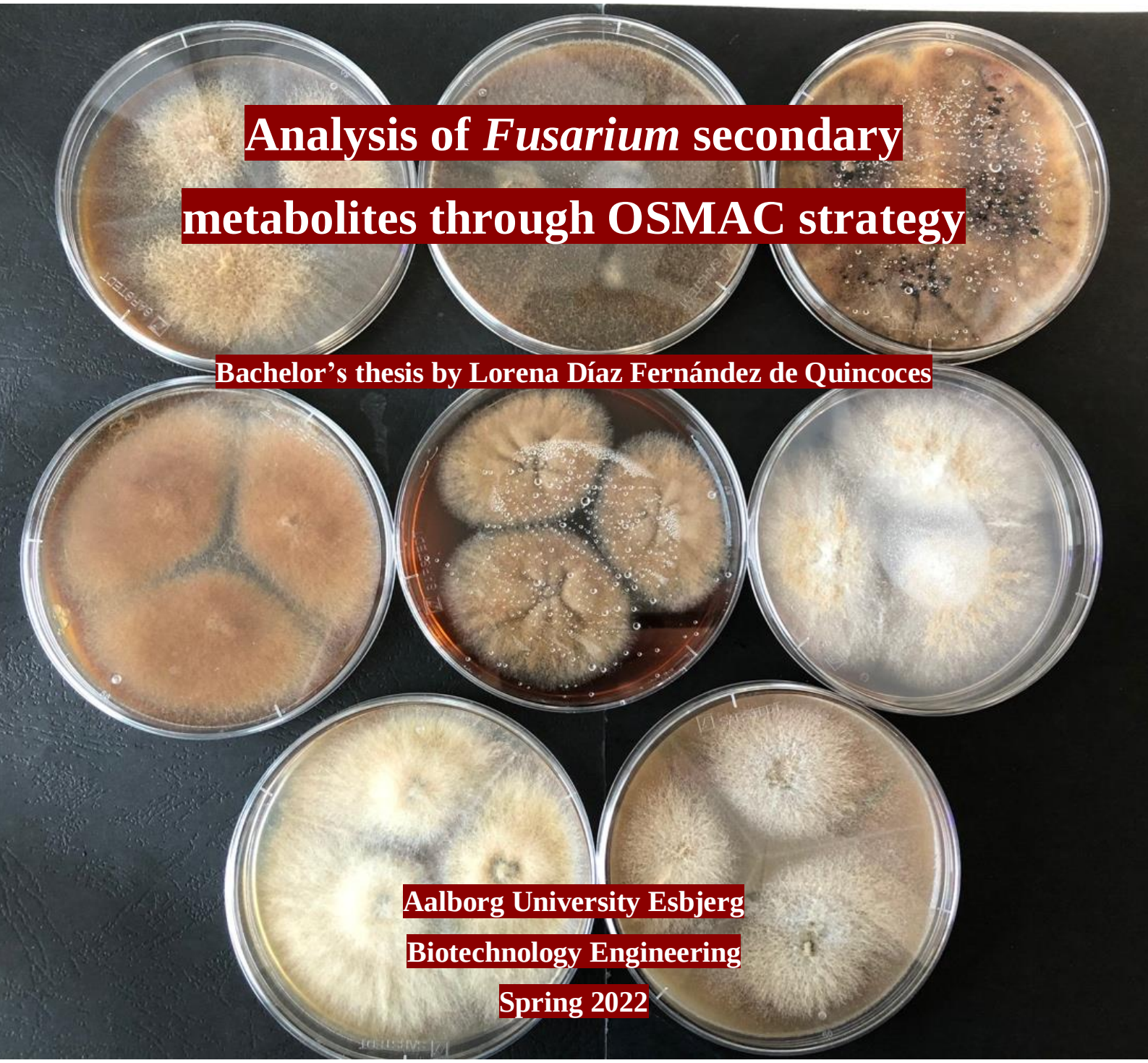


Analysis of *Fusarium* secondary metabolites through OSMAC strategy

Bachelor's thesis by Lorena Díaz Fernández de Quincoces

Aalborg University Esbjerg

Biotechnology Engineering

Spring 2022

AALBORG
UNIVERSITY

STUDENT REPORT

Title Page

Title: Analysis of *Fusarium* secondary metabolites through OSMAC strategy

Bachelor thesis period: Spring 2022

Study program: Bioengineering

Supervisor: Mikkel Rank Nielsen

Page number: 64

Date of completion 31/05/2022

Author: Lorena Díaz Fernández de Quincoces

Abstract

Fungal secondary metabolites (SMs) have been brought to the spot light due to their numerous promising properties in different fields of the industry, and therefore the research focused on the discovery of novel compounds has considerably increased through the last decades. However, the challenge does not only rely on discovering the SMs, but also optimizing their production, and to do so, it is essential to link them to their respective biosynthetic gene clusters (BGCs). The expression of *PKS35* in *Fusarium solani* is still under study as it seems to be a complicated BGC to trigger due to its inactivity in usual growth conditions. To overcome this issue, the present study aimed to study the production of *PKS35* compounds through an OSMAC strategy and several genetically engineered mutants from both *F. solani* and *F. graminearum*. Through phenotypical and metabolical resulting data obtained in this study, it has been possible to draw conclusions regarding the activity of *PKS35* in several media and conditions, pointing to rice agar, YES and PDA as optimum growth media for the study of this BGC, and 3 candidate compounds are proposed. Additionally, UV light seemed to trigger the expression of *PKS35* and it was further linked to perithecia pigmentation in *F. solani*. Still, there were some factors that complicated the study of *PKS35*'s production rates, such as the connected regulation with *PKS3* and the observed interaction with *PKS12*, since a high rate of one of the pathways' intermediates rubrofusarin was yielded when *PKS35* was found active in *F. graminearum* mutants.

Preface

The overall experience I had through completing this report has been very satisfying, as the development of new skills both inside and outside the laboratory have helped to give the best shape to this thesis. I want to thank to Aalborg University and especially my supervisor Mikkel Rank Nielsen for accepting my proposal for this thesis work and helping me along the whole way with advice and recommendations. This has been a great opportunity to learn how to manage new situations and face challenges along the way, which I know will be helpful in the future. This research has indeed offered me a deeper view into the wide range of possibilities the work with microorganisms offers and I'm happy to say it has as well been a great source of motivation to continue with my further studies in the field.

This report does not only represent the effort I put into my bachelor's thesis, but also an important step on my academical path that gets me more engaged towards the biotechnology field and all its promising applications. Thank you.

Aalborg University Esbjerg, May 31st, 2022

INDEX

1 Introduction

Within the last few decades, due to the increasing growth of biotechnologically produced compounds, a further view on different biological products has given the chance to discover promising natural molecules and study their diverse applications for society. Microorganisms of all sorts currently represent a biological factory of promising products that can be obtained through several scientific strategies. Filamentous fungi produce a multitude of low molecular weight bioactive compounds and thanks to the progress done with numerous bioinformatic tools, the increasing number of fungal genome sequences available for study have demonstrated how these microorganisms' potential is far from being exploited (Boruta, 2018). Among others, fungal secondary metabolites (SMs) have gained value in the scientific field due to the diverse novel properties they possess. These are small biocompounds that are not associated directly with growth or reproduction of the concerned microorganism, but rather related to the survival of this one under diverse environmental conditions. These SMs can vary in structure and purpose, while some possess beneficial pharmaceutical properties, others can work as toxins against animals and/ or plants (Li et al., 2020).

Fungi produce SMs varying among polyketides (PKs), non-ribosomal peptides (NRPs) and terpenes (TPs) and, among those, they are known for their production of valuable PKs. PKs derive from a precursor molecule consisting of a chain of alternating ketone and methyl groups. These compounds currently stand in the scientific hotspot due to their chemical and biological diversity, besides the ongoing recent research where increasingly more novel products with promising properties are being discovered. Within the several genus in the fungal kingdom, *Fusarium* represents one of the main producers of valuable SMs in fields such as pharmacy, agriculture or health. This is one of the most economically important genera of plant-pathogenic fungi and includes two species considered among the top 10 plant pathogens based on scientific and economic considerations (Brown et al., 2022). Many studies have revealed the capability of diverse *Fusarium spp.* to yield a wide variety of secondary metabolites (SMs) with a broad spectrum of bioactivities such as alkaloids, jasmonates, anthranilates, cyclic peptides, cyclic depsipeptides, xanthones, quinones, and terpenoids with activities such as phytotoxicity, antimicrobial, cytotoxicity, insecticidal, antioxidant, and antiangiogenic

(Ibrahim et al., 2021). Some popular examples of *Fusarium spp.* SMs are for instance pigments aurofusarin and bikaverin, and mycotoxins equisetin, fumonisin, fusarin and zearalenone. It is not only the discovery of novel SMs that matters, but also the challenge of linking those to their original gene cluster so that further research of their expression can be carried out. The responsible enzymes for the production of these compounds are polyketide synthases (PKS), whose genes are usually organized in a unique biosynthetic gene cluster (BGC).

Research on the linking of PKS genes to their final products has so far offered a closer understanding of the secondary metabolic activity of *Fusarium*.. A recent study (Brown et al., 2022) analyzed a total of 2974 PKS gene sequences from the genomes of 206 *Fusarium spp*. in order to study this genus' fungal products' potential. These PKSs were resolved through a phylogenetic analysis, additional to other 118 previously described PKS from other fungi, into 123 clades. This research proposes each clade to maintain a similarity within its PKSs, which will differ from the ones of other clades. Thus, once again the potential of natural products synthesized by *Fusarium* has been proved to offer future prospects into medical or industrial novel products' development.

Still, there are several cases where the biosynthetic gene clusters (BGCs) haven't been yet linked to their produced compound, and thus can be doomed on the failure on triggering the expression of the gene under laboratory conditions, which remains silent. Such case is of the *PKS35* gene cluster present in *F. solani*, which up to the date has previously been related to the pigmentation of the perithecia in *F. solani* but still hasn't been proved (Graziani et al., 2004).

For this reason, current research is directed towards the study of those silent BGCs and the linking to their produced compounds. Accordingly, fungal genomic analysis has the potential to critically contribute to the exploration of the chemical space of *Fusarium* SMs. It can be complicated to mimic the natural conditions under which certain gene clusters are expressed, and therefore many of those remain silent in laboratory conditions. To overcome this problem, OSMAC (One Strain Many Compounds) strategies rely on the culture of one strain under different medias and conditions, aiming to activate the targeted gene. It is a relatively simple, quick and effective tool to activate those silent BGCs to produce novel SMs. Furthermore, the combination of omic technology and the

OSMAC strategy has future promising prospects in the discovery of new valuable compounds. Genetical engineering as well plays an important role in this research field, enabling the expression of silent BGCs through the application of different strategies (Li et al., 2020).

The present report aims to uncover the identity of the produced compounds by *PKS35* gene cluster present originally in *F. solani*. To trigger the expression of this BGC, both wild type and diverse genetically modified strains from *F. graminearum* and *F. solani* will be cultured. This approach will be done through an OSMAC strategy, where every strain will be cultured in different medias and conditions in order to study whether any of the samples prove to have the concerned BGC triggered. After the growth period, the samples will be processed to proceed an HPLC-UV analysis where the produced SMs will be compared among samples. Apart from the search and identification of final products of *PKS35*, one of the main goals of this research is the chance of further specialization of the optimal growth conditions of *Fusarium* so that the expression rate of this BGC could be higher and extraction and study of its products may become easier in the future.

2 Literature Review

2.1 Fungal Secondary Metabolites

In nature, fungi are challenged by multiple biotic and abiotic stressors, ranging from other microorganisms to nutrient deprivation, pH and temperature. As one physiological response, they produce some bioactive compounds known as secondary metabolites (SMs). This group of chemically diverse molecules is heterogeneous and consists of substances with low molecular weight, characteristically produced by large multimodular polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs) or terpene synthases (TPSs) (Macheleidt et al., 2016). SMs, unlike primary metabolites, are not directly linked to the growth of microorganisms that produce them, but they are usually related to the environmental interactions of the microorganisms, thus producing a selective advantage by increasing their survivability or fecundity (Li et al., 2020).

Several researches have demonstrated how the large number of genes encoding SMs are regulated in a connected manner with fungal development or in response to stressors (both abiotic and biotic). Thus, this SMs will be produced when the fungi is required to protect itself from an exterior stressor, such as the expression of pigments that act as antimicrobial agents for the survival of the producer fungi (Neubauer et al., 2017). The loss or overproduction of specific SMs can alter fungal development, survival or interkingdom and intrakingdom encounters (Keller, 2019). Currently, fungal SMs are in the scientific hotspot due to the big interest they represent for further research in many different fields, such as the food industry, cosmetics industry, medical field etc. In Table 1 some of the most biotechnologically relevant fungal SMs are presented, along with their application and producing fungus.

Table 1 Examples of biotechnologically relevant fungal metabolites (Boruta, 2018)

Secondary Metabolite	**Producing fungus**	**Application**
Astaxanthin	*Phaffia rhodozyma*	Pigment
ß-carotene	*Blakeslea trispora*	Pigment
Cephalosporin C	*Acremonium chrysogenum*	Resource for the production of cephalosporins
Cyclosporin A	*Tolypocladium inflatum*	Immunosuppressants
Gibberelic acid	*Gibberella fujikoruoi*	Plant growth regulator
Griseofulvin	*Penicillium griseofulvum*	Antifungal agent
Lovastatin	*Apergillus terreus*	Cholesterol-lowering drug
Monascin, Ankaflavin, Monascorubin, Rubropunctatin	*Monascus sp.*	Pigments
Mycophenolic acid	*Penicillium sp.*	Immunosuppressant
Penicilin G	*Penicillium rubens*	Antibiotic
Taxol	*Taxomyces andreanae*	Anticancer drug

There are several classes of fungal SMs, from toxins, which can be used to protect plants from possible plagues in the agricultural industry, to relevant biocompounds used as drugs in the medical industry, such as penicillin. In this scenario, it is clear the relevance that these bioactive compounds represent for the development of several fields, and therefore it is unquestionable the priority that has been given to further research on the discovery of novel compounds that could possess new properties (Brakhage, 2013). Up to the date, some of the most relevant biotechnological fungal metabolites include penicillin, a β-lactam antibiotic; lovastatin, a cholesterol-lowering drug; or cyclosporine, a molecule with a immunosuppressive effect that inhibits the production of cytokines involved in T-cell activation (Russell et al., 1992a). The study (Schueffler et al., 2014) presents and discusses the fungal natural products that were discovered between 2009 and 2013, showing the wide range of products based on fungal SMs that have been brought to the market and the many more that are expected.

2.2 Secondary metabolites of the *Fusarium* genus

Since the discovery of penicillin in Fleming's experiments, and many other popular drugs that have been found on the way, the overall potential of fungus' SMs has slowly been unraveled through further research during the last few decades. However, the wide amount of biosynthetic gene clusters (BGCs) in a single filamentous fungal genome combined with the historic number of sequenced genomes suggests that the SM wealth is still largely untapped. Although the underlying interest in fungal SMs is multivariate, most reviews highlight the predominant interest in fungal SMs destined to drug discovery (Keller, 2019), for which mining algorithms and scalable expression platforms have greatly expanded access to the chemical repertoire of fungal-derived SMs. One of the most popular fungal genus that has been described and studied up to the date is *Fusarium*. Besides functions as mycotoxins, other of their fungal specialized metabolites play roles in host infection, interspecies competition and defense against predators (Hijri et al., 2018). So far, at least 272 compounds have been reported to be exclusively produced by *Fusarium* genus, setting this species as a treasure trove of SMs with unique chemical properties (Li et al., 2020).

Member of the genus *Fusarium* are well known for the great source of bioactive compounds that they represent in current research, belonging most of these SMs to the chemical groups of polyketides (PKSs) and non-ribosomal peptides (NRPs) (Hansen et al., 2015). So far, out of the whole *Fusarium* spp., 67 PKS BGCs and 52 NRPS BGCs have been identified across its genome and, out of these, only 16 PKS and 13 NRPS genes have so far been linked to their product (Figure 1 Identified PKSs and NRPs metabolites from Fusarium in the study (Nielsen et al., 2019)) (Nielsen et al., 2019).

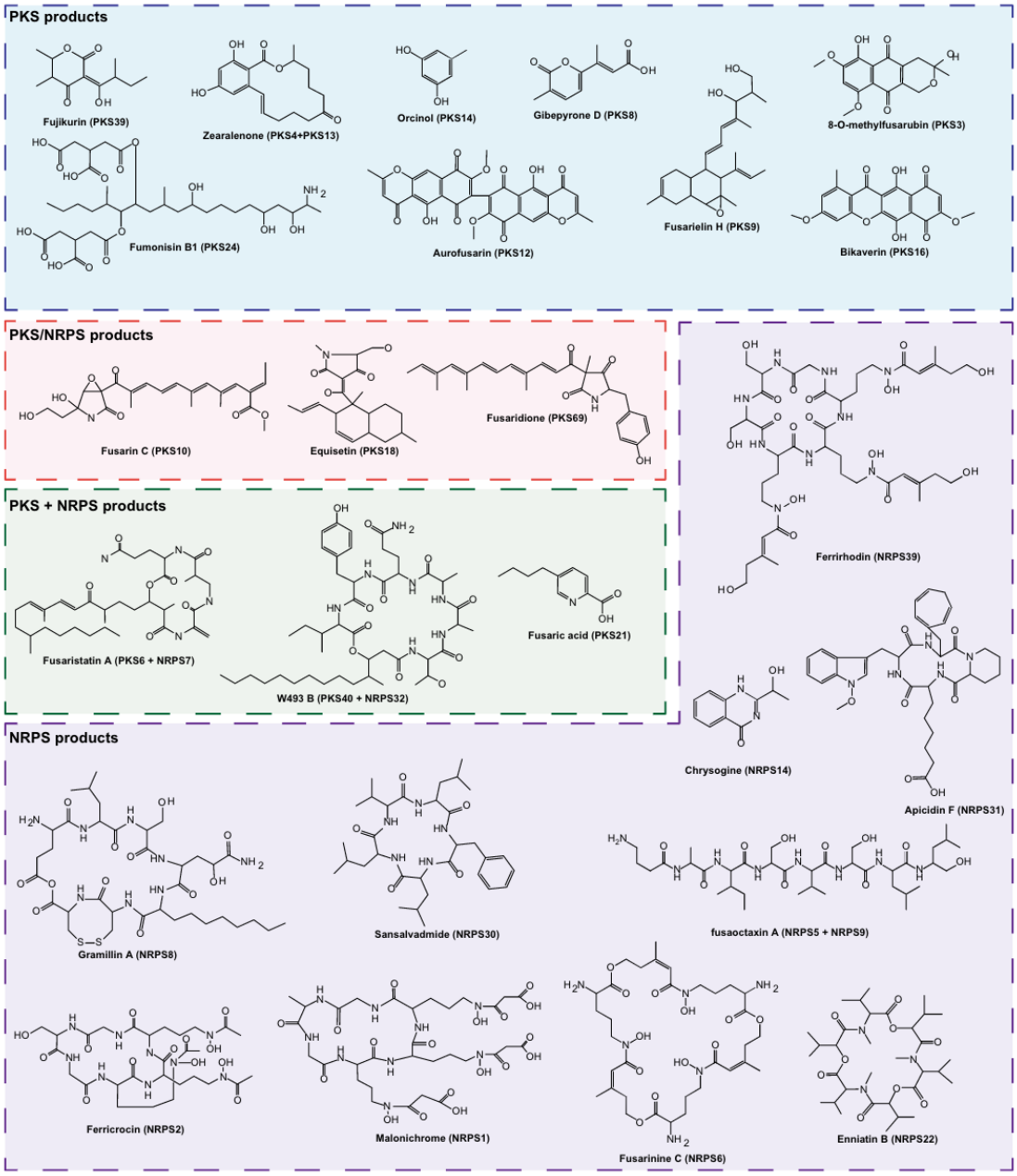


Figure 1 Identified PKSs and NRPs metabolites from Fusarium in the study (Nielsen et al., 2019)

Some studies have examined the presence of different known BGCs in 31 species of *Fusarium* and their results offer an overall view of the diversity of these gene clusters among the subjected species (Brown et al., 2016; Hansen et al., 2012, 2015; Hijri et al., 2018). Analyzing the graph represented in Figure 2, it is possible to observe the frequency of each *PKS* gene cluster in the different *Fusarium spp*. For instance, *PKS3*, responsible for the expression of fusarubins and bostrycoidin, is found in every *Fusarium spp*.. This compound is known to be related to the perithecium pigmentation in most species, and it

has been found responsible for its black coloring (Frandsen et al., 2016). Along with this, also other *PKS* are commonly found in different *Fusarium spp.*, such as *PKS10*, responsible for the synthesis of fusarins, or *PKS16*, which expresses the known compound bikaverin. There are some BGCs that, on the other hand, are not so frequently found in many *Fusariums spp.*, and therefore their function gains another sort of interest, since it may represent some sort of unique advantage. Gene clusters such as *PKS12*, (responsible for aurofusarin), or the *PKS4* and *PKS13* (which synthesis zearalenone) were not found to be expressed by many *Fusarium spp*. (Hansen et al., 2012).

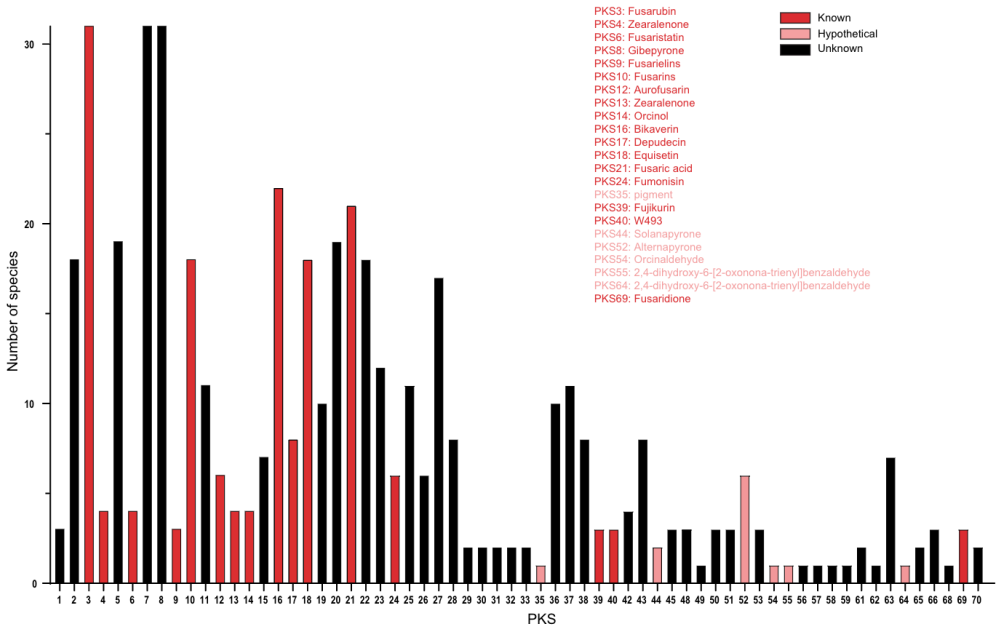


Figure 2 Collected information about the rate of PKS gene clusters in 31 different Fusarium spp in the study (Nielsen et al., 2019)

Currently, several *PKS* products have been unraveled and studied in numerous *Fusarium spp.*, while many others remain unknown due to the unawareness of their activation conditions, as it is the case for *PKS35*. This BGC is present so far only in *F. solani* and is related to its perithecial pigmentation, while in the rest of *Fusarium spp.*, *PKS3* is responsible for this role (Graziani et al., 2004). Fusarubins and bostrycoidin, products of *PKS3*, function as a protection tool of the ascospores inside the perithecium from harming conditions for the fungi, such as UV light or oxidation from reactive oxygen species (Frandsen et al., 2016; Keller, 2019). However, in *F. solani* this gene cluster is believed

to be behind mycelial pigmentation. This peculiar change of roles in the case of *F. solani* has drawn the attention of the science community and has directed the research towards the study of the still unknown bioproduct of *PKS35* and whether its metabolic pathway is somehow similar or connected to the one of *PKS3*.

2.3 Functional crosstalk of different biosynthetic pathways

The biosynthesis of SMs are given by certain pathways that proceed through a series of intermediates which accumulate for a finite period of time as the reactions continue towards the production of the final compound. Eventually, all flux through the pathway would end and only the final product would exist, but this seems quite unlikely as in reality the situation is complicated by varying rates of reaction for the different steps. As many genes are parallelly expressed in one microorganism, it is very likely that these several pathways will converge at different points, with multiple units made in parallel before assembly into the final product. Thus, in practice, any biosynthetic pathway can lead to the accumulation of a mixture comprising the final product and the additional varying concentrations of pathway intermediates and shunt metabolites. The composition of such a mixture will further vary when alternating growth conditions are used (Senges et al., 2018). The identification of this mixture may provide a further understanding of the subjected biosynthetic pathway and the possible additional interactions (Qin et al., 2019). Additionally, the functional crosstalk between two different biosynthetic pathways is not only of considerable value in increasing the structural diversity of NPs, but also represents a new way to construct new drug of natural origin.

The study of novel SMs is indeed complicated by this convergence of pathways, where the formation of the targeted product might be intersected by another product's biosynthetic pathway. Filamentous fungi are popular for the valuable source of NPs they represent, which is supported by a complex underlying mechanism that is still poorly understood and needs further exploration (Dai et al., 2022). There have been several studies with *Fusarium spp*. where the study of one certain SM would lead to the discovery of converging pathways, which represents the complexity of the regulatory systems that take place within the fungi. For instance, in the study (Rank et al., 2020), it was observed how the overexpression (OE) of the transcription factor of *PKS35* led to the OE of

additional *PKS3* compounds. This phenomenon leads to the believe of the regulation of both BGCs being connected, which would add further challenge to the study of one single pathway.

2.4 Biosynthesis of fungal PKs

In fungi, PKs are synthesized by large multi-domain enzymes called polyketide synthases (PKSs) that catalyze the iterative condensation of simple carboxylic acids. These enzymes will build the scaffold of the compounds, which after being release from the PKS, can be further modified by oxygenases, reductases, and/or cyclases or by other enzymes that catalyze addition of terpenes, fatty acids or even other PKs. The final compound can vary from single aromatic-ring compounds, sucg as orsellinic acid, or multiring compounds like aflatoxins, to simpler compunds as linear structures with an amine or sugar substituent like fumonisin or fusapyrone, respectively (Brown et al., 2022). The set of enzymes responsible for the whole synthesis of one PK is encoded altogether by a biosynthesis gene cluster (BGC), which is organized in modules. Each module will be responsible for one discrete chain elongation step and this can be subdivided into domains that control the choice of extender unit. Diverse modifications can take place on each of the intermediates of the overall process. Several studies have researched the possibility of modular NRPS–PKS hybrid systems working together in order to form hybrid products such as rapamycin or aspyridones (Bergmann et al., 2007; Cane et al., 1999).

2.4.1 PKS synthesis

The PKs family is popular for both its structural diversity and biological activity, and some of their examples are widely known clinically important molecules (the cholesterol lowing drug lovastatin, antifungal drug griseofulvin, etc.) (Minami et al., 2020). These molecules are constructed from simple carboxylic acid-derived compounds and to do so filamentous fungi employs proteins known as polyketides synthases (PKS), multienzyme domains consisting of numerous domains of covalently-linked catalytic domains. Each PKS module is made up of a set of three core domains. These domains together are

responsible for the central structural reactions of PK biosynthesis, additional to a diverse set of auxiliary domains that mediate several modifications (reduction, dehydration and methylation) in order to obtain the final product (Cane et al., 1999).

The fungal PKSs always include three core domains, as mentioned: a keto-acyl synthase (KS), acyl transferase (AT), and acyl carrier protein (ACP) domain, since these are the minimal set of domains required for a carboxylic acid condensation. This core biosynthesis involves the condensation of a starter unit, such as an acetyl-CoA or propionyl-CoA, with an extender unit (malonyl-CoA or methylmalonyl-CoA). This condensation reaction is followed by the decarboxylation of the extender unit, where a beta-keto functional group is yielded and a carbon dioxide is released. Each PKS will be unique of a PK chain, as they contain diverse combinations of domains that will ultimately generate the final product (Moretto et al., 2019).

Additional to the common reactions for every PKs, the PKS can also induce supplementary domains for further modification of the product, such as the ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains, which enable the reduction of carbonyl groups after each cycle of condensation. Some fungal PKSs can as well include a methyl transferase (MT) domain, which catalyzes the incorporation of one or more methyl groups into the PK carbon skeleton (Proctor et al., 2007). This is the reason why there's no one unique final product for some BGCs, but they can produce a variety of different SMs as a result of this phenomena. An example of this is the expression of the BGC *PKS3*, a gene cluster capable of producing several products in the same fungi, such as in *F. solani* where *PKS3* is capable of producing bostrycoidin, fusarubin, javanicin or matricin among others.

BGCs can also contain genes that encode regulatory proteins responsible for the activation of the transcription of the cluster genes and proteins that transport the PKs across the cell membrane. The set of genes that encode the PKS and modifying enzymes that work in one unique PK are most of the times located next to each other in BGCs, which are often coordinately regulated. As a result, they exhibit patterns of expression that are correlated with production of the corresponding PKs (Proctor et al., 2003).

Depending on the structure of the PKS and the resulting molecule, they produce two different subgroups: type I PKS and type II PKS. While type I PKSs are single-chain proteins composed of different domains, type II PKSs are multi-enzyme compexes consistant of multiple monofunctional proteins. The type I PKS can be further classified into two subgroups, known as the iterative type I PKS (iPKS) and modular type I PKS (mPKS). The iPKS are common in fungi and the domains catalyze repeated rounds of elongation, while mPKS are rather found in bacteria and their working activity is similar to this of NRPs. The type II PKSs produce highly diverse PK chains by sequential condensation of the starter units with extender units, followed by reduction, cyclization, aromatization and tailoring reactions. These are usually responsible for the biosynthesis of aromatic PKs, polycyclic compounds containing at least one aromatic ring. Aromatic PKs have demonstrated a big potential in the clinical and environmental fields due to their antibacterial, anticancer and antiviral bioactivities, and therefore have attracted increasing attention to this research line (Wang et al., 2020).

2.5 Strategies to activate silent gene clusters

The analysis of fungal genomes through the past decades has revealed that the number of SMs that fungi can produce is much higher than what can be isolated and identified using classical approaches. To isolate and study those metabolites, it is first necessary to link them to their original BGC in order to study their biosynthetic pathways and expression patterns, which sometimes can be complicated as some BGCs remain silent under laboratory conditions. Numerous techniques are employed to face this challenge, from the use of bioinformatic tools, to the application of popular genetical engineering techniques (knock-out gene, overexpression...) or variation of growth conditions (Keller, 2019). It is also possible to combine different techniques to approach the matter, as it was done for instance in the study (Scherlach et al., 2006), where the aspoquinolones A-D was discovered by combining bioinformatic data with variation of the cultivation parameters.

2.5.1 Knock-out gene technique

Among the genetical engineering techniques that are currently employed for the mining of new SMs, the "knock-out" strategy relies on the inactivation of a BGC (by deletion of its core gene) followed by a comparative metabolic profile analysis of the mutant and the wild type through an HPLC or LC-MS analysis (Chiang et al., 2008). The goal of this technique is to link one product or activity to its BGC through a targeted deletion of that gene cluster. A comparative study between the wild type and the mutant where a gene was knocked out is carried out, where it's possible to study the functional differences between both strains. This way, the metabolic difference that is pointed out in the analysis is related to the gene that was initially knocked out in the mutant.

Besides the mining of new NPs, the knock-out technique can also enhance the activation of silent gene clusters through the inactivation of negative regulatory genes or the deletion of genes whose activity could cast a shadow over the expression of the silent ones (Zhou et al., 2021).

2.5.2 Overexpression

Other alternative strategies have been developed in order to activate silent BGCs, such as overexpression (OE) (Corre et al., 2008). When working with fungal BGCs, OE is quite a promising strategy that relies on the manipulation or addition of regulators of a BGC, as these will act upon all the genes required for the biosynthesis of the compound which are clustered together. After the OE of a BGC, an increase on its expression is expected, which therefore eases the possible study of its products. This approach is feasible since one single regulator controls the expression of all members of the BGC to a certain extent (Brakhage et al., 2011). In the study carried out by (Bergmann et al., 2007), a pathway-specific regulatory gene of *A. nidulans* designated *apdR* which encodes a Zn_2Cys_6 transcription factor was OE. This OE led to the activation of a silent BGC that produced two novel PKS–NRPS hybrid metabolites, named aspyridones A and B.

2.5.3 Heterologous expression

Alternatively, another common approach for the activation of silent gene clusters is through the heterologous expression (HE) in a different host, which avoids the complexity of the regulatory network in the host cells. Therefore, reconstruction and HE of the biosynthetic pathways can release the products from the complex metabolic network. However, when the BGC is controlled by promoters of different strengths the complexity of the engineering reconstruction becomes more challenging (Zhou et al., 2021). Also, the need of post-translational modifications of some of the regulators needs to be considered, as otherwise the experiment may not work or the activity could considerably be decreased. Post transcriptional mechanisms will always be an additional difficulty when working with HE and fungi: intron splicing, misfolding of peptide, lack of precursors or cellular trafficking remain as challenges to be overcome in the development of this strategy (Keller, 2019).

2.5.4 OSMAC strategy

Apart from the methods that involve genetical engineering of the strains, it is as well possible to induce the expression of gene clusters through the introduction of the fungi to difficult cultivation conditions. The formation of a particular SM proceeds under specific circumstances, in other words, it is typical for a fungus to reveal only a fraction of its chemical diversity under a given set of environmental conditions. Hence, both the biochemical capabilities of the producer itself and the encountered environmental stimuli determine whether the given SM is produced or not. Therefore, it is complicated to predict under which conditions may the synthesis of the SMs of fungi be triggered, as there are several factors that lie behind the activation of a BGC.

Fungi are one of the main mediators among different organisms and the ecosystem, besides their interactions also with pathogens or endosymbiosis with different microorganisms in their surroundings (Bahram et al., 2022). In the study of (Daniel, 2004), metagenomes isolated from environments such as soil, were observed to be rich sources for novel natural products, proving the view that these close interactions of microorganisms that take place in their natural habitats are favorable for fungal SMs

production. Several studies have supported this theory by proving how the cultivation of various microorganisms together under different conditions turned out to give a wide variety of novel SMs (Cueto et al., 2001; Losada et al., 2009; Oh et al., 2007).

There are a variety of existing methods in order to approach the mining of new SMs, but it can sometimes be difficult when the producer microorganism is taken out of its natural environment. Hence, the development and application of strategies that simulate the natural habitat of cluster-bearing microorganisms, such as co-cultivation of one or more microorganisms or the breeding under different conditions (temperature, lighting, pH…), are required and have already demonstrated their potential when studying novel fungal SMs. The number, diversity and varied distribution in *Fusarium* PKSs reflect a diversity of habitats occupied by the different members of the genus (Brown et al., 2022). Thus, variations in the conditions and the media have great impact on the profile of SMs produced (Bode et al., 2002a).

Through the OSMAC approach, it is possible to study which factors may induce certain metabolic pathways. The idea behind this strategy is the alteration of easily accessible cultivation parameters such as media composition, lighting conditions, temperature or others in order to increase the variety of different fungal SMs synthesized by the fungi (Bode et al., 2002).

So far, different studies have shown the potential of the OSMAC technique working with all sorts of microorganisms. In the study carried out by (Bode et al., 2002b) over 100 new compounds were isolated belonging to more than 25 different structural classes from only 6 different microorganisms. The OSMAC strategy has as well been previously applied with the functional analysis of *PKS* genes of *F. graminearum*, where the fungi were subdued to 18 different conditions, considering numerous media and growth periods. It was possible to analyze the expression level of each *PKS* gene in every condition through a RT-PCR of the obtained samples: gene-specific primers were used to amplify regions of each PKS gene to identify the conditions under which they were expressed. The results presented by these studies do not only help on analyzing the synthesized PKs, but also represent a source of valuable information for the preference of growth conditions choice in further research regarding any of the analyzed BGC. For instance, *PKS3* was found to be expressed only in some media (carrot agar, rice agar and DON-inducing media), which

would indicate that in further research about this *PKS* any of these could be a recommendable choice. Besides studying the expression level of the *PKS* genes, it was as well possible to analyze the function of each of them. Each gene was disrupted independently and the resulting mutants were examined for changes in development and pathogenicity. This is another example of a study where different strategies were combined: additionally to the OSMAC strategy, the targeted genes were also knocked-out for functional analysis purposes (Gaffoor et al., 2005).

New media that enhance the synthesis of novel fungal SMs have also been discovered through experiments involving OSMAC studies. For instance, in the study (Pérez et al., 2017), fruit and vegetable juices were used for first time for an OSMAC approach and demonstrated to be promising on the research of new bioactive SMs of *F. tricinctum*. In light of this, when it comes to the statement of the protocol to follow with OSMAC, the metabolic pathways are still a complex field of study, thus a wide range of new conditions and media are allowed to be employed in order to find novel natural bioproducts.

2.6 HPLC-MS analysis of metabolites

Current analytical methods for identification of molecules have been developed through new technologies and are continuously improving. Among the best well-known and most used techniques, high performance liquid chromatography (HPLC) represents an easy and efficient methods for characterization and quantification in the study of SMs synthesis. HPLC is a highly sensitive method for detection, identification and quantification of chemicals, especially samples using UV and visible absorbance. The compounds are separated based on their interaction with the stationary phase, which is essential to any chromatographic separation. In the case of reverse phase HPLC, the stationary phase is less polar than the mobile phase: thus, the retention time of a compound increases with the decrease of polarity. If this retention times are compared with settled standards, it is possible to identify the different molecules (Hanachi, 2009).The sample must be prepared previous to the HPLC analysis, for the purpose of eliminating contamination and get the clearest results possible.

The HPLC method can be supported by an additional UV detection spectroscopy. Thus, additional to the resulting chromatogram with the corresponding retention time of each compound represented in peaks, a UV spectrum of the compound will be provided. UV-Vis spectroscopy is a well-known analytical technique that measures the number of discrete wavelengths of UV or visible light that are absorbed by or transmitted through a sample in comparison to a reference or blank sample. The UV maximum of a molecule is dependent upon the extent of conjugation in the molecule. Conjugation is possible by means of alternating single and double bonds: as long as each contiguous atom in a chain has an available p-orbital, the system can be considered conjugated. These conjugated systems have exclusive properties that lie behind the pigmentation of a molecule. When it comes to polyketides, the aromatic rings present in their structures are usually responsible for their UV absorbance.

The information revealed by this technique is often presented as a graphic of absorbance on the vertical *y* axis and wavelength on the horizontal *x* axis. This graphic is commonly referred to as an absorption spectrum, and each molecule have their specific one. Therefore, it is possible to identify known metabolites whose UV spectrum are already known and corroborated in the science field (Han et al., 2014). Therefore, HPLC-UV eases the separation of metabolites according to their polarity in the column and gives an initial identification step through the UV detection of the analyzed compounds. An additional MS study will provide further information on the masses of the compounds and can be used in order to support any contemplated hypothesis.

In previous studies in the field, this characterization method has been widely used to identify SMs of *Fusarium spp*., which is directly connected to the goal of the present research. In the study by (Lebeau et al., 2019) bikaverin and its various intermediates were identified by an HPLC-MS analysis, which helped with the study of bikaverin's metabolic pathway as well as some of its regulatory patterns in *F. oxysporum*. In other studies this technique also enhanced the discovery of novel potential metabolites, where several yet undescribed putative naphthoquinone/anthraquinone analogue compounds produced by *F. graminearum* were observed in an OE mutant (Westphal et al., 2018). Thus, this method does not only help on the identification of known compounds, but also

embraces the discovery of new potential SMs that are initially unknown and can lead to further research.

3 Problem Statement

Filamentous fungi are known to be a great source of potential metabolites for the industrial, medical or agricultural field. With biotechnology's development through the years, it is possible to improve the ongoing techniques and products to a next level. During the last decades, science research has been very focused in the discovery of fungal secondary metabolites (SMs) that possess a wide diversity of properties and activites, making them attractive in every field. For instance, multiple fungal statins are used to lower cholesterol; paclitaxel and cyclosporin are immunosuppressants; chaetoviridin and fusafungin are antibacterial, while echinocandins and azoxystrobin are antifungal. Polyketide-derived metabolites represent a majority of the fungal natural products characterized to date. Within all the promising fungi that have proved their worth in this field, *Fusarium* has demonstrated to be one of the most economically and scientifically valuable ones due to the great variety of *PKS* its numerous species possess.

The expression of these *PKS*s have the ability to yield metabolites with large structural variation and different promising uses in the industry. However, the production of these compounds is not always obtained easily, as SMs are often expressed in nature under stressful factors as defense mechanisms for the fungi. Therefore, the triggering of the expression of the BGCs that produce those metabolites is one of the essential steps regarding this scientific field. A big research is still ongoing concerning the expression of the "silent" BGCs and the linking to their corresponding products. There are different approaches to this matter but, up to date, genetical engineering of the strains has proved promising strategies to trigger their expression. The different techniques are employed to create mutants that may, or may not if unlucky, be able to unravel finally the identity of the metabolite that is produced by a certain gene.

Besides the obtention of mutants, the development and application of strategies that simulate the natural habitat of cluster-bearing microorganisms have already proved their potential when studying the production of fungal SMs. The different techniques are all gathered within the OSMAc strategy, where a unique strain is cultured in many different conditions or media and the posterior expression levels are studied in order to see whether there are any optimum growing cues.

In the present study, a combination of both approaches will be employed in order to trigger the expression of *PKS35* BGC found in *F. solani*, which has previously been related to the perithecia pigmentation in this species. There have been diverse approaches for the study of *PKS35*, but so far, its final product remains still unidentified due to the lack of expression of the BGC in previous research, where *PKS3* was found to interact with the expression of *PKS35*, thus complicating the study of the latter one's products. Up to this date, the identification of this BGC's products remains quite challenging and therefore, in this project an OSMAC strategy involving genetically engineered mutants will be employed to unravel *PKS35*'s compounds identity and further information about optimum conditions for other SMs formation. The wild types and mutants of both *F. gramienarum* and *F. solani* facilitated by Aalborg University (AAU) Esbjerg will undergo an OSMAC strategy with a posterior metabolites' extraction and HPLC-UV analysis.

Previous researchers from AAU created mutants of both *F. graminearum* and *F. solani* for the same purpose of the study of *PKS35*, which will be employed in the present research. With these mutants, the goal was to trigger the expression of *PKS35* either in its original host *F. solani*, where the mutants OE the transcription factor of PKS35 (Fs O-tfPKS35) or to heterologously express it in *F. graminearum* (Fg::*PKS35*). On the other hand, after the observation of the connected regulation of *PKS35* and *PKS3* in previous experiments, other mutants were generated to avoid this matter both in *F. graminearum* (Fg*::PKS35*Δ*pgl1*), but especially in *F. solani*, since the OE had first been observed in the mutants where the tf*PKS35* was OE, thus creating a new mutant Fs O-tf*PKS35*Δ*fsr1*. With the mentioned and some additional mutants, and the several growth conditions, a new approach to trigger the expression of *PKS35* is carried out in the present report.

4 Materials and Methods

4.1 Fungal Strains

For the present study, different strains of *Fusarium graminearum* and *Fusarium solani* were employed. These were provided by the Aalborg University (Esbjerg) laboratory, where they were maintained as mycelia in 30% glycol at -80 ºC. Apart from the wild type for both species, the other strains used for the current research were mutants that had been previously created by the research department in the university.

The mutation of these strains was approached through different genetical engineering techniques. Among those, the knock-out strategy made possible the deletion of the target gene in order to inactivate the activity of a certain PKS of interest (cases for Fg*Δpgl1*, Fg::*PKS35Δpgl1::*, Fs O-tf*PKS35Δfsr1* and Fs O-tf*PKS35ΔPKS35*). In other cases, OE of the targeted BGC (*PKS35*) was induced through the OE of its transcription factor (Fs O-tf*PKS35*, Fs O-tf*PKS35Δfsr1* and Fs O-tf*PKS35ΔfPKS35*). Those mutation were carried out through *Agorbacterium tumefaciens* mediated transformation, a method that relies on the capability of the bacterial pathogen *A. tumefaciens* in transferring foreign genes into a wide variety of host plants. *A. tumefaciens* is also capable of infecting filamentous fungi when induced by acetosyringone, which makes possible the integration of foreign DNA into the fungal genome (Nielsen et al., 2022). For the strains employed in this study, this method proved to be effective for targeted gene deletion mediated by homologous recombination.

The correct insertion of the plasmid was validated for each of the mutants in previous research. A brief description of each of the strains is provided in Table 2. For the culturing phase, two different mutants of Fg*::PKS35Δpgl1* were employed for higher rate of success.

Table 2 Different strains of F. graminearum and F. solani used for the study followed by the employed nomenclature, genotype and expected activity of gene clusters PKS3 and PKS35

Strain	Function
Fg PH-1 (wt)	Control of Fg::*PKS35*
Fs 77-13-4 (wt)	Control of Fs O-tf*PKS35*
Fg∆*pgl1*	Control of Fg::*PKS35*∆*pgl1*
Fg*::PKS35*∆*pgl1* (M1)	Study of *PKS35* in *F. graminearum*
Fg*::PKS35*∆*pgl1* (M2)	Study of *PKS35* in *F. graminearum*
Fg::*PKS35*	Study of *PKS35* in *F. graminearum* with possible interaction of *PKS3*
Fs O-tf*PKS35*	Study of OE of PKS35 in *F. solani* with possible interaction of PKS3
Fs O-tf*PKS35*∆*fsr1*	Study of OE of PKS35 in *F. solani*
Fs O-tfP*KS35*∆*PKS35*	Control of Fs O-tfP*KS35*∆*PKS35*

Before starting with the definitive cultures, the *Fusarium* strains were first inoculated in PDA plates in order to awaken them. Once it was proved the good state of all the strains and they showed no abnormalities in the growth, the definitive cultures were inoculated in order to take off with the research.

4.2 Culture conditions

For the OSMAC strategy, a total of 8 media were selected for the growth of the *Fusarium* strains. The selection was based on previous researches regarding the production of SMs by the fungi (Table 3). The followed recipes to make the media are added in the Appendix. Each medium was prepared in duplicate, as for each, two different conditions were established: growth in darkness and 28 ºC (C1) and growth under UV light and room temperature (C2). Therefore, taking into account the 8 media, 2 condition per each media and the culture of 9 strains (presence of two mutants for strain Fg*::PKS35*∆*pgl1*) in each of the 16 condition, an amount of 18 plates of each media were produced, an overall total number of 144 plates produced. The cultures were grown for a period of 2 weeks under the respective condition for each case, after which it was proceeded to the metabolites' extraction step. In Figure 3 it is possible to observe the settings that were prepared for both condition 1 and 2 in order to grow all the *Fusarium* samples.

Table 3. Listed the 8 media that were used for the study. In the continuous column, the scientifical references from which the media was decided in each case.

Media	Source
Carrot agar	(Gaffoor et al., 2005; Urban et al., 2003)
Rice agar	(Gaffoor et al., 2005; Pérez Hemphill et al., 2017)
YES (Yeast extract sucrose)	(Hestbjerg et al., 2002)
PDA (Potato dextrose agar)	(Gaffoor et al., 2005; Pradeep et al., 2013)
SDA (Sabouraud dextrose agar)	(Imamura et al., 2008)
DFM (Defined Fusarium medium)	(Frandsen et al., 2016)
CM (complete medium)	(Malz et al., 2005; Westphal et al., 2021)
V8 juice	(Choi et al., 2009)

Figure 3. Pictures of plates growing when initially cultured both under the set ups for C1 in the left (darkness and 28 ºC) and under C2 in the righ (UV light and room temperature). Pictures taken in the AAU laboratories (Esbjerg).

4.3 Secondary metabolite extraction

For the screening of SMs produced by the different *Fusarium* strains, the extraction procedure followed in this project was based on the same one that was used in the study

(Kristensen et al., 2021a), as it also worked on the study of SMs produced by *Fusarium*. Per each culture plate, 10 agar plucks were sampled and transferred to a glass tube with 2.5 mL of extraction solvent (3 parts ethyl acetate; 2 parts dichloromethane; 1 part methanol; 1 % formic acid). The samples were then treated with an ultrasonic bath for 15 min at room temperature. The solvent left was afterward transferred into a new vial and completely evaporated under a steady stream of nitrogen. In the last step, each sample was dissolved in 800 μL of methanol and left in ultrasonic bath for 10 additional min. The final samples were filtered through a 0.22 μm micro-filter into an HPLC vial. Those were finally analyzed in an HPLC-UV analysis in the Aalborg University (AAU) of Esbjerg.

4.4 HPLC-UV analysis

The extracts were analyzed using the same HPLC settings that were employed in the study of (Kristensen et al., 2021b) and the wavelengths were measured at 230 nm, 276 nm, 340 nm and 495 nm. Previous to the analysis of the results, basic data from the most common *F. graminearum* and *F. solani* SMs was collected (usual retention times in similar studies, UV spectrum) in order to make easier the identification of the peaks through the data analysis. For this purpose, the collected information from several studies in the *Fusarium* SMs research field was gathered (Droce et al., 2016; Kristensen et al., 2021b; Nancy N., 1979; Russell et al., 1992b; Westphal et al., 2018). With the collected data it was possible to identify the peaks that corresponded to known SMs from both *F. graminearum* and *F. solani* and therefore study their production patterns in the different strains and media. The unknown compounds that were observed to follow an interesting pattern of expression were listed as metabolites of interest for further discussion in the report.

For further comparison of the samples, it was additionally noted the production rate of the compounds of interest. This was done according to the area of the respective peak. The whole analysis process was done using the software *OpenLab CDS* from *Agilent*, a chromatography data system.

5 Results and Discussion

The *Fusarium* strains were grown in a total amount of 16 different conditions: 8 media, each one with 2 different conditions of growth regarding lighting and temperature: darkness and 28ºC (Condition 1, C1) and UV light and room temperature (Condition 2, C2). In this section, tables and figures will help summarize the collected information on the expression of different compounds (both known and unknown potential SMs), producer strains, medias and growth conditions. Results are sometimes compared depending on the strain employed, while in other occasion it will be the media that triggers different expression patterns. The results will as well be discussed in this same section for a smoother connection to the drawn conclusions. In order to understand the images of the plates, and not to interrupt the picture observation, a scheme of the location of each media plate is facilitated in Figure 4. A. Scheme followed when showing the plates cultured in a unique media and condition and the variable are the strains. B. Scheme followed when showing the plates cultured in a unique strain and condition and the variable are the growth medias.

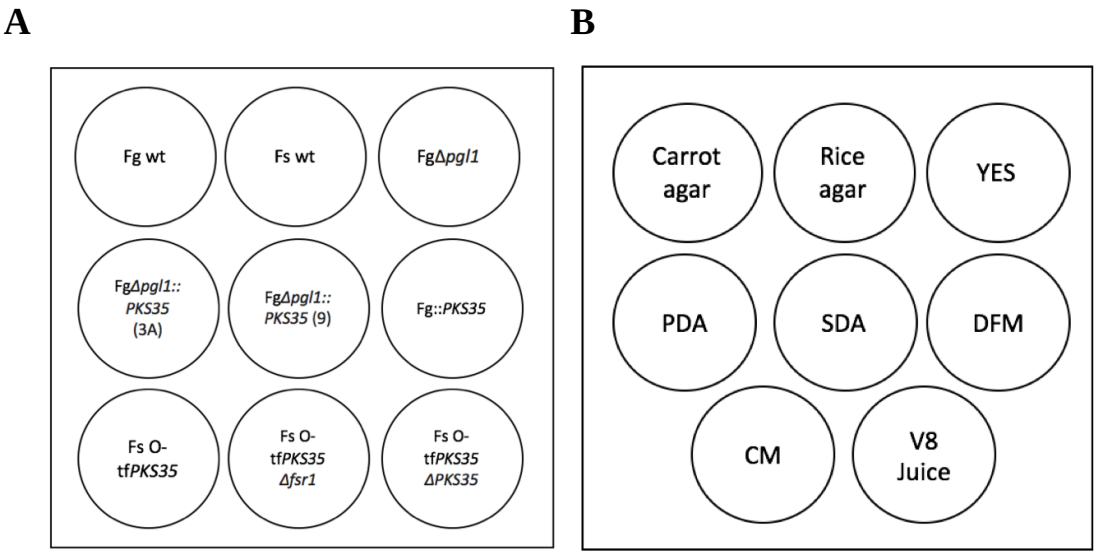


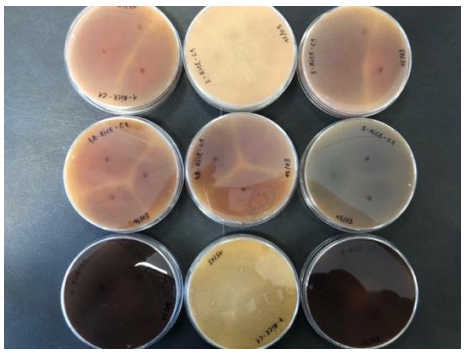
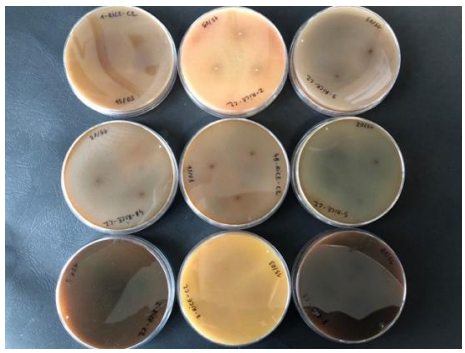
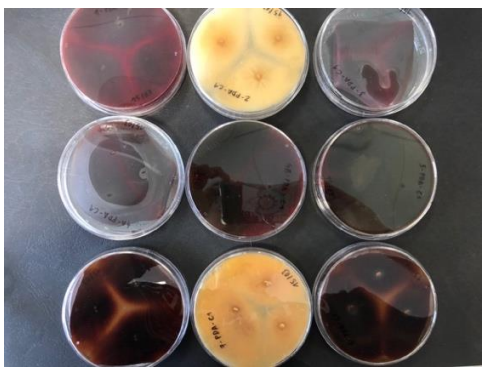
Figure 4. A. Scheme followed when showing the plates cultured in a unique media and condition and the variable are the strains. B. Scheme followed when showing the plates cultured in a unique strain and condition and the variable are the growth medias.

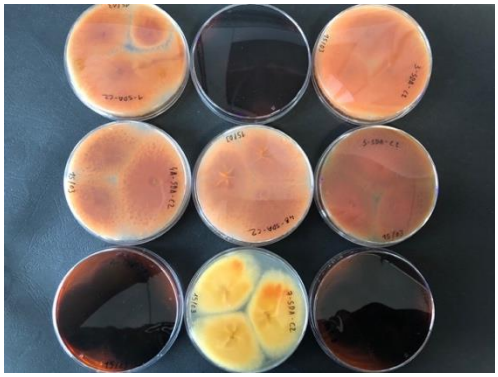
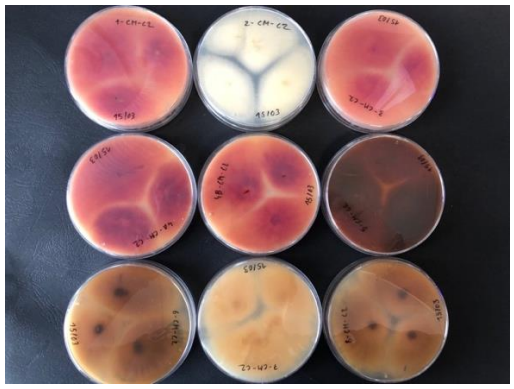
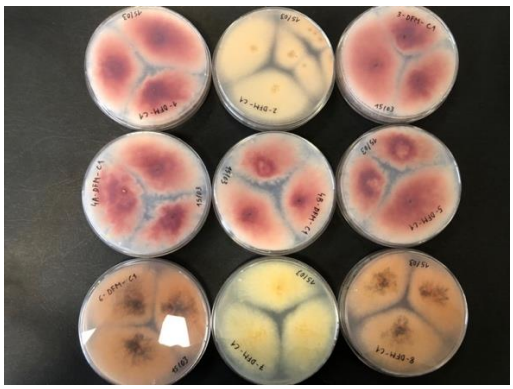
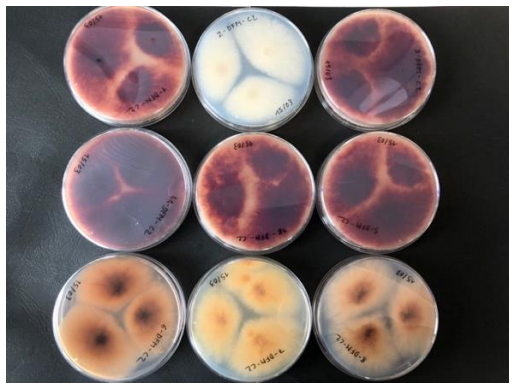
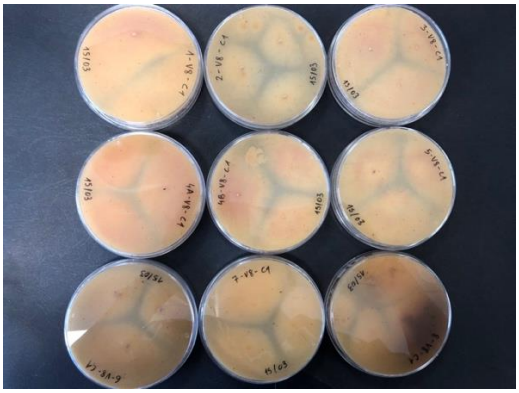
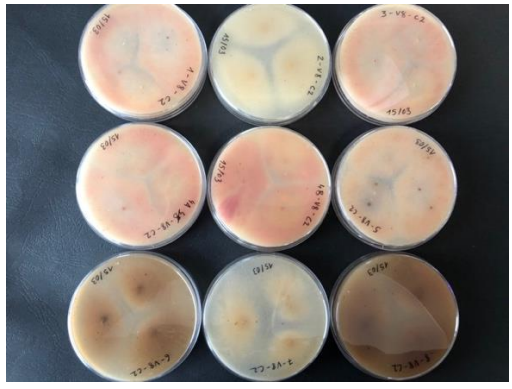
5.1 Culture of strains under the different media and conditions

The *Fusarium* strains were grown for a period of 2 weeks in 8 different media and under the previously specified condition. In Figure 5 the different media and conditions are organized to give an overall view of the produced plates. These exhibited some phenotypical differences, which can be deemed to the different mutants' genotypes, media composition differences or due to growth conditions (C1 or C2). These samples were afterwards subdued to an extraction method where the produced compounds were extracted for their further study through an HPLC-UV analysis.

In Figure 5 it can be observed the formation of mycelium under both C1 and C2. The *PKS* responsible for the pigmentation of the mycelium in each *Fusarium* species is different, and this is why the different coloration among strains from *F. graminearum* and *F. solani* is so visible (check Figure 4 to identify the genotype of each plate). In *F. graminearum*, *PKS12* is responsible for the characteristic red pigmentation observed, as this *PKS* produces aurofusarin as its final product. On the other hand, the mycelium pigmentation in *F. solani* relies on the expression of *PKS3*. While most *F. graminearum* strains look fairly similar, the phenotypical difference among *F. solani* strains is more remarkable. This pigmentation difference is due to both the deletion of *fsr1*, the core gene of *PKS3* in *F. solani*, and the OE of the tf*PKS35* (pigmentation loss visible in strain Fs O-*PKS35*Δ*fsr1*). Since the goal of this research is to study *PKS35*, and *PKS3* was found to interact with its expression, its core gene was deleted in some of the mutants. This deletion does not make much of a difference in *F. graminearum* mutants, but it does represent a phenotypical difference for the *F. solani* ones, as the *PKS* responsible for the mycelium coloration of the fungi is no longer active. The whole matter regarding the phenotypical differences found among mutants from *F. solani* are further discussed in "5.3 Evidence of *fsr1* deletion by pigmentation loss in Fs O-*PKS35*Δ*fsr1*".

Concerning the impact of the different growth conditions, in some of the medias it is possible to observe a slight difference depending on the growth condition the culture was subdued to. The observations made concerning the growth conditions the cultures were subdued to will further be discussed in the report along with additional data from the metabolite production rate results obtained in the HPLC analysis (5.6 The impact of growth conditions on metabolites' expression).

Media	Condition 1	Condition 2
Carrot		
Rice		
YES		
PDA		

SDA		
CM		
DFM		
V8 juice		

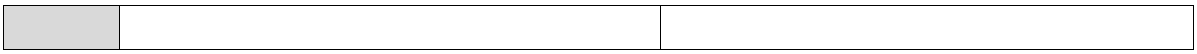


Figure 5. All the produced plates after a period of 2 weeks since culture under growth C1 in the left and C2 in the right. All the medias are represented and indicated in the table. For strain identification, follow the scheme facilitated in Fig. 4.

5.2 Identification of compounds

Once the HPLC-UV results were analyzed, the identification of the peaks was possible through the observation of the strains that produced them (analysis of species or certain gene deletion) and the additional UV spectra analysis, which could be compared to standards of known molecules. A color code is settled for the different *Fusarium* strains, which will be kept through the whole results figure presentation for the purpose of an easier and faster understanding of the information. In Figure 6 the individual chromatograms measured at 276 nm of every strain belonging to the samples grown in PDA under C1 are represented along with the identified peaks.

The identified compounds were named with their metabolite name in Figure 6. Those would be 6 SMs: aurofusarin (PKS12), rubrofusarin (PKS12), bostrycoidin (PKS3), fusarielin H (PKS9), fusarubin (PKS3) and zearalenone (PKS4 and PKS13). Fusarubin was not identified as such at the beginning of the analysis, so through the project this metabolite will be referred to in the figures as compound 4. Additionally, 8 unknown compounds have been identified as possible interest metabolites for the present research. Among those, compounds 1, 2 and 3 are candidate compounds for *PKS35* and therefore special attention is payed to their analysis in further approaches through the report. All the known and unknown metabolites are listed in Table 4 along with their retention time and producer strains.

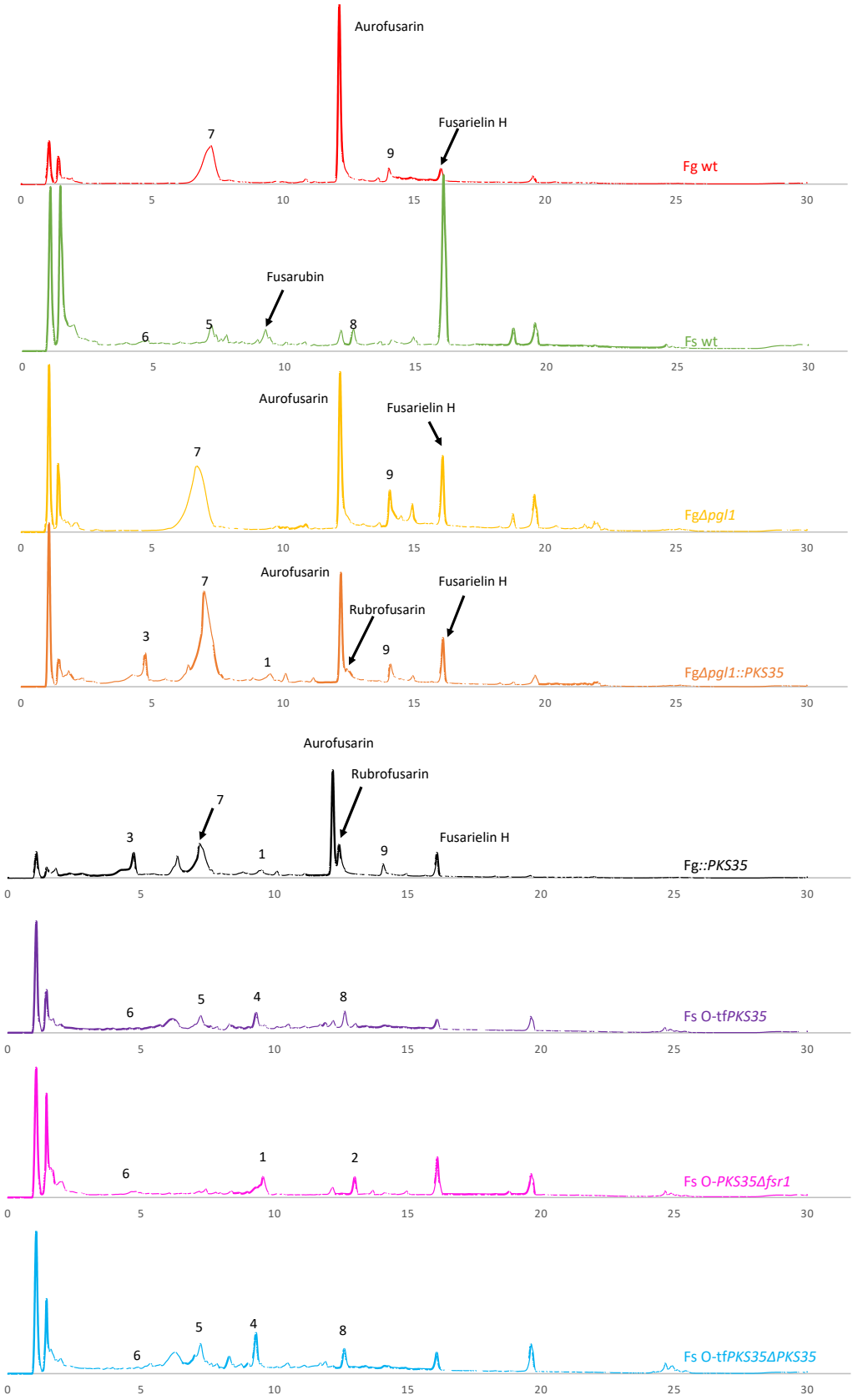


Figure 6. HPLC chromatograms of the extractions grown in PDA under C1 detected at UV 276 nm. Every strain displays its individual chromatogram according to the color code established. The peaks are identified according to the naming established in Table 1.

Table 4. Identified compounds in the resulting data obtained through HPLC-UV analysis ordered according to their retention time. Information about the retention time (min) and the strains by which the compound is expressed are given. Out of all the compounds, 6 are identified with the corresponding metabolite (compound 4 was further identified as fusarubin during the research), and 8 remain yet unknown.

Compound	Retention time	Present in strains
Compound 3	4.7	Fg*::PKS35*Δ*pgl1* Fg*::PKS35* Fs O-tf*PKS35* Fs O-tf*PKS35*Δ*fsr1*
Compound 6	4.8	Fs wt Fs O-tf*PKS35* Fs O-tf*PKS35*Δ*fsr1* Fs O-tf*PKS35*Δ*PKS35*
Compound 5	7.2	Fs wt Fs O-tf*PKS35* Fs O-tf*PKS35*Δ*PKS35*
Compound 7	7.3	Fg wt FgΔ*pgl1* Fg*::PKS35*Δ*pgl1* Fg*::PKS35*
Compound 4 (Fusarubin)	9.2	Fs wt Fs O-tf*PKS35* Fs O-tf*PKS35*Δ*PKS35*
Compound 1	9.3	Fg*::PKS35*Δ*pgl1* Fg*::PKS35* Fs O-tf*PKS35*Δ*fsr1*
Zearalanone	10.5	Fg wt FgΔ*pgl1* Fg*::PKS35*Δ*pgl1* Fg*::PKS35*
Bostrycoidin	10.5	Fs wt Fs O-tf*PKS35* Fs O-tf*PKS35*Δ*PKS35*
Aurofusarin	12	Fg wt FgΔ*pgl1* Fg*::PKS35*Δ*pgl1* Fg*::PKS35*
Rubrofusarin	12.4	Fg*::PKS35*Δ*pgl1* Fg*::PKS35*
Compound 8	12.6	Fs Fs O-tf*PKS35* Fs O-tf*PKS35*Δ*PKS35*

Compound 2	13	Fg*::PKS35*Δ*pgl1* Fg*::PKS35* Fs O-tf*PKS35*Δ*fsr1*
Compound 9	14	Fg wt Fgδ*pgl1* Fg*::PKS35*Δ*pgl1* Fg*::PKS35*
Fusarielin H	16	Fg wt Fgδ*pgl1* Fg*::PKS35*Δ*pgl1* Fg*::PKS35*

5.2.1 Identification of *PKS35* candidate compounds

Three compounds were characterized as possible candidate compounds for *PKS35*: Compound 1, Compound 2 and Compound 3. This hypothesis was built due to their frequent appearance in strains Fg*::PKS35* and Fs O-tf*PKS35*Δ*fsr1*, and sometimes as well in Fg*::PKS35*Δ*pgl1* and in Fs O-tf*PKS35*. These producing strains either had the BGC genetically engineered into their genome (*F. graminearum* strains) or the transcription factor (tf) of *PKS35* was OE (*F. solani* strains). Thus, their UV spectra was studied for further information about the compounds. Quite a similarity was observed among the three compounds' spectra: Compound 1 (λmax): 222, 274, 356; Compound 2 (λmax): 218, 276, 342; Compound 3 (λmax): 218, 272, 346. In Figure 7 it is possible to observe the individual chromatograms of different producer strains compared to Fg wt, which wouldn't be expected to produce any of these compounds as naturally it does not possess *PKS35* in its genome, and those 3 unknown metabolites are believed to be products of that BGC. The UV spectra for each compound are as well displayed in Figure 8.

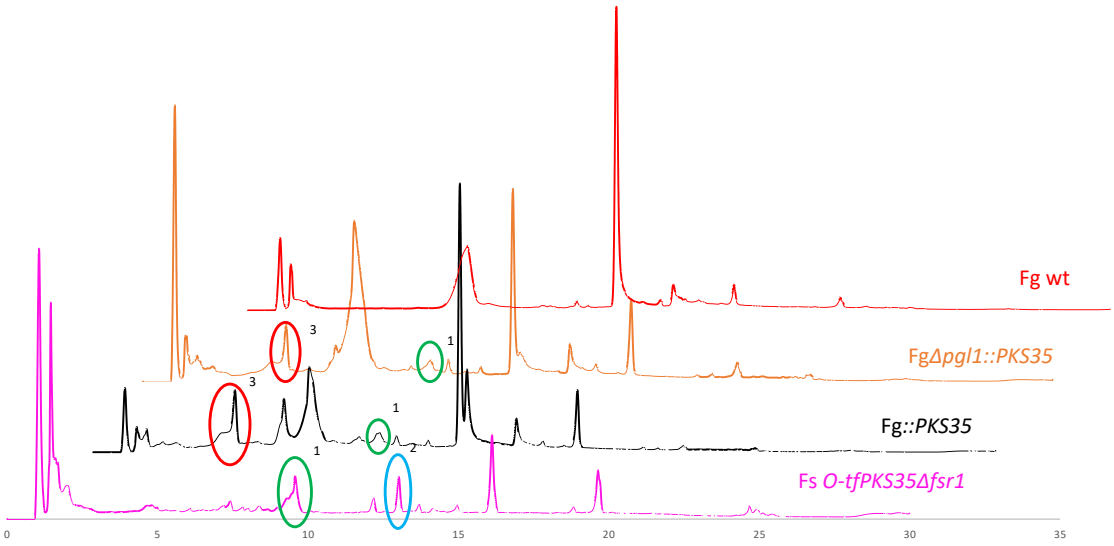


Figure 7. HPLC chromatograms of the extractions grown in PDA under C1 detected at UV 276 nm from strains Fg wt, Fg::PKS35Δpgl1, Fg::PKS35 and Fs O-tfPKS35Δfsr1. In circles the peaks identified as the candidate compounds for PKS35: in green compound 1, in blue compound 2 and in red compound 3

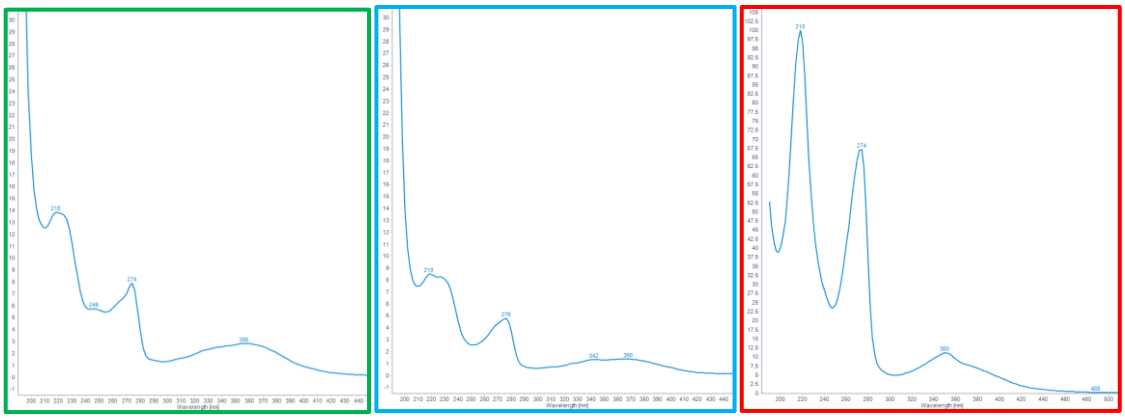


Figure 8. UV-spectrum of the candidate compounds for PKS35 that are circled with the same color in Figure 7 (green for compound 1, blue for compound 2 and red for compound 3).

5.3 Evidence of *fsr1* deletion by pigmentation loss in Fs O-*PKS35Δfsr1*

The resulting plates showed a clear phenotypical difference among *F. solani* strains, especially Fs O-tf*PKS35* and Fs O-tf*PKS35Δfsr1*, where the complete loss of mycelium pigmentation was observed in the second one. This mutant strain suffered the deletion of *fsr1*, the core gene of *PKS3*, which is responsible for the mycelium pigmentation in *F. solani*. This coloration loss phenomenon was not observed when *pgl1*, core gene of PKS3

of *F. graminearum*, was deleted in the strains Fg*Δpgl1 and* Fg::*PKS35Δpgl1* (view the *F. graminearum* plates in Figure 5. All the produced plates after a period of 2 weeks since culture under growth C1 in the left and C2 in the right. All the medias are represented and indicated in the table. For strain identification, follow the scheme facilitated in Fig. 4. and using Figure 4 identify the different genotypes of the mutants to see the comparison). The difference in *F. gramienarum* is that *PKS3* is no longer the responsible for the mycelium pigmentation, but the perithecia one, and there was no formation of perithecia observed in the *Fusarium* cultures carried out in this report. However, in the *F. solani* strains, where *PKS3* is indeed responsible for the mycelium pigmentation, a clear pigmentation loss was observed between the Fs O-tfP*KS35Δfsr1* strain and the other *F. solani* strains (Figure 9).

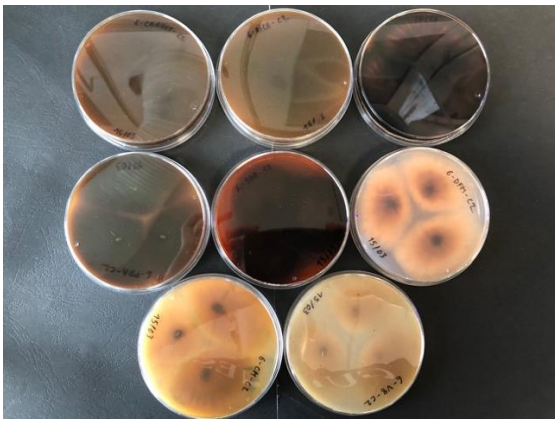
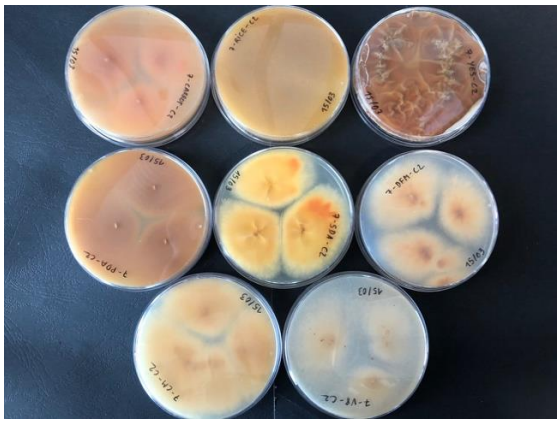
Fs O-tf*PKS35*	**Fs O-tf*PKS35Δfsr1***
	
	

Figure 9. Plates after a period of 2 weeks growth under C2. All the medias are represented. The left column shows the growth of Fs O-tfPKS35 in the different media and the right column shows the growth of Fs O-tfPKS35Δfsr1 in the different media. Pigmentation loss is observed for the cultures of Fs O-tfPKS35Δfsr1. For media identification, follow the scheme facilitated in Fig. 4.

5.4 Increasing rubrofusarin production of Fg*::PKS35*

A coloration difference was observed among Fg*::PKS35* and the rest of *F. gramienarum* strains, giving this first one a slightly darker pigmentation as visible in Figure 10. The difference is not as evident as the one observed previously in Figure 9, but it still represents a pattern repeated for most media. Aditional to this data, Fg*::PKS35* also was observed to produce an unexpectedly high rate of rubrofusarin in most media (Figure 11). Rubrofusarin is one of the intermediates in the biosynthetic pathway of aurofusarin, the final product of *PKS12*. This compound possess a caracteristic orange-brown color darker than the usual red from aurofusarin. Thus, this could be the reason behind the slightly darker coloration of Fg*::PKS35*, as *PKS12* is responsible for the mycelium pigmentation in *F. graminearum*.

Apart from producing rubrofusarin, Fg::*PKS35* also produced some of the *PKS35* candidate compounds (compounds 1, 2 and 3) in most of the growth conditions (Figure 14). As this comes as un-natural activity for *F. graminearum* (since *PKS35* is not originally found in this *Fusarium sp*.), it could be linked to the unexpected high expression rates of rubrofusarin, which is not observed so much in the strains where there is no production of *PKS35* candidate compounds (Figure 11).

Fg*::PKS35Δpgl1*	**Fg*::PKS35***
	

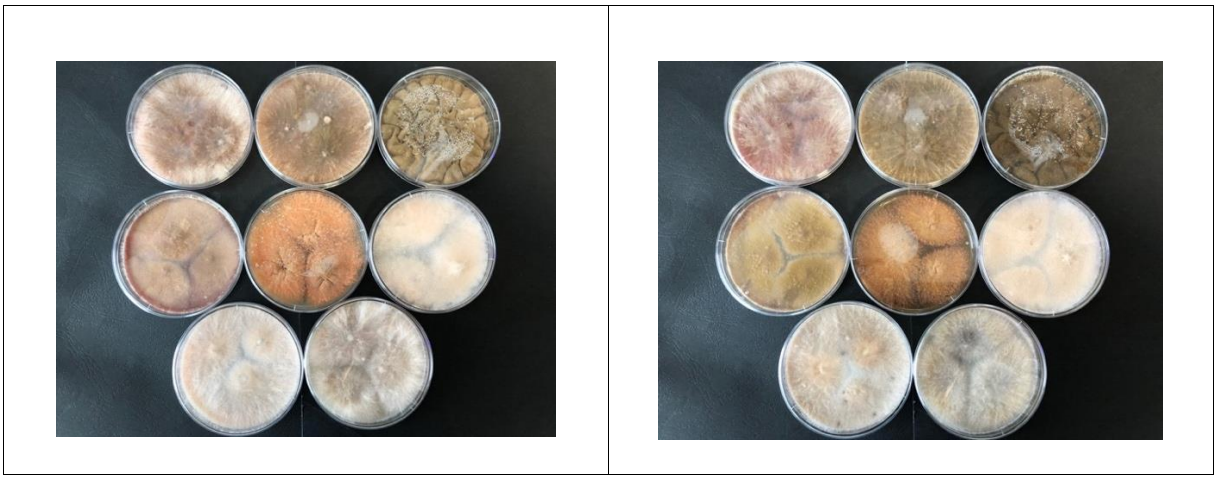


Figure 10. Plates after a period of 2 weeks growth in all different media under C2. All the medias are represented. Strain Fg::PKS35Δpgl1 visible in the left and strain Fg::PKS35 in the right. For media identification, follow the scheme facilitated in Fig. 4.

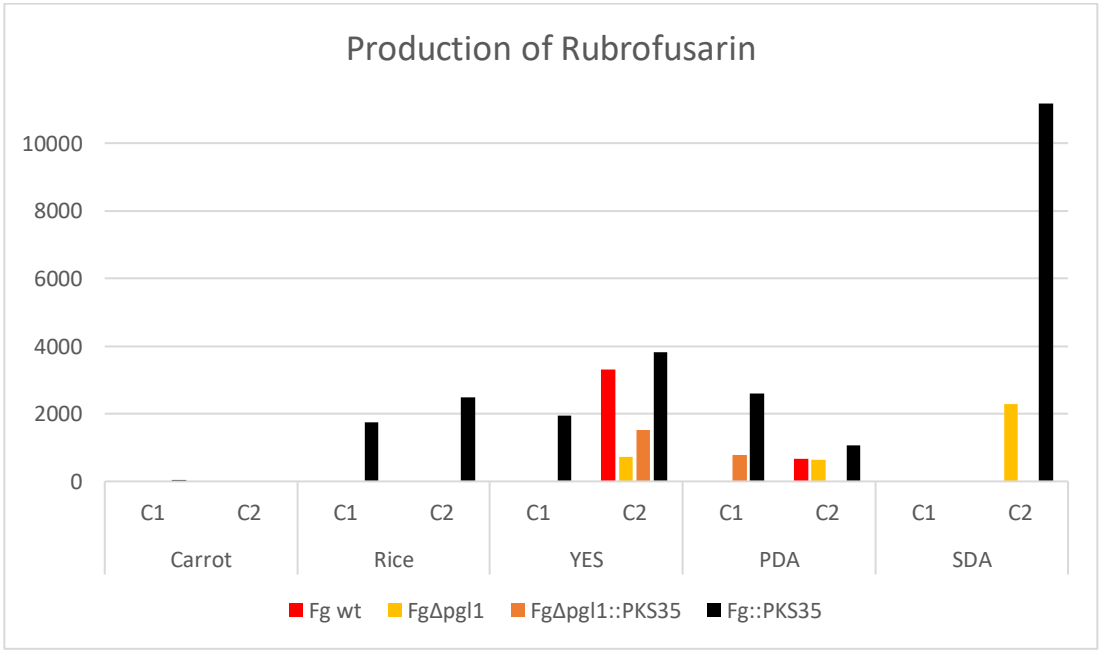


Figure 11. Comparison graphic where the different levels of rubrofusarin expression are represented for each F. gramienarum strain in the different medias and both growth conditions. DFM, CM and V8 juice medias are not represented as no levels of rubrofusarin synthesis were identified. For comparison purposes, the values of YES C2 have to be multiplied by 10.

In a previous comparative study by (Frandsen et al., 2006) where mutants of *F. gramienarum* were employed to study the biosynthetic pathway of aurofusarin, the deletion of the putative transcription factor *aurR2* resulted in an increased level of

rubrofusarin relative to aurofusarin, the same phenomenon that takes place in the strain Fg*::PKS35*. In both cases, the expression of aurofusarin stayed the same both in the Fg wt and in the mutant, but in this second one a considerable increase in the synthesis of rubrofusarin was observed. In Figure 11 it is visible the increase of rubrofusarin production in most of the cases for Fg*::PKS35*. It could be hypothezised that *PKS35* somehow interrupts the action of *aurR2*, leading therefore to the accumulation of rubrofusarin. This is indeed to go too far into unknown ground, however it could be an interesting approach for further study into the *PKS35* biosynthetic pathway and its intermediates. All in all, it is believed that there is some sort of interaction between the pathways of both *PKS35* and *PKS12*. One possible approach to this matter could be through a RT-PCR of the mutants with the accummulation of rubrofusarin in order to study the expression of *aurR2* in *PKS12* and see if there are any unusual expression patterns when *PKS35* is also being expressed in the strain.

5.5 Possible connection of *PKS3* and *PKS35* regulation

During the analysis of the results, there was some data that related the activity of both *PKS3* and *PKS35* in the *F. solani* strains where the tf of *PKS35* was OE. The figures that are displayed in this section aim to highlight the phenotypically and metabolically differing aspects among *F. solani* and its mutants Fs O-tf*PKS35* and Fs O-tf*PKS35Δfsr1*, and to connect those phenotypical differences with the expression of the compounds that were identified with the HPLC-UV results (Figure 12).

When Fs wt and Fs O-tf*PKS35* are compared, considering both the phenotype and the metabolites production, there seems to be some differences that point towards the interconnection between *PKS3* and *PKS35*. In the first place, Fs O-tf*PKS35* presents a slightly darker pigmentation compared to the one of the wild type (Figure 12). As it is known, *PKS3* is responsible for the mycelium pigmentation in *F. solani* by the synthesis of compounds such as fusarubins (compound 4), and the darker coloration of Fs O-tf*PKS35* could be deemed on the OE of the tf*PKS35* also overexpressing *PKS3,* pointing to a connected regulation of both BGCs. This is supported by the fact that the production of *PKS3* related compounds (4, 5 and 6) is increased considerably in the mutant compared

to the wild type's expression rates (Figure 12). Additionally, in Fs O-tf*PKS35* a new compound related to *PKS35* (Compound 3) is identified, which is not present in the wild type and could indicate that *PKS35* is also being OE.

It is interesting to see that when *PKS3* is also being expressed, the *PKS35* activity rate seems to be lower than when the first BGC is inactive. This is the case for Fs O-tf*PKS35Δfsr1*, where, after the inactivation of *PKS3*, the three candidate compounds of *PKS35* are produced (compounds 1, 2 and 3), in comparison with Fs O-tf*PKS35* where only compound 3 was observed to be expressed (Figure 12). It is believed that the high production rate of *PKS3* compounds in Fs O-tf*PKS35* could be covering a much lower expression rate of *PKS35*. However, in Fs O-tf*PKS35Δfsr1* where *PKS3* is inactivated, these new *PKS35* candidate compounds are produced. This information supports a previously made hypothesis in the study (Rank et al., 2020), where the regulation of *PKS3* and *PKS35* was stated to maybe be connected when similar results as these ones were observed.

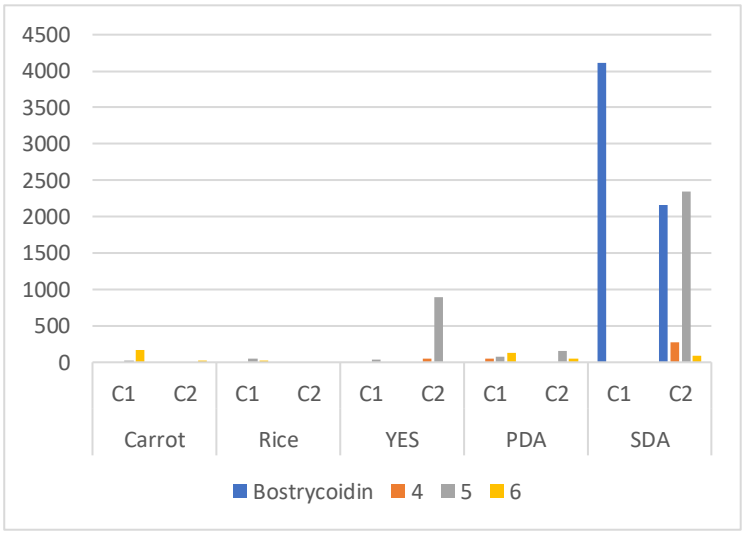
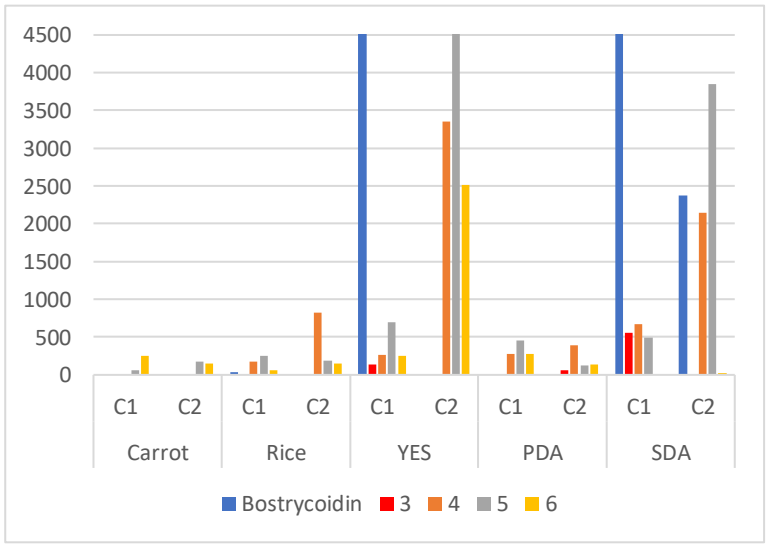
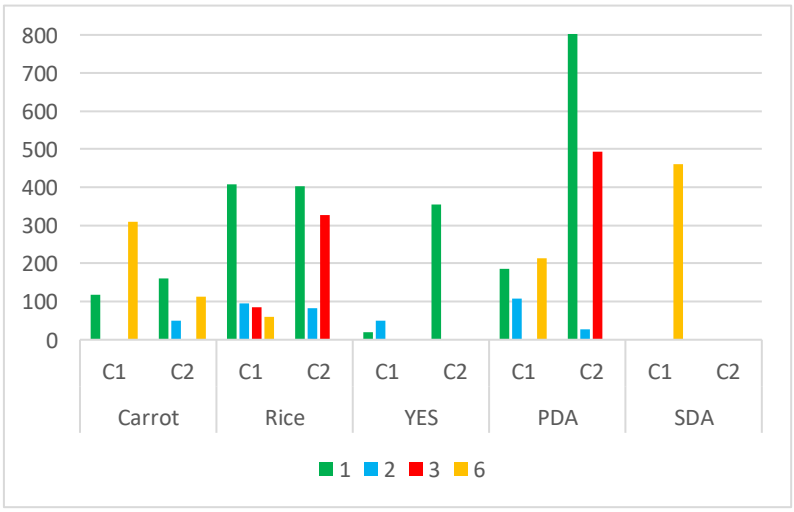
Fs wt	Production of metabolites in each strain
	
Fs O-tf*PKS35*	
	
Fs O-tf*PKS35Δfsr1*	
	

Figure 12. In the left side, images of the indicated strain growing in all 8 media under C1 in the next order from up to down: Fs wt, Fs O-tfPKS35 and Fs O-tfPKS35Δfsr1. For media identification, follow the scheme facilitated in Fig. 4. In the right, comparison graphics where the different expression rates are represented for each strain in the different medias and both growth conditions. DFM, CM and V8 juice medias are not represented as no levels of metabolites' expression were observed. Consider difference in y axis for the graphics.

The three *PKS35* candidate compounds are believed to be very closely related molecules, especially in the case of compound 1 and 2, as their retention times are very close, they are produced in the same strains and their UV spectra is very similar (Figure 8). Compound 1 is considered to be the main candidate for the final product of *PKS35*, as it is produced in most samples and presents the highest expressions rates out of the 3 candidates (Figure 17). Compounds 2 and 3 are therefore considered to be intermediates in the *PKS35* biosynthetic pathway. As it is observed in Figure 17, compound 1 was nearly uniquely produced in Fs O-tf*PKS35Δfsr1*, with the exception of the samples from PDA C1 where the compound presented very high expression rates for all producing strains (Fg *Δpgl1::PKS35*, Fg*::PKS35* and Fs O-tf*PKS35 Δfsr1*). It's interesting to mention that precisely in that same sample (PDA C1), the production of compounds from *PKS3* (compounds 4 and 5) was much lower compared to other media (Figure 12) and this could be the reason why *PKS35* seems to be expressed in a higher level. This would state PDA under C1 (darkness and 28ºC) as potential growth conditions for *PKS35* SMs extraction, as *PKS3* doesn't seem to interrupt *PKS35*'s expression.

Regarding the still unknown function of compounds from *PKS35*, mycelia pigmentation is discarded, since the mutants Fs O-tf*PKS35Δfsr1* lose all their coloration due to *PKS3* inactivation, regardless of *PKS35* compounds production (Figure 12). In previous researches the idea of *PKS35* being related to perithecia pigmentation in *F. solani* has been considered (Graziani et al., 2004), however, in these samples there hasn't been perithecia observation, therefore it not possible to draw any conclusions regarding visual evidence on the matter.

5.6 The impact of growth conditions on metabolites' expression

Besides the effect of the strains' genotype, the production of metabolites was also affected by the different medias and conditions in which the fungi were cultured. This is not only

supported by phenotypical differences, but also by the compounds' expression rates that were collected from the HPLC-UV data. Every media was subjected to two different growth conditions concerning temperature and lighting. The following figures show pictures of the same strain cultured in the 8 different media compared between both growth conditions (Figure 13 and Figure 15). Both strains Fg*::PKS35* and Fs O-tf*PKS35Δfsr1* are displayed as they have additional interest due to their *PKS35* candidate compounds higher rate production. Additional comparison graphics are as well displayed, where the production of several compounds is compared from one condition to the other for five media (Carrot agar, Rice agar, YES, PDA, SDA). The additional 3 media (DFM, CM, V8 juice) are not represented as there wasn't any apparent production of compounds of interest (Figure 14 and Figure 16)

The phenotypical difference between conditions can be outlined in the Fg*::PKS35* cultures media for instance, were a darker pigmentation is observed for C2 samples compared to C1 (Figure 13). This darker coloration of the mycelium could be related to a higher production rate of a certain metabolite, which in this case could be rubrofusarin, as this molecule is known to have a characteristic brown-ish color. This idea is supported by data represented in Figure 14 where a higher production rate of rubrofusarin is observed in samples grown under C2 for most cases. The only differing case is the growth in PDA, where the visible darker pigmentation under C1 is once again supported by nearly a doubled production of rubrofusarin under this condition (Figure 13).

Fg*::PKS35* under C1	**Fg*::PKS35* under C2**
	

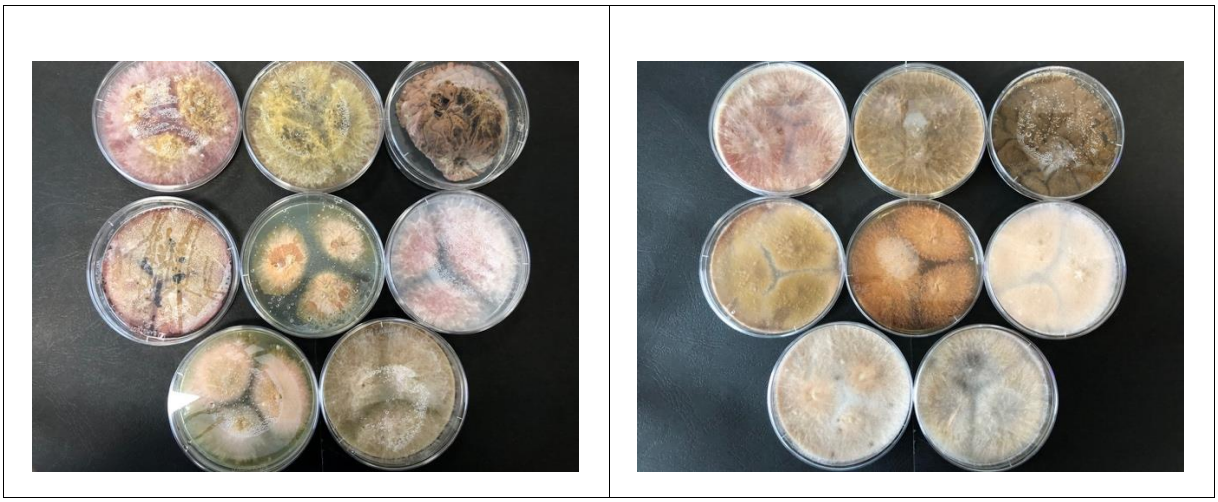


Figure 13.Plates after a period of 2 weeks growth of Fg::PKS35 in all the different media. The left column shows the growth of Fg::PKS35 under C1 (darkness and 28ºC) and the right column shows the growth of Fg::PKS35 under C2 (UV light and room temperature). For media identification, follow the scheme facilitated in Fig. 4.

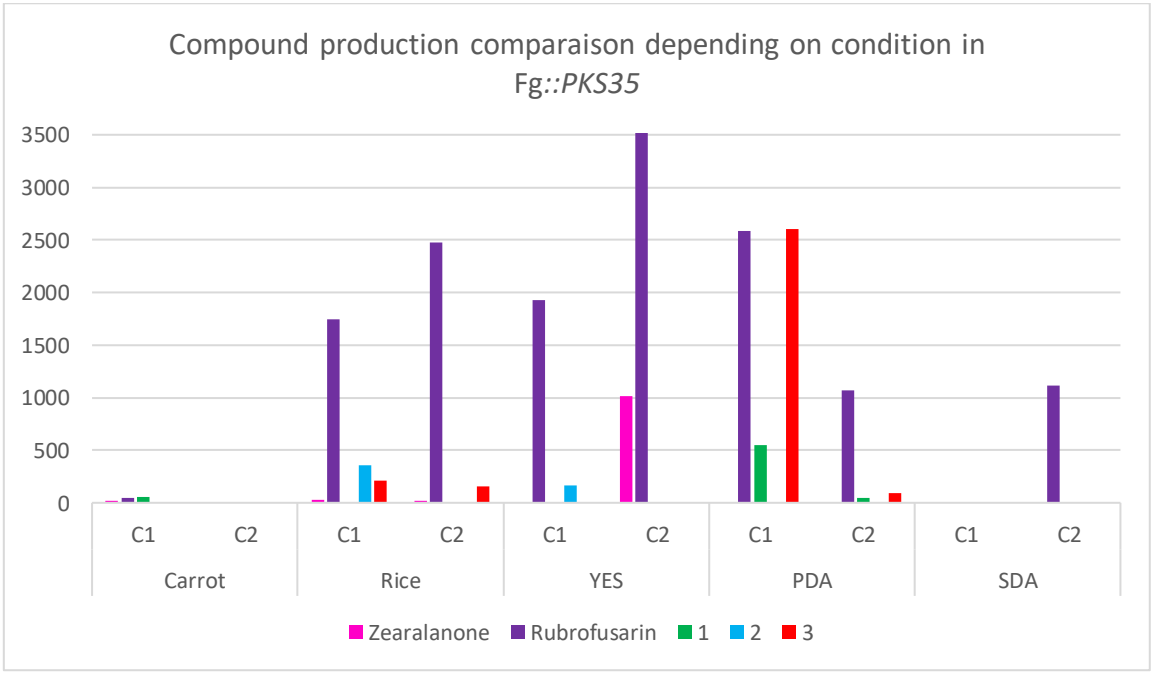


Figure 14. Production of different metabolites (see the legend) by Fg::PKS35 in 5 different media (Carrot agar, Rice agar, YES, PDA and SDA) and both conditions. CM, DFM and V8 juice have not been added as there were no metabolites identified for this strain.

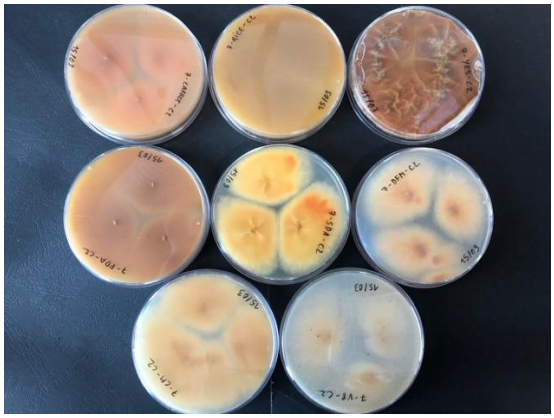
Fs O-tf*PKS35Δfsr1* under C1	Fs O-tf*PKS35Δfsr1* under C2
	
	

Figure 15. Plates after a period of 2 weeks growth of Fg::PKS35 in all the different media. The left column shows the growth of Fg::PKS35 under C1 and the right column shows the growth of Fg::PKS35 under C2.For media identification, follow the scheme facilitated in Fig. 4.

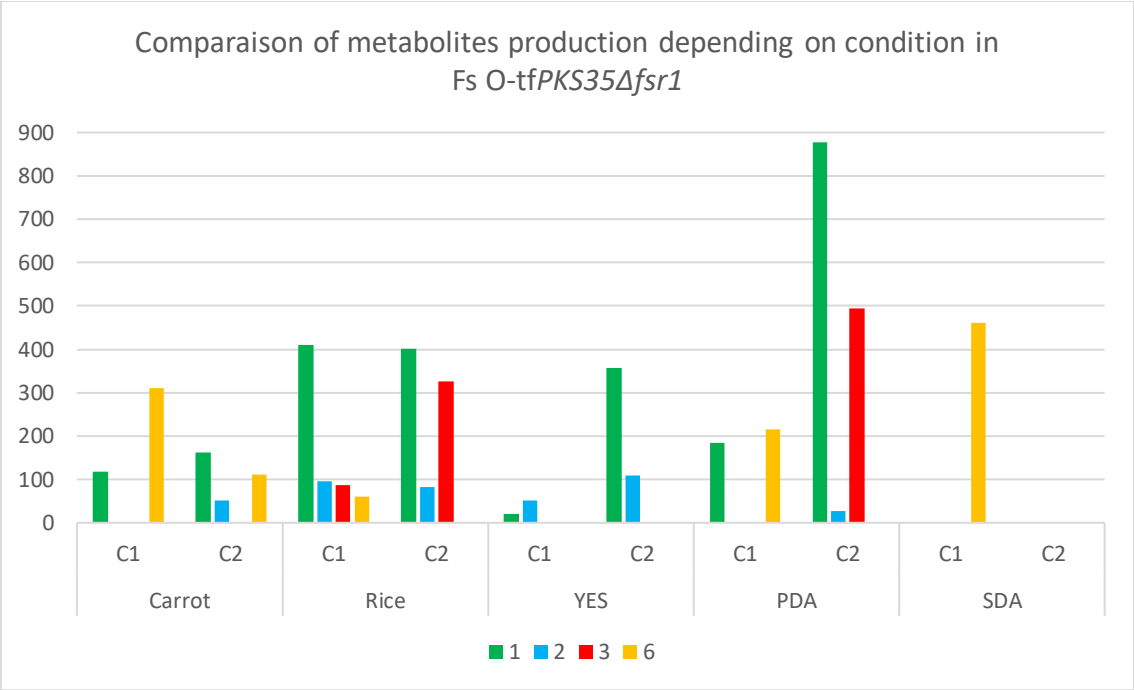


Figure 16. Production of different metabolites (see the legend) by Fg::PKS35 in 5 different media (Carrot agar, Rice agar, YES, PDA and SDA) and both conditions. CM, DFM and V8 juice have not been added as there were no metabolites identified for this strain. Figure repeated from Fig. 12, but this time highlighting its relevance.

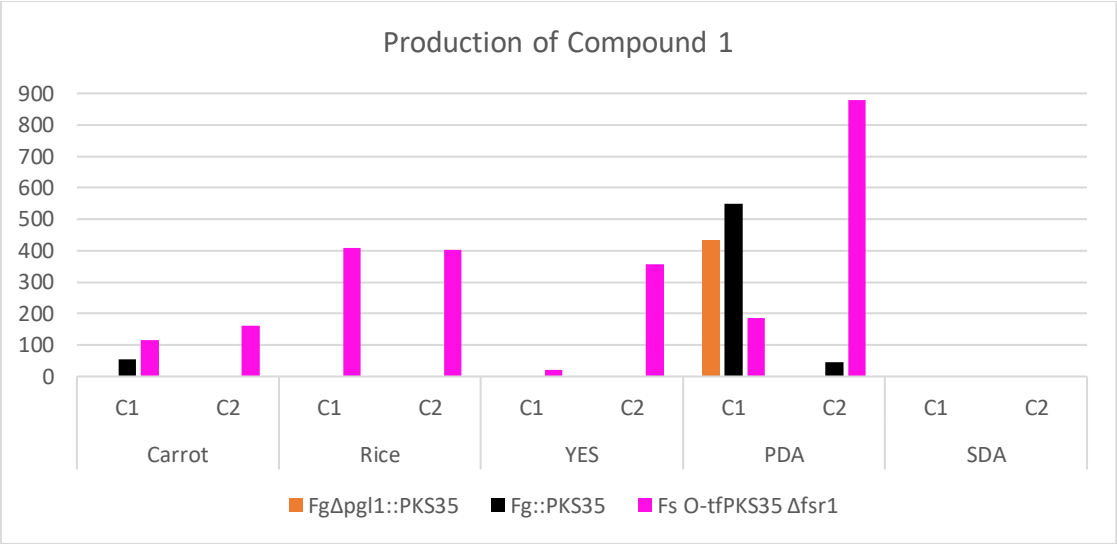
The phenotypical difference for Fs O-tf*PKS35Δfsr1* is not as evident, as there are no big pigmentation differences among the samples from both conditions (Figure 15). If the comparison graphic is observed, the expression rates of the 4 unknown compounds is in this quite irregular, with the only difference pointing towards a higher expression of compound 1 (*PKS35* candidate compound) under C2. However, there is no visual evidence of this phenomenon in neither of the plates. This could either mean that *PKS35* candidate compounds are not related to pigmentation, or that pigmentation does not take place in these samples for some reason.

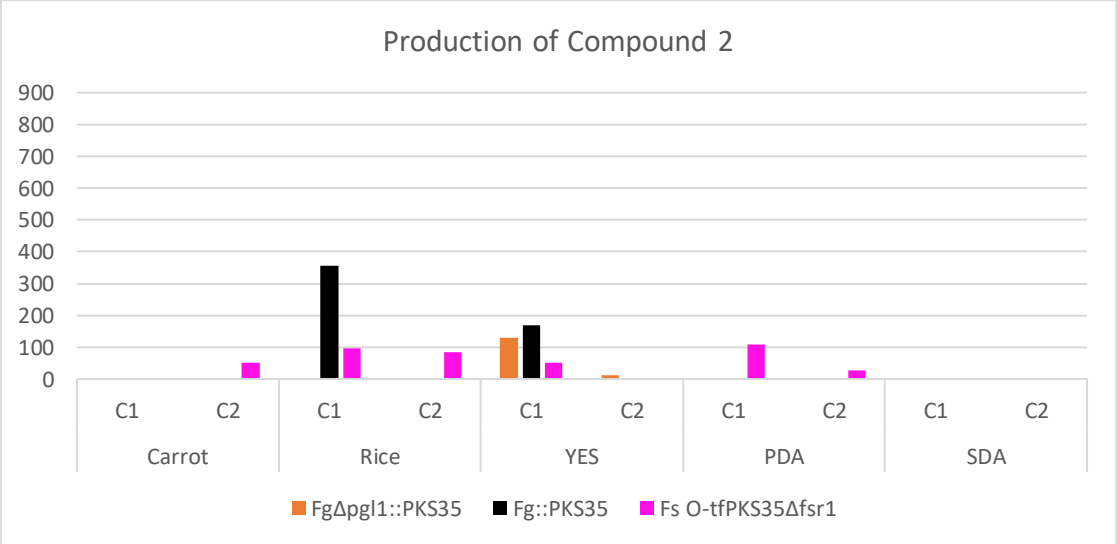
In previous studies, UV light along with other harmful conditions were used in order to generate perithecia in *F. graminearum*, where the responsible BGC for perithecia pigmentation is *PKS3* (Frandsen et al., 2016). However, in *F. solani* this *PKS* is responsible for the mycelia pigmentation, which makes possible the chance of *PKS35* being linked to the perithecia pigmentation. Therefore, this could be the reason why phenotypical difference was not observed in Figure 15, as peritehcia was not formed.

A feasable approach for the *PKS35* study could be the induction of perithecia and posterior metabolites' extraction. The chosen medias could be those that showed the highest expression rates for *PKS35* candidate compounds in the present report, such as rice agar, YES and PDA. Even if in this study perithecia was not formed, UV light already seemed to trigger the expression of *PKS35*, so further stressful factors (i.e. addtion of detergents such as TWEEN 60) could induce the formation of perithecia and therefore also increase the expression rate of *PKS35*.

5.7 Potential media for fungal SMs extraction

Regarding *PKS35* SMs production, the expression levels of the candidate compounds (compound 1, 2 and 3) have been analyzed in the different media and conditions, in order to find out whether there may be some optimum production conditions. The C2 (UV light and room temperature), especially in PDA media, seems to enhance the production of compounds 1 and 3, although there aren't any visible expression patterns for compound 2 indicating a higher level of expression in neither of the two conditions (Figure 17). Concerning growth media, there are some specific media that seem to yield higher production levels of the three candidate compounds compared to the rest of them. Those are, in both C1 and C2 circumstances, rice agar, YES and PDA. The expression rates comparisons for each compound in the different media and conditions are visible in Figure 17. As well, it is important to mention that DFM, CM and V8 juice didn't seem to yield any metabolites of interest, neither from *PKS35* nor many other *PKSs* in *F. solani* and *F. graminearum*. Thus, these medias wouldn't be recommended for the study of *Fusarium's* SMs in further research.





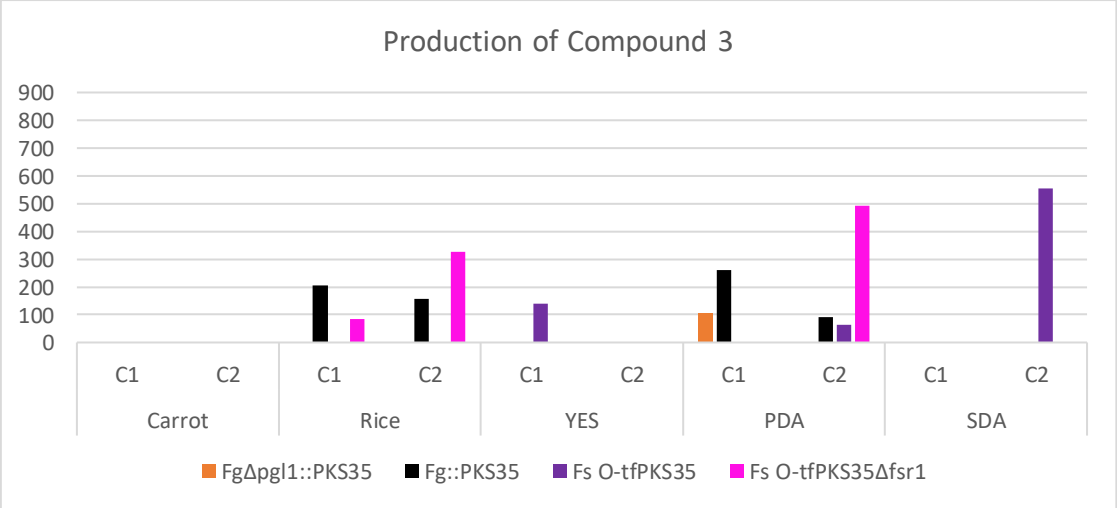


Figure 17. Comparison graphics where the different levels of PKS35 candidate compounds' expression are represented for each producer strain in the different medias and both growth conditions. DFM, CM and V8 juice medias are not represented as no production of the interest compounds was identified. For comparison purposes, in the production graphic of compound 3, the values of PDA C1 have to be multiplied by 10.

6 Overall Discussion

The present report has addressed several fields regarding *Fusarium* SMs and the possible optimum conditions and media for their production. Although the main goal was to trigger the expression of *PKS35* and identify possible candidate compounds, the OSMAC strategy also offered the possibility to analyze the production rate of several other SMs in the different media that have been used, as well as to study if either of the conditions enhanced the expression of any specific BGCs. The drawn conclusions were supported by both data collected from the HPLC analysis (chromatograms and production rate graphics), but also visual evidence of phenotypes observed in the culture plates, which indeed has been a crucial fact when pointing out differing aspects among samples. The following aspects were pointed out through the analysis of the results.

- 3 candidate compounds of *PKS35* have been identified, all of them with similar UV spectrum but very different retention times. While candidate 1 was believed to be the final product of *PKS35*, compounds 2 and 3 could be intermediates in the biosynthetic pathway. This is stated according to their expression rates, since compound 1 presents the higher rates and is usually only produced when *PKS3* is not active, which brings us to the next point.
- The possible connection in the regulation of both *PKS35* and *PKS3* is proposed. In the *F. solani* strains where the tf of *PKS35* was OE and *PKS3* was as well active, a much higher expression rate of *PKS3* was observed. It is possible that the high production rate of the products of *PKS3* casted a shadow over the much lower production of *PKS35*.
- The connection between *PKS3* and *PKS35* was not the only connection observed between BGCs. *PKS12*, responsible for the production of aurofusarin and mycelia pigmentation in *F. graminearum* strains, seemed to be intersected by *PKS35* in Fg*::PKS35*, a strain where the production of rubrofusarin was unexpectedly increased. The accumulation of rubrofusarin was spotted phenotypically due to a darker pigmentation in Fg::*PKS35* strains. These strains were as well observed to be producing some of the *PKS35* compounds, which supports the idea of this BGC being active and thus intersecting somehow the *PKS12* pathway.
- An hypothesis regarding *PKS35*'s function was as well proposed considering the obtained results. The mechanism of this BGC seems to be activated by harming

conditions such as UV light, as higher production rates were obtained under C2. As this BGC has previously been linked to the perithecia pigmentation in *F. solani*, and perithecia is produced in stressful conditions, the link between UV light and higher production rate of *PKS35* further supports this idea of *PKS35* being related to the pigmentation or the formation of perithecia. However, since formation of perithecia was not observed in this study, this can only be proposed for future experiments in this study field.

- As this study was based on an OSMAC strategy, information about the optimum growth medias has as well been collected. For the optimum production of *PKS35* compounds, rice agar, YES and PDA are proposed for further experiments, while medias CM, DFM and V8 juice are not recommended as they didn't yield interest compounds. In the previous point UV light was mentioned to trigger the expression of this BGC, although there were cases where medias grown under C1 (darkness and 28 ºC), such as PDA, yielded high production of *PKS35* candidate compounds as well.

7 Conclusions

So far, this approach to the study of *PKS35* has offered some new perspectives about the metabolic pathway of the BGC. The previously proposed connected regulation with *PKS3* has further been proved with both phenotypical and metabolical evidence of the metabolite expression rate from different strains. It has been observed that the OE of the tf of *PKS35* triggered the OE of *PKS3*, as a higher expression rate of *PKS3* products was analyzed. However, the targeted gene *PKS35* would hardly be noticeable in these Fs O-tf*PKS35* strains, since its rate expression would be zero or very low. When this is contrasted with the mutant Fs O-tf*PKS35Δfsr1*, a big increase in the *PKS35* compounds production was observed, which is believed to be deemed in the inactivation of *PKS3*. Additional to this interaction, *PKS35* also seems to be somehow enhancing the accumulation of rubrofusarin in the aurofusarin pathway, therefore interacting in this case with *PKS12*. It can be assumed that *PKS35* was active in those strains as some of its compounds were identified in the samples.

So far, *PKS35* seems to interact with other PKSs (at least *PKS3* and *PKS12*) that are being expressed in the employed *Fusarium* strains, which makes harder the study of the targeted BGC. Therefore, in future approaches the inactivation of the concerned *PKS*s could ease the study of *PKS35*, as it has already been observed in the mutant Fs O-tf*PKS35Δfsr1*, which yielded the highest production rate of PKS35 compounds. Regarding these metabolites, 3 candidates have been contemplated during the study of this research, due to both their expression patterns and their UV spectra similarities among each other. Out of the 3 candidates, compound 1 is considered the main candidate to be the BGC's final product, while compound 2 and 3 are contemplated as possible intermediates in *PKS35*'s biosynthetic pathway. The further isolation and purification of these compounds could offer a closer study of these metabolites and the possible linkage among them.

As it was initially stated, one of the goals of this study was to analyze the impact of different media and conditions in the growth and metabolite production of the different strains of *Fusarium*. This OSMAC strategy has indeed offered valuable information for further study of *PKS35*, as some conditions have been observed to yield higher rates of metabolite expression, which would ease the future study of *PKS35* compounds. Rice

agar, YES and PDA are highlighted as optimum media for *PKS35* metabolite formation, while CM, DFM and V8 juice are not recommended for the study of this BGC, as the expression rate of SMs in general seemed to be very low or none. Carrot and SDA are considered to be average growth media for *Fusarium* metabolite formation, as their production levels weren't either the highest but still interesting compounds were identified.

Additional to media composition, external conditions also seemed to have effect on the expression of metabolites. UV light embraced higher levels of *PKS35* compounds expression in most media, especially in the ones that were considered as optimum. As this BGC has been related to perithecia pigmentation before, it would be interesting a future approach with additional stressful factors (addition of detergent) that could induce the formation of perithecia and therefore also maybe increase the expression rate of *PKS35*.

8 Bibliography

Bahram, M., & Netherway, T. (2022). Fungi as mediators linking organisms and ecosystems. *FEMS Microbiology Reviews*, *058*, 1–16. https://doi.org/10.1093/femsre/fuab058

Bergmann, S., Schümann, J., Scherlach, K., Lange, C., Brakhage, A. A., & Hertweck, C. (2007). *Genomics-driven discovery of PKS-NRPS hybrid metabolites from Aspergillus nidulans*. https://doi.org/10.1038/nchembio869

Bode, H. B., Bethe, B., Hˆfs, R., & Zeeck, A. (2002a). Big Effects from Small Changes: Possible Ways to Explore Nature's Chemical Diversity. In *ChemBioChem* (Vol. 3). WILEY. http://www.sanger.ac.uk/Projects/S

Bode, H. B., Bethe, B., Hˆfs, R., & Zeeck, A. (2002b). Big Effects from Small Changes: Possible Ways to Explore Nature's Chemical Diversity. In *ChemBioChem* (Vol. 3). WILEY. http://www.sanger.ac.uk/Projects/S

Boruta, T. (2018). Uncovering the repertoire of fungal secondary metabolites: From fleming's laboratory to the international space station. *Bioengineered*, *9*(1), 12–16. https://doi.org/10.1080/21655979.2017.1341022

Brakhage, A. A. (2013). Regulation of fungal secondary metabolism. In *Nature Reviews Microbiology* (Vol. 11, Issue 1, pp. 21–32). https://doi.org/10.1038/nrmicro2916

Brakhage, A. A., & Schroeckh, V. (2011). Fungal secondary metabolites – Strategies to activate silent gene clusters. *Fungal Genetics and Biology*, *48*(1), 15–22. https://doi.org/10.1016/J.FGB.2010.04.004

Brown, D. W., Kim, H.-S., Mcgovern, A. E., Probyn, C. E., & Proctor, R. H. (2022). Genus-wide analysis of Fusarium polyketide synthases reveals broad chemical potential. *Fungal Genetics and Biology*, *160*, 103696. https://doi.org/10.1016/j.fgb.2022.103696

Brown, D. W., & Proctor, R. H. (2016). Insights into natural products biosynthesis from analysis of 490 polyketide synthases from Fusarium. *Fungal Genetics and Biology*, *89*, 37–51. https://doi.org/10.1016/j.fgb.2016.01.008

Chiang, Y. M., Szewczyk, E., Nayak, T., Davidson, A. D., Sanchez, J. F., Lo, H. C., Ho, W. Y., Simityan, H., Kuo, E., Praseuth, A., Watanabe, K., Oakley, B. R., & Wang, C. C. C. (2008). Molecular Genetic Mining of the Aspergillus Secondary Metabolome: Discovery of the Emericellamide Biosynthetic Pathway. *Chemistry and Biology*, *15*(6), 527–532. https://doi.org/10.1016/j.chembiol.2008.05.010

Choi, H.-W., Kim, J.-M., Hong, S. K., Kim, W. G., Chun, S.-C., & Yu, S.-H. (2009). Mating Types and Optimum Culture Conditions for Sexual State Formation of Fusarium fujikuroi Isolates . *Mycobiology*, *37*(4), 247. https://doi.org/10.4489/myco.2009.37.4.247

Corre, C., Song, L., O'rourke, S., Chater, K. F., & Challis, G. L. (2008). *2-Alkyl-4-hydroxymethylfuran-3-carboxylic acids, antibiotic production inducers discovered by Streptomyces coelicolor genome mining*. www.pnas.org/cgi/content/full/

Cueto, M., Jensen, P. R., Kauffman, C., Fenical, W., Lobkovsky, E., & Clardy, J. (2001). *Notes Pestalone, a New Antibiotic Produced by a Marine Fungus in Response to Bacterial Challenge*. https://doi.org/10.1021/np0102713

Dai, G., Shen, Q., Zhang, Y., & Bian, X. (2022). *Biosynthesis of Fungal Natural Products Involving Two Separate Pathway Crosstalk*. https://doi.org/10.3390/jof8030320

Daniel, R. (2004). The soil metagenome - A rich resource for the discovery of novel natural products. In *Current Opinion in Biotechnology* (Vol. 15, Issue 3, pp. 199–204). https://doi.org/10.1016/j.copbio.2004.04.005

David E Cane and Christopher T Walsh. (1999). *Polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs) catalyze chain elongation from simple building blocks to create a diverse array of natural products. PKS and NRPS proteins share striking architectural and organizational similarities that can be exploited to generate entirely new natural products*.

Droce, A., Saei, W., Jørgensen, S. H., Wimmer, R., Giese, H., Wollenberg, R. D., Sondergaard, T. E., & Sørensen, J. L. (2016). Functional analysis of the fusarielin biosynthetic gene cluster. *Molecules*, *21*(12). https://doi.org/10.3390/molecules21121710

Frandsen, R. J. N., Nielsen, N. J., Maolanon, N., Sørensen, J. C., Olsson, S., Nielsen, J., & Giese, H. (2006). *The biosynthetic pathway for aurofusarin in Fusarium graminearum reveals a close link between the naphthoquinones and naphthopyrones*. https://doi.org/10.1111/j.1365-2958.2006.05295.x

Frandsen, R. J. N., Rasmussen, S. A., Knudsen, P. B., Uhlig, S., Petersen, D., Lysøe, E., Gotfredsen, C. H., Giese, H., & Larsen, T. O. (2016). Black perithecial pigmentation in Fusarium species is due to the accumulation of 5-deoxybostrycoidin-based melanin. *Scientific Reports*, *6*. https://doi.org/10.1038/srep26206

Gaffoor, I., Brown, D. W., Plattner, R., Proctor, R. H., Qi, W., & Trail, F. (2005). Functional analysis of the polyketide synthase genes in the filamentous fungus Gibberella zeae (Anamorph Fusarium graminearum). *Eukaryotic Cell*, *4*(11), 1926–1933. https://doi.org/10.1128/EC.4.11.1926-1933.2005

Graziani, S., Vasnier, C., & Daboussi, M. J. (2004). Novel polyketide synthase from Nectria haematococca. *Applied and Environmental Microbiology*, *70*(5), 2984–2988. https://doi.org/10.1128/AEM.70.5.2984-2988.2004

Han, Z., Tangni, E. K., Huybrechts, B., Munaut, F., Scauflaire, J., Wu, A., & Callebaut, A. (2014). Screening survey of co-production of fusaric acid, fusarin C, and fumonisins B1, B2 and B3 by Fusarium strains grown in maize grains. *Mycotoxin Research*, *30*(4), 231–240. https://doi.org/10.1007/s12550-014-0207-1

Hanachi, P. (2009). *Using HPLC to Determination the Composition and Antioxidant Activity of Berberis Vulgaris*. https://www.researchgate.net/publication/326519963

Hansen, F. T., Gardiner, D. M., Lysøe, E., Fuertes, P. R., Tudzynski, B., Wiemann, P., Sondergaard, T. E., Giese, H., Brodersen, D. E., & Sørensen, J. L. (2015). An update to polyketide synthase and non-ribosomal synthetase genes and nomenclature in Fusarium. In *Fungal Genetics and Biology* (Vol. 75, pp. 20–29). Academic Press Inc. https://doi.org/10.1016/j.fgb.2014.12.004

Hansen, F. T., Sørensen, J. L., Giese, H., Sondergaard, T. E., & Frandsen, R. J. N. (2012). *Quick guide to polyketide synthase and nonribosomal synthetase genes in Fusarium*. https://doi.org/10.1016/j.ijfoodmicro.2012.01.018

Hestbjerg, H., Nielsen, K. F., Thrane, U., & Elmholt, S. (2002). *Production of Trichothecenes and Other Secondary Metabolites by Fusarium culmorum and Fusarium equiseti on Common Laboratory Media and a Soil Organic Matter Agar: An Ecological Interpretation*. https://doi.org/10.1021/jf020432o

Hijri, M., Zheng, J., van der Lee, T. A. J., Medema, M. H., Hoogendoorn, K., Barra, L., Waalwijk, C., & Dickschat, J. S. (2018). Evolution and Diversity of Biosynthetic Gene Clusters in Fusarium. *Frontiers in Microbiology | Www.Frontiersin.Org*, *1*, 1158. https://doi.org/10.3389/fmicb.2018.01158

Ibrahim, S. R. M., Sirwi, A., Eid, B. G., Mohamed, S. G. A., & Mohamed, G. A. (2021). Bright Side of Fusarium oxysporum: Secondary Metabolites Bioactivities and Industrial Relevance in Biotechnology and Nanotechnology. *Industrial Relevance in Biotechnology and Nanotechnology*. *J. Fungi*, *7*. https://doi.org/10.3390/jof711

Imamura, Y., Chandra, J., Mukherjee, P. K., Lattif, A. A., Szczotka-Flynn, L. B., Pearlman, E., Lass, J. H., O'Donnell, K., & Ghannoum, M. A. (2008). Fusarium and Candida albicans biofilms on soft contact lenses: Model development, influence of lens type, and susceptibility to lens care solutions. *Antimicrobial Agents and Chemotherapy*, *52*(1), 171–182. https://doi.org/10.1128/AAC.00387-07

Keller, N. P. (2019). Fungal secondary metabolism: regulation, function and drug discovery HHS Public Access. *Nat Rev Microbiol*, *17*(3), 167–180. https://doi.org/10.1038/s41579-018-0121-1

Kristensen, S. B., Pedersen, T. B., Nielsen, M. R., Wimmer, R., Muff, J., & Sørensen, J. L. (2021a). *toxins Communication Production and Selectivity of Key Fusarubins from Fusarium solani due to Media Composition*. https://doi.org/10.3390/toxins13060376

Kristensen, S. B., Pedersen, T. B., Nielsen, M. R., Wimmer, R., Muff, J., & Sørensen, J. L. (2021b). *toxins Communication Production and Selectivity of Key Fusarubins from Fusarium solani due to Media Composition*. https://doi.org/10.3390/toxins13060376

Lebeau, J., Petit, T., Dufossé, L., & Caro, Y. (2019). Putative metabolic pathway for the bioproduction of bikaverin and intermediates thereof in the wild Fusarium oxysporum LCP531 strain. *AMB Express*, *9*(1). https://doi.org/10.1186/s13568-019-0912-4

Li, R., Npr, /, Zhang, H., Li, M., Yu, R., Bai, X., & Wang, H. (2020). *Natural Product Reports Fusarium: a treasure trove of bioactive secondary metabolites Fusarium: a treasure trove of bioactive secondary metabolites †*. *37*, 1551–1702. https://doi.org/10.1039/d0np00038h

Losada, L., Ajayi, O., Frisvad$, J. C., Jiujiang, Y., & Nierman, W. C. (2009). *Effect of competition on the production and activity of secondary metabolites in Aspergillus species*. https://doi.org/10.1080/13693780802409542

Macheleidt, J., Mattern, D. J., Fischer, J., Netzker, T., Weber, J., Schroeckh, V., Valiante, V., & Brakhage, A. A. (2016). Regulation and Role of Fungal Secondary Metabolites. In *Annual Review of Genetics* (Vol. 50, pp. 371–392). Annual Reviews Inc. https://doi.org/10.1146/annurev-genet-120215-035203

Malz, S., Grell, M. N., Thrane, C., Maier, F. J., Rosager, P., Felk, A., Albertsen, K. S., Salomon, S., Bohn, L., Schäfer, W., & Giese, H. (2005). Identification of a gene cluster responsible for the biosynthesis of aurofusarin in the Fusarium graminearum species complex. *Fungal Genetics and Biology*, *42*(5), 420–433. https://doi.org/10.1016/j.fgb.2005.01.010

Minami, A., Ugai, T., Ozaki, T., & Hideaki, &. (2020). *predicting the chemical space of fungal polyketides by phylogeny-based bioinformatics analysis of polyketide synthase-nonribosomal peptide synthetase and its modification enzymes*. *10*, 13556. https://doi.org/10.1038/s41598-020-70177-w

Moretto, L., Heylen, R., Holroyd, N., Vance, S., & Broadhurst, R. W. (2019). Modular type I polyketide synthase acyl carrier protein domains share a common N-terminally extended fold. *Scientific Reports*, *9*(1). https://doi.org/10.1038/S41598-019-38747-9

Nancy N. (1979). *New antibiotic pigment related to fusarubin from Fusarium solani*.
Neubauer, P., Craig Crampton, M., Li, W.-J., Rao, N. M., W-j, L., Prabhu Narsing Rao, M., & Xiao, M. (2017). Fungal and Bacterial Pigments: Secondary Metabolites with Wide Applications. *Front. Microbiol*, *8*, 1113. https://doi.org/10.3389/fmicb.2017.01113
Nielsen, M. R., Evelyne, S., Kaniki, K., & Sørensen, J. L. (2022). *Chapter 6 Targeted Genetic Engineering via Agrobacterium-Mediated Transformation in Fusarium solani*. https://doi.org/10.1007/978-1-0716-2273-5_6
Nielsen, M. R., Teis, ·, Sondergaard, E., Giese, H., & Sørensen, J. L. (2019). Advances in linking polyketides and non-ribosomal peptides to their biosynthetic gene clusters in Fusarium. *Current Genetics*, *65*, 1263–1280. https://doi.org/10.1007/s00294-019-00998-4
Oh, D.-C., Kauffman, C. A., Jensen, P. R., & Fenical, W. (2007). Induced Production of Emericellamides A and B from the Marine-Derived Fungus Emericella sp. in Competing Co-culture. *J. Nat. Prod*, *70*. https://doi.org/10.1021/np060381f
Pérez Hemphill, C. F., Sureechatchaiyan, P., Kassack, M. U., Orfali, R. S., Lin, W., Daletos, G., & Proksch, P. (2017). OSMAC approach leads to new fusarielin metabolites from Fusarium tricinctum. *The Journal of Antibiotics*, *70*, 726–732. https://doi.org/10.1038/ja.2017.21
Pradeep, B. v, Pradeep, F. S., Shakila Begam, M., & Palaniswamy, M. (2013). Influence of Culture Media on Growth and Pigment Production by Fusarium moniliforme KUMBF1201 Isolated from Paddy Field Soil. *World Applied Sciences Journal*, *22*(1), 70–77. https://doi.org/10.5829/idosi.wasj.2013.22.01.7265
Proctor, R. H., Brown, D. W., Plattner, R. D., & Desjardins, A. E. (2003). *Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in Gibberella moniliformis q*. https://doi.org/10.1016/S1087-1845(02)00525-X
Proctor, R. H., Butchko, R. A. E., Brown, D. W., & Moretti, A. (2007). Functional characterization, sequence comparisons and distribution of a polyketide synthase gene required for perithecial pigmentation in some Fusarium species. *Food Additives and Contaminants*, *24*(10), 1076–1087. https://doi.org/10.1080/02652030701546495
Qin, Z., Devine, R., Hutchings, M. I., Wilkinson, B., & Hutchings, M. (2019). *Aromatic polyketide biosynthesis: fidelity, evolution and engineering*. https://doi.org/10.1101/581074
Rank, M., Bruun, T., Kabemba Kaniki, E., Karolina Rilana, A., Esben, T., & Laurids, J. (2020). *Aalborg Universitet Solving the Polyketide Pigmentation Puzzle in Fusarium solani Linking biosynthetic genes to compounds SOLVING THE POLYKETIDE PIGMENTATION PUZZLE OF FUSARIUM SOLANI*.
Russell, G., Graveley, R., Seid, J., Al-Humidan, A.-K., & Skjodt, H. (1992a). *Mechanisms of Action of Cyclosporine and Effects on Connective Tissues*.
Russell, G., Graveley, R., Seid, J., Al-Humidan, A.-K., & Skjodt, H. (1992b). *Mechanisms of Action of Cyclosporine and Effects on Connective Tissues*.
Scherlach, K., & Hertweck, C. (2006). *Discovery of aspoquinolones A-D, prenylated quinoline-2-one alkaloids from Aspergillus nidulans, motivated by genome mining †*. https://doi.org/10.1039/b607011f
Schueffler, A., & Anke, T. (2014). *Fungal natural products in research and development*. https://doi.org/10.1039/c4np00060a
Senges, C. H. R., Al-Dilaimi, A., Marchbank, D. H., Wibberg, D., Winkler, A., Haltli, B., Nowrousian, M., Kalinowski, J., Kerr, R. G., & Bandow, J. E. (2018). The secreted metabolome of Streptomyces chartreusis and implications for bacterial

chemistry. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(10), 2490–2495. https://doi.org/10.1073/pnas.1715713115

Urban, M., Mott, E., Farley, T., & Hammond-Kosack, K. (2003). The Fusarium graminearum MAP1 gene is essential for pathogenicity and development of perithecia. *Molecular Plant Pathology*, *4*(5), 347–359. https://doi.org/10.1046/j.1364-3703.2003.00183.x

Wang, J., Zhang, R., Chen, X., Sun, X., Yan, Y., Shen, X., & Yuan, Q. (2020). Biosynthesis of aromatic polyketides in microorganisms using type II polyketide synthases. In *Microbial Cell Factories* (Vol. 19, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s12934-020-01367-4

Westphal, K. R., Bachleitner, S., Severinsen, M. M., Brundtø, M. L., Hansen, F. T., Sørensen, T., Wollenberg, R. D., Lysøe, E., Studt, L., Sørensen, J. L., Sondergaard, T. E., & Wimmer, R. (2021). Cyclic, Hydrophobic Hexapeptide Fusahexin Is the Product of a Nonribosomal Peptide Synthetase in Fusarium graminearum. *Journal of Natural Products*, *84*(8), 2070–2080. https://doi.org/10.1021/acs.jnatprod.0c00947

Westphal, K. R., Wollenberg, R. D., Herbst, F. A., Sørensen, J. L., Sondergaard, T. E., & Wimmer, R. (2018). Enhancing the production of the fungal pigment aurofusarin in Fusarium graminearum. *Toxins*, *10*(11). https://doi.org/10.3390/toxins10110485

Zhou, J., Pan, G., Liu, Z., Luo, Y., Liu, Z., Zhao, Y., & Huang, C. (2021). *Recent Advances in Silent Gene Cluster Activation in Streptomyces*. https://doi.org/10.3389/fbioe.2021.632230

9 Appendix

The purpose of this section is to provide the essential information in order to produce the growth media that has been used throughout the experimental procedure.

Carrot Agar

- 200 g carrot infusion (autoclaving required)
- 20 g agar
- Distilled water up until 1000 ml

Rice Agar

- 20 g white rice extract (Wholemeal rice flour from *Urtekram)*
- 20 g agar
- Distilled water up until 1000 ml

YES

- 20 g yeast extract
- 150 g sucrose
- 0,5 g $MgSO_4 \cdot 7H_2O$
- 20 g agar
- 1 ml trace metal solution (SM)
- Distilled water up until 1000 ml

Trace metals solution (SM)

- 1 g $ZnSO_4 \cdot 7\ H_2O$
- 0,5 g $CuSO_4 \cdot 5\ H_2O$
- 100 ml MQ water

Everything is heated to boiling point in order to dissolve all the components completely. Afterwards it is autoclaved

PDA

- 39 g potato dextrose broth
- 20 g agar
- 1 mL trace metals solution
- Distilled water up until 1000 ml

SDA (Sabouraud dextrose agar)

- 40 g dextrose
- 10 g peptone
- 20 g agar
- Distilled water up until 1000 ml

DFM

- 62,5 ml 20% glucose
- 200 ml 50 mM asparragine
- 10 ml 0,21 M $MgSO_4 \cdot 7\ H_2O$ (26 g/ 500 ml)
- 10 ml 1,12 M KH_2PO_4 + 0,7 M KCl (pH 6)
- 20 g agar
- 1 ml 1000x trace solution
- Distilled water up until 1000 ml

1000x Trace solution

- 40 mg $Na_2B_4O_7 \cdot 10\ H_2O$,
- 400 mg $CuSO_4 \cdot 5\ H_2O$
- 1,2 g $FeSO_4 \cdot 7\ H_2O$
- 700 mg $MnSO_4 \cdot H_2O$
- 800 mg $NaMoO_2 \cdot 2\ H_2O$
- 10 g $ZnSO_4 \cdot 7\ H_2O$
- MQ water up until 1000 ml

CM

- 2,4 g NaNO3
- 0,6 g $MgSO_4 \cdot 7H_2O$
- 0,1 g NaCl
- 0,1 CaCl2
- 10 g sucrose
- 7,5 g malt extract
- 2,5 g yeast extract
- 20 g agar
- 1 ml trace element mixture
- Distilled water up until 1000 ml

Trace element mixture for CM

- 9 mg H_3BO_3
- 58,5 mg $CuSO_4 \cdot 5\ H_2O$
- 1.95 mg KI
- 9 mg $MnSO_4 \cdot H_2O$
- 7.6 mg $NaMoO_4$
- 822 mg $ZnSO_4 \cdot 6\ H_2O$
- 139.8 mg $FeCl_3 \cdot 6\ H_2O$
- 300 ml MQ water

V8 Juice

- 175 ml Campbell's V8-juice (American Company, Peder Hvitfeldts Str.13, 1173 Kbh K)
- 3 g $CaCO_3$
- 20 g agar
- Distilled water up until 1000 ml