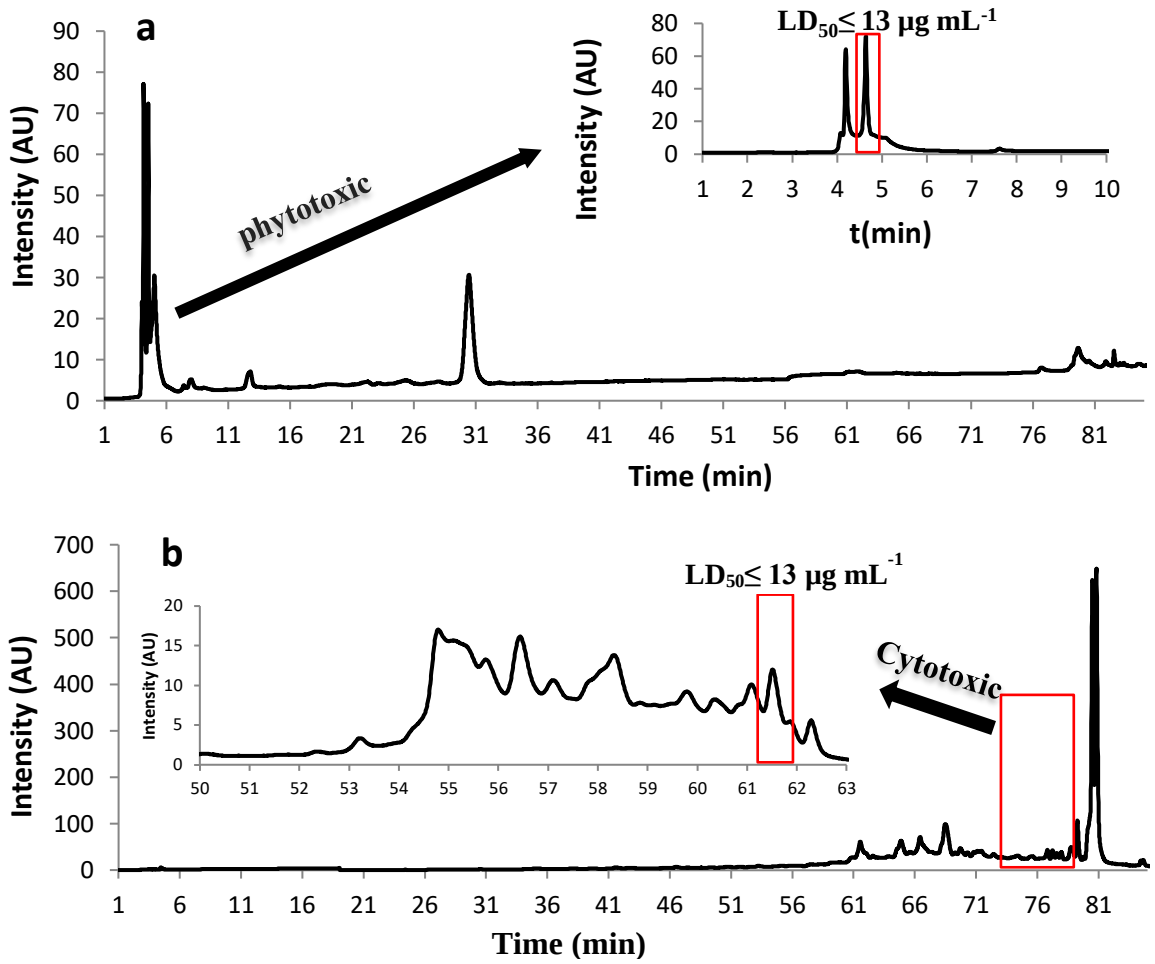
Supplementary Figure 5. Body length gain by the mealworm *T. molitor* fed on *M. nivale* (Mn) and *M. majus* (Mm) mycelium. *Microdochium* strain mycelium on agar plugs were offered to springtails at 22°C in the dark. After three weeks, the length of the worms was recorded (n= 20, one worm per replicate). *F. graminearum* (Fg) and *R. oryzae* (Ro) served as positive and negative control, respectively. Wilcoxon.test. pairwise comparison against Ro as a reference in multiple comparisons. $P > 0.05^{ns}$; $P \leq 0.05$*; $P \leq 0.01$**; $P \leq 0.001$***; $P \leq 0.0001$****. The horizontal lines represent means



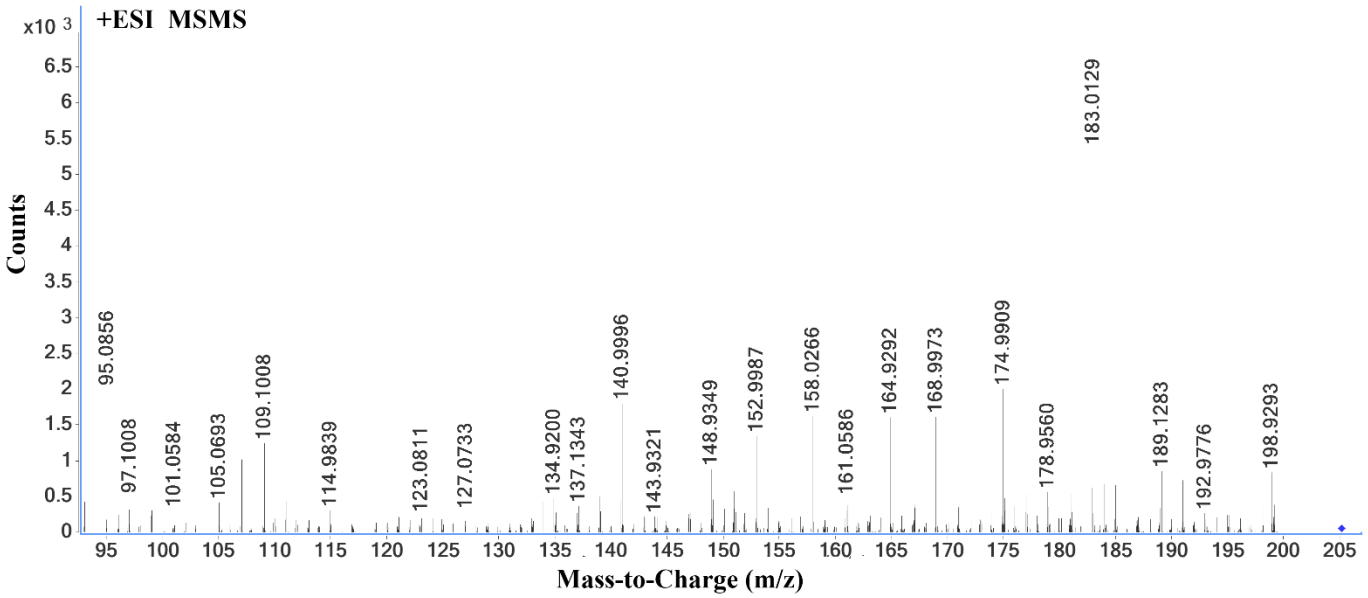
Supplementary Figure 6. Dose-response curves of the ethyl acetate medium extract caused mortality on brine shrimps. Curve fitting of a two-parameter log-logistic model of binomially distributed data with lower limit is fixed at 0, and the upper limit is fixed at 1. Black dots represent replicates at different concentrations (n= 10)



Supplementary Figure 7. Bioassays-guided fractionation by flash chromatography. Potato broth cultured with *M. nivale* strain Mn8 was filtered and extracted with ethyl acetate, then fractionated by flash chromatography. a) Fractions isolated by flash chromatography; b) The inhibition of radish seeds as a response to applying various concentrations of the phytotoxic fraction (n= 4, four seeds per replicate); c) The mortality of brine shrimps as a response to applying various concentrations of the cytotoxic fraction (n= 8). The curve fitting of a two-parameter log-logistic model of binomially distributed data with a lower limit is fixed at 0, and the upper limit is fixed at 1. Black dots represent replicates at different concentrations



Supplementary Figure 8. Purification of flash fractions by prep. HPLC. The fractions were purified two times consecutively. a) the phytotoxic fraction, b) the cytotoxic fraction. each with LD50≤ 13 µg mL^{-1} against brine shrimps and radish seeds



Supplementary Figure 9. MS/MS spectrum of the yet unidentified cytotoxic compound (TOF/MS in positive mode)

Chapter 6. General discussion

This work focused on two topics; 1) Studying the properties of aurofusarin (solubility and stability) and Optimizing selected parameters, including culture media and extraction solvents to enhance aurofusarin yield determining the influence of the cultural conditions on its biosynthesis by *Fusarium* spp. 2) Screening the biological activity of secondary metabolites produced by *Microdochium nivale* and *M. majus* and shed light on the commonalities between all the studies that have been conducted.

Fungi are an important source of the biologically active compounds. It is noteworthy that almost one-third of the natural, effective molecules are supplied by fungi (Demain & Sánchez, 2009). Filamentous fungi belong to the *Ascomycete* (namely *Pezizomycotina* class), and several *Basidiomycete* classes are topping the list in this domain (Keller, 2019). However, evidence of the significance of fungal secondary metabolites for adaptation of their producers to their environment is increasing continuously (Lim & Keller, 2014). The role of secondary metabolites to facilitate the life cycles of their producers, particularly sporulation (Lind et al., 2018) and colonization (Lysøe et al., 2011), or as defense compounds against their competitors (Spraker et al., 2018) has been explored.

Our studies observed a variation in the production of aurofusarin among strains in the same *Fusarium* species, even though all strains were exposed identical cultural conditions. In the same context, we found that one strain in the *M. nivale* (Mn8) was very efficient against mealworms, springtails, brine shrimps, and radish seeds, whilethe other strains influenced the fitness of the tested organisms in some, but not in all, bioassays. Here the question arises, is the biosynthesis of secondary metabolites strain-dependent? In fact, strains are subtypes of the same species which differ slightly at the genetic level (Meyer et al., 1991). But it is well known that the polymorphism in the secondary metabolites gene clusters can change the chemical end-products. For example, genetic variations within the gene (*TRI8*) involved in the trichothecene synthesis in *F.graminearum* result in populations possessing either 3ADON or 15ADON chemotypes (Crippin et al., 2019). In this regard, Lind et al. investigated the polymorphisms of secondary metabolic gene clusters in 66 strains of *Aspergillus fumigatus* and categorized the variation within five distinct types. These

variations affect the genes involved in the production of many mycotoxins such as fumigaclavine, gliotoxin, and helvolic acid, in addition to the production of unidentified compounds; however, it has been challenging to understand the mechanisms that generate the variation of the genes cluster (Lind et al., 2018).

Besides genetic polymorphism, epigenetics may also play a role in the difference in secondary metabolism among strains. The term epigenetics refers to the processes that affect the expression of genes and lead to fully or partially heritable modifications without changing the DNA sequence (Chang et al., 2019; Mukherjee et al., 2019; Weinhold, 2006). The first study in the epigenetic regulation of fungal secondary metabolism was published in 2007 (Shwab et al., 2007). Since then, techniques like chromatin remodeling proteins and inducing silent gene clusters using chemicals have been used intensively, especially in the genera *Aspergillus* and *Fusarium* (Pfannenstiel & Keller, 2019).

Nevertheless, despite the huge number of fungal secondary metabolites, only a relatively small number of the enzymes have been characterized. Thus, the enzymes are less specific compared to the primary metabolism. Furthermore, secondary metabolic pathways usually display wide branching, and the same end product can result from alternative reactions. (Crippin et al., 2019; Vining, 1990). These factors make the puzzle of the differences in the secondary metabolism among strains difficult to resolve.

As mentioned previously, the genes encoding secondary metabolite biosynthesis are sorted into groups known as a biosynthetic gene cluster (BGC). A few classes of fungal secondary metabolites have been defined according to the backbone enzymes involved (Keller, 2019). One of the most diverse classes is the polyketide class, from which aurofusarin is derived. Aurofusarin was extracted and purified from several species in the genus *Fusarium* (Chapter2 and 3). Furthermore, the repellent effect of aurofusarin against fungivores has been proved (Xu et al., 2019).

Away from the aurofusarin, streptol was isolated from a strain in *M. nivale* (Mn8). Streptol is a phytotoxic compound that was isolated from actinobacteria (Isogai et al., 1987; Kizuka et al., 2002). It is derived from the biosynthetic pathway of the acarbose in actinobacterium, *Actinoplanes* spp (Zhao et al., 2020). To our knowledge, streptol has never been isolated from fungi thus far. If the streptol is a bacterial secondary metabolite, how then does it occur in *M. nivale*? The horizontal gene transfer (HGT) mechanism may explain this situation. The genetic material is transferred between micro-organisms, commonly between prokaryotes (Eisen, 2000; Kück et al., 2014).

However, it was reported that *Saccharomyces cerevisiae* received DNA from *Escherichia coli* (Heinemann & Sprague, 1989) and *Agrobacterium tumefaciens* (Bundock et al., 1995). Moreover, it was supposed that HGT could occur indirectly between phylogenetically and geographically independent organisms (Kunin et al., 2005).

The integration of genetic material transferred from prokaryotic to eukaryotes and their collaboration in the genome evolution of recipient strains has been proved, granting fungal strains unique metabolism (Wisecaver & Rokas, 2015). For instance, *S. cerevisiae* can synthesize uracil anaerobically through the acquired bacterial DNA (Hall et al., 2005). Another example is beta-lactam antibiotic genes that have a bacterial origin were detected in *Aspergillus nidulans*, *Penicillium chrysogenum*, and *Acremonium chrysogenum* (Kück et al., 2014). The sequencing of several fungal genomes has eased the detection of HGT that are involved in the fungal evolution. Despite the various barriers, the probability of HGT occurrence is still there (Fitzpatrick, 2012).

What factors can alter the biosynthesis of fungal secondary metabolites?

The biosynthesis of aurofusarin in the same fungal strain was induced or suppressed according to the ambient conditions. On the other hand, strains of *M. nivale* had been recorded as a mycotoxin producer; however, later researches revealed the disability of these strains to secrete toxic metabolites. Many factors can change the secondary metabolism in fungi:

Abiotic factors

Fungal secondary metabolites biosynthesis is responsive to the ambient conditions (Brown, 1926). Many abiotic factors can influence the secretion of secondary metabolites, such as the media composition, temperature, and pH value. Depending on the media composition, the secretion of fungal secondary metabolites can be stimulated or not (Kalinina et al., 2017). A remarkable yield of aurofusarin was obtained in the Raulin-Thom medium compared to the Czapek-Dox medium (Ashley et al., 1937). Noteworthy, providing the last medium with various percentages of glucose-stimulated *Fusarium* sp. to produce different colors (Brierley, 1917). We demonstrated that the artificial PDB medium inhibited the secretion of aurofusarin in *F. graminearum*. In contrast, the self-made PDB was an efficient medium for aurofusarin. Another important factor that influences

the biosynthesis of fungal secondary metabolites is the pH value. We quantified a considerable amount of aurofusarin in *F. graminearum* in neutral cultures at 23°C and the darkness. It was reported that the pH values of 5.5–6.6 (Medentsev et al., 2005) and > 4 (Ashley et al., 1937) enhanced the production of aurofusarin depending on the other ambient conditions. Exposure to light and the thermal effect is important abiotic factors affecting the accumulation of secondary metabolites (Bayram et al., 2008; Joffe & Lisker, 1969; Lind et al., 2018; Nazari et al., 2016; Pruss et al., 2014). Maintaining aurofusarin stabile during the storage and analysis intervals is a challenge in the quantification analysis (Jarolim et al., 2018; Xu et al., 2019). Aurofusarin is a light-susceptible molecule, which degraded quickly when stored in transparent vials. Notably, aurofusarin was stable for few months at -20°C, whereas it was lost at 37°C (Jarolim et al., 2018). The synthesis of aurofusarin by *F. graminearum* was promoted at > 10°C in naturally contaminated wheat during storage (Garcia-Cela et al., 2018). We found that aurofusarin content in pure methanol remains constant overnight. In contrast, aurofusarin content is remarkably decreased in the presence of water. Noteworthy, the biosynthesis of aurofusarin was stimulated by mechanical damage (Xu et al., 2019); conversly, it was suppressed after treatment with H_2O_2 (Medentsev et al., 2005).

Biotic factors

Fungi are exposed to many biotic stresses in their niche. Commonly, the co-cultivation of several organisms influences the production of natural products. For example, *F. sporotrichioides* released T-2 toxin excessively in the presence of *A. flavus* and *P. verrucosum* (Ramakrishna et al., 1996). Likewise, the accumulation of fumonisin B1 in *F. moniliforme* and *F. proliferatum* increased in a shared environment with *F. graminearum* (Velluti et al., 2000). The biosynthesis of aurofusarin by *Fusarium* spp. is inhibited if the culture which contaminated with antimycin A-producing bacteria (Medentsev et al., 2005). In contrast, predation of *F. graminearum* induced the biosynthesis pathway of aurofusarin (Xu et al., 2019).

Finally, the important question is, what benefits do fungi get from the biosynthesis of secondary metabolites like aurofusarin or streptol? Costs of the biosynthesis of secondary metabolites are cheap in the autotrophs (plants) that can recover the loss in their carbons reservoir within a very short time (Foyer et al., 2007). But what about fungi? Fungi are heterotrophs, and the biosynthesis of secondary metabolites is costly. For example, the growth rate of *Gibberella*

zeae mutants, deficient of some genes in polyketide synthase PKS, was enhanced compared to the wild type. (Gaffoor et al., 2005). Particularly, disruption of the genes responsible for the biosynthesis of aurofusarin in *F. graminearum* and G. zeae is accompanied by a robust growth and conidia production in the mutant than the wild type (Kim et al., 2005; Malz et al., 2005). On the other hand, although producing a metabolite like aurofusarin is biologically very costly, especially since the aurofusarin fungal producers synthesize a considerable amount of it (Ashley et al., 1937; Gray et al., 1967; Xu et al., 2019), this single metabolite can protect their producers from being grazed by fungivores (Xu et al., 2019). The advantage of the secondary metabolites for their producer may compensate the costs of biosynthesis.

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Summary and outlook

In this study, we concentrated our concerns on; aurofusarin, the secondary metabolite produced by several filamentous fungal genera, mainly by species in *Fusarium* genera, and on bioactive secondary metabolites produced by *Microdochium nivale* and *M. majus*.

The key characteristic of aurofusarin is the repellent effect against fungivores. We confirmed the previous finding that the red pigment aurofusarin is a water-insoluble metabolite, and it is preferably soluble in non-polar solvents. Aurofusarin dissolved readily in m-cresol and chloroform/methanol. The last solvent mixture is preferable to extract aurofusarin from the fungal mycelium. During the chemical analysis, aurofusarin was protected from degradation by dissolving it in a water-free solvent. Some studies revealed that the biosynthesis of fungal secondary metabolites is dependent on the ambient conditions. Here we demonstrated the inhibition effect of the commercial PDB medium on the accumulation of aurofusarin. Simultaneously aurofusarin was produced generously on freshly prepared PDB. Moreover, the molar extinction coefficient of aurofusarin was determined to be 21,600 $M^{-1}cm^{-1}$ at λ_{max} of 266 nm.

On the other hand, the toxicity of *M. nivale* and *M. majus* against mealworms, springtails, brine shrimps, radish seeds, and duckweeds was investigated. All *M. majus* strains displayed a non-toxic effect, whereas some strains in *M. nivale* strains were toxic to the tested organisms; among them, one strain reduced the fitness of the organisms in all bioassay. Consequently two bioactive compounds (phytotoxic and cytotoxic) were isolated from this strain. The phytotoxic compound has been determined as to bestreptol ((1*S*,2*S*,3*S*,4*R*)-5-(hydroxymethyl)cyclohex-5-ene-1,2,3,4-tetrol). This compound is known as a bacterial secondary metabolite possessing a phytotoxic effect. However, this is the first time to report this compound in fungi.

Recommendations for future research

In this work, we provided an overview of the physical and chemical properties of aurofusarin. Concomitantly, we isolated streptol (phytotoxic agent) from *M. nivale*. However, it remains to be seen if:

• It is possible to identify the inhibitory agents of aurofusarin in the commercial PDB if it is not a lack of the stimulation case.

• The culture aeration at various rates affects the accumulation of aurofusarin.

• all *M. nivale* and *M. majus* strains are able to produce streptol and at which quantity.

• The conserved gene(s) responsible for the biosynthesis of streptol in bacteria is expressed in the *M. nivale* streptol producer strains.

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"***وَمَا أُوتِيتُمْ مِنَ الْعِلْمِ إِلَّا قَلِيلًا***" سورة الإسراء﴿٨٥﴾

"***And mankind have not been given of knowledge except a little***"

Surah al-Isra (85)

Statutory declaration

1. I, hereby, declare that this Ph.D. dissertation has not been presented to any other examining body either in its present or a similar form. Furthermore, I also affirm that I have not applied for a Ph.D. at any other higher school of education.

Göttingen,

.....
Albatol Alsarrag

2. I, hereby, solemnly declare that this dissertation was undertaken independently and without any unauthorized aid.

Göttingen,

.....
Albatol Alsarrag