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Computational and molecular study of terpene synthase genes in *Trichoderma*

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ABSTRACT

Trichoderma is a fungal genus comprising a large number of species of great interest for plant protection and industry. The broad spectrum of lifestyles of the isolates belonging to this genus is likely supported by the diversity of their Secondary Metabolites (SMs) arsenal. In the present work, we combined genome mining and comparative genomic approaches to provide an extensive view of the SMs potential in this genus. Assessment of the core-genes and Biosynthetic Gene Clusters (BGCs) involved in SMs biosynthesis in the genomes of 21 isolates of 17 *Trichoderma* species showed that closest phylogenetic species tend to have similar SMs inventories, while lifestyle diversity could explain differences found among clades. *Trichoderma* contains a striking number of terpenoid synthases (TSs) genes, whose almost half is included in clusters. Characterization based on conserved sequence features and phylogenetic analysis with known TS proteins revealed the putative functions of 15 groups of prenyl transferases, terpene cyclases and chimeric proteins, providing an overview of the diversity of these enzymes in the genus. Trichodiene synthases (TRI5)-encoding genes were found in different genomic contexts in the non-trichothecene producer species. In particular, *tri5* genes from *T. gamsii* isolates are embedded in a 21.3 Kb putative cluster including a transcription factor, some tailoring enzymes and a transporter. Since *T. gamsii* lacks the other *TRI* genes required for trichothecene production, we hypothesize that *tri5* might be involved, in this species, in the biosynthesis of sesquiterpene/s other than trichothecenes, therefore participating in not yet defined metabolic pathways in *Trichoderma*. The characteristics of the isolate T6085 of *T. gamsii* (*Tgam)*, known both to antagonize *Fusarium* spp. and to reduce Fusarium Head Blight (FHB) on wheat, and for its ability to colonize the rhizosphere and, endophytically, the wheat roots, enabled to study the regulation of the TS genes in different environments. Overall, oxidative and saline stresses, N starving, and availability of C source differentially affected TS genes expression, and results suggest that production of indole diterpenes could occur in response to oxidative stress. TS genes expression did not change significantly when the fungus was growing on wheat spikes in presence/absence of *F. graminearum*. In contrast, an evident reprogramming of terpene biosynthesis seems to take place when the fungus colonizes the roots (on PDA) compared to when it grows on PDA alone. Specifically, if we consider the expression of *tri5*, results suggest this gene in *T. gamsii* has a different type of regulation compared to what is known for *tri5* of *T. brevicompactum*. The strong up-regulation of *tri5* found when the fungus colonizes the wheat roots suggests this gene could have an important role in the interaction with the plant. In addition, metabolic profiles of *Tgam* revealed the absence of trichothecene compounds, and the ability of the fungus to produce diterpenes and high amounts of pyrones. In summary, we provide i) an extensive view of the SMs potential in *Trichoderma*, ii) a genomic characterization of the TS inventory in the genus, iii) a picture of TS gene regulation in *Tgam* in different environments, iv) a step forward to deciphering the regulation of *tri5* in *Tgam* and its relevance in the relation with the plant, and v) interesting questions about the biological significance of *tri5* in beneficious *Trichoderma* species.

RESUMEN

Trichoderma comprende un amplio número de especies, de gran interés en el manejo de enfermedades de las plantas de cultivo y la industria. El amplio rango de estilos de vida de estas especies está respaldado por su diversidad de Metabolitos Secundarios (MSs). En este trabajo, se ha utilizado una combinación de enfoques de minería genómica y genómica comparativa generando una extensa visión sobre el potencial de biosíntesis de MSs en *Trichoderma*. La evaluación del contenido en genes core y clusters de genes involucrados en la producción de MSs en 21 cepas de 17 especies de *Trichoderma* muestra que las especies filogenéticamente cercanas tienen arsenales de MSs similares, y que las diferencias en los estilos de vida de estas especies podrían explicar las diferencias encontradas entre clados. *Trichoderma* contiene un sorprendente número de terpeno sintasas (TSs). La caracterización de estas enzimas, en base a elementos de secuencia conservados y análisis filogenético junto con TSs de función conocida, permitió asignar funciones putativas a 15 grupos de prenil-transferasas, terpeno ciclasas y proteínas quiméricas, proporcionando una visión general de la diversidad de esta familia génica dentro del género. Las enzimas trichodieno sintasas (TRI5) se encontraron en distintos contextos genómicos en especies no productoras de trichotecenos. En *T. gamsii*, *tri5* se encuentra incluido en un cluster de 21.3 kb junto con un factor de transcripción, algunas encimas de modificaciones secundarias y un transportador. Dado que *T. gamsii* carece de los genes TRI necesarios para la biosíntesis de trichotecenos, *tri5* podría estar involucrado en esta especie, en la biosíntesis de sesquiterpeno/s diferentes a los trichotecenos, participando así en vias metabólicas aun no definidas en *Trichoderma*. Las características de *T. gamsii* T6085 (*Tgam*), antagonista de *Fusarium* spp. y capaz de controlar la Fusariosis de la Espiga (FHB) en trigo, así como de colonizar la rizosfera y endofitar las raíces de las plantas de trigo, permitieron estudiar la regulación de los genes TSs en diferentes condiciones ambientales. El estrés oxidativo, salino y por baja disponibilidad de N, así como la presencia/ausencia de una fuente de C, afectaron diferencialmente la expresión de los genes TSs, y los resultados sugieren que la producción de diterpenos de indol podría ocurrir en respuesta al estrés oxidativo. La expresión de los genes TSs no cambió cuando el hongo crecía en las espigas de trigo en presencia/ausencia de *F. graminearum*. Sin embargo, una evidente reprogramación en la biosíntesis de terpenos parece ocurrir cuando el hongo coloniza las raíces de las plantas de trigo. Si se considera la expresión de *tri5*, los resultados sugieren que su regulación sería distinta en *T. gamsii* y *T. brevicompactum*. La fuerte expresión de *tri5* durante la colonización de las raíces sugiere que este gen podría tener un papel importante en la interacción del hongo con la planta. Por otro lado, los perfiles metabólicos de *Tgam* revelaron la ausencia de trichotecenos, y la habilidad del hongo para producir diterpenos y grandes cantidades de pironas. Este trabajo proporciona i) una visión extensa del potencial de biosíntesis de MSs en *Trichoderma*, ii) una caracterización genómica detallada de la diversidad en la familia de TSs en *Trichoderma*, iii) una fotografía de la regulación de las TSs en *Tgam* en diferentes situaciones ambientales, iv) algunas claves sobre la regulación de *tri5* en *Tgam* y su relevancia en la relación con la planta, y v) preguntas interesantes sobre el significado biológico de *tri5* en especies beneficiosas de *Trichoderma*.

RIASSUNTO

Trichoderma è un genere fungino che comprende un grande numero di specie d'interesse per la difesa delle piante e per l'industria. L'ampia varietà degli stili di vita degli isolati appartenenti a questo genere è supportata dalla diversità dei Metaboliti Secondari (MSs) che sono in grado di produrre. Nel presente lavoro, la combinazione di un approccio di *genome-mining* e di genomica comparata ha permesso di ottenere una visione ampia del potenziale biosintetico, in termini di MS, del genere *Trichoderma*. La valutazione dei geni *"core*" e dei *cluster* genici coinvolti nella biosintesi dei MSs presenti nei genomi di 21 isolati appartenenti a 17 specie di *Trichoderma*, ha rivelato che le specie filogeneticamente vicine tendono ad avere un arsenale di MSs simile, mentre la diversità dello stile di vita potrebbe spiegare le differenze riscontrate tra i *clade*. *Trichoderma* contiene una grande quantità di enzimi appartenenti alla classe delle terpene sintasi (TSs), la cui caratterizzazione, sulla base delle sequenze conservate e dell'analisi filogenetica effettuata su quelle con funzione nota, ha rivelato le funzioni putative di 15 gruppi di prenil-transferasi, terpene ciclasi ed enzimi chimerici, fornendo una panoramica della diversità di questi enzimi all'interno del genere. Gli enzimi tricodiene sintasi (TRI5) sono stati trovati in diversi contesti genomici anche in specie non produttrici di tricoteceni. In *T. gamsii*, *tri5* appartiene ad un cluster genico di 21.3 Kb che include un fattore di trascrizione, diversi enzimi responsabili di modificazioni secondarie, ed un trasportatore. Poiché *T. gamsii* non presenta altri geni TRI necessari per la biosintesi dei tricoteceni, *tri5* potrebbe essere coinvolto, in questa specie, nella biosintesi di sesquiterpeni diversi, partecipando quindi a vie metaboliche non ancora definite in *Trichoderma*. Le caratteristiche dell'isolato T6085 di *T. gamsii* (*Tgam*), noto sia per antagonizzare *Fusarium* spp. e ridurre l'incidenza della fusariosi sul frumento, che per la sua capacità di colonizzare la rizosfera e, endofiticamente, le radici di questa specie vegetale, ha permesso di studiare la regolazione dei geni TS in diverse condizioni ambientali. In generale, gli stress ossidativi e salini, la bassa disponibilità di N e la presenza/assenza di fonti di C, hanno effetti differenziali sull'espressione dei geni TS che, tuttavia, non cambia quando il fungo cresce nelle spighe di frumento in presenza/assenza di *F. graminearum*. Un'evidente riprogrammazione nella biosintesi dei terpeni sembra verificarsi, invece, quando il fungo colonizza le radici di frumento (su PDA), rispetto a quando cresce da solo su mezzo agarizzato. In particolare, si se considera l'espressione di *tri5*, i risultati suggeriscono che in *T. gamsii* questo gene è regolato diversamente rispetto a quanto noto per *T. brevicompactum*. La forte espressione di *tri5* rilevata quando il T6085 colonizza le radici di frumento suggerisce che questo gene potrebbe avere un ruolo importante nell'interazione con la pianta. Inoltre, i profili metabolici di *Tgam* rivelano l'assenza di tricoteceni e la capacità di questo fungo di produrre diterpeni ed un'elevata quantità di pirone. Questo lavoro fornisce: i) una visione ampia del potenziale biosintetico dei MSs e; ii) una caratterizzazione delle TSs in *Trichoderma*; iii) un quadro sulla regolazione dei geni TS in *Tgam* in diverse condizioni ambientali; iv) nuove informazioni sulla regolazione di *tri5* in *Tgam* e il suo coinvolgimento nelle interazioni tra il fungo e la pianta e; v) lascia aperti alcuni quesiti, meritevoli di indagini, sul significato biologico di *tri5* in specie benefiche di *Trichoderma*.

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Abbreviations

µL	microliter
µm	micrometer
µM	micromolar
^{13}C-NMR	carbon 13 – nuclear magnetic resonance
^{1}H-NMR	proton – nuclear magnetic resonance
6-PP	6-pentyl-pyrone
aa	amino acids
Acetyl-coA	acetyl-coenzyme A
act	actin gene
ANOVA	analysis of variance
ATP	adenosine triphosphate
BCA	biocontrol agent
BCAs	biocontrol agents
BGC	biosynthetic gene cluster
BGCs	biosynthetic gene clusters
BLAST	*Basic Local Alignment Search Tools*
BLASTp	BLAST protein
bp	base pair
C	carbon
°C	Celsius degree
cal	calmodulin gene
cDNA	complementary DNA
cm	centimeter
Ct	cycling threshold
C-terminal	carboxylic-terminal
DEPC	diethyl pyrocarbonate
DI	disease index
diTS	diterpene synthases

DMAPP	dimethylallyl-pyrophosphate
DMATS	dimethylallyl-tryptophane synthases
DNA	deoxyribonucleic acid
DON	deoxynivalenol
DS	disease severity
EDTA	ethylenediaminetetraacetic acid
ESI–TOF	electrospray ionization-time of flight
eV	electron volt
Fgra	*Fusarium graminearum* ITEM 124
FHB	fusarium head blight
FPP	farnesylpyrophosphate
g	gram
gDNA	genomic DNA
GGPP	geranylgeranylpyrophosphate
GPP	geranylpyrophosphate
h	hours
HA	harzianum A
HAD	haloacid dehalogenase
HGT	horizontal gene transference
HPLC	high performing - liquid chromatography
IA	isoamyl alcohol
indolediTS	indole-diterpene synthases
IPP	isopentenyl-pyrophosphate
IPR	InterPro
IPS	isoprenyl-pyrophosphate synthase
JGI	*Joint Genome Institute*
kb	kilobase
kDa	kilodalton
L	liter
LC-MS	liquid chromatography-mass spectrometry

mA	milliampere
MES	2-(*N*-morpholino) ethanesulfonic acid
mg	milligram
mL	milliliter
mm	millimeter
mM	millimolar
monoTS	monoterpene synthases
N	nitrogen
NCBI	*National Center for Biotechnology Information*
ng	nanogram
NIV	nivalenol
NRPS	non-ribosomal-peptide synthase
NTC	non template control
N-terminal	amine-terminal
P	p value
PCR	polymerase chain reaction
Pfam	protein family
PKS	polyketide synthase
PT	prenyltransferases
PTHR	panther
qPCR	quantitative real-time PCR
Q-TOF	quadrupole-time of flight
RiPPs	ribosomally-synthesized and post-transcriptionally modified peptides
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
sesquiTS	sesquiterpene synthases
sesterTS	sesterterpene synthases
SM	secondary metabolites
sp.	one species

spp.	more than one species
TBE	tris-borate-EDTA
TC	terpene cyclase
tef-1	transcription-elongation factor 1 gene
tetraTS	tetraterpene synthases
TF	transcription factor
Tgam	*Trichoderma gamsii* T6085
TRI5	trichodiene synthase
Tris	tris (hydroxymethyl) aminomethane
triTS	triterpene synthase
TS	terpene synthase
UV	ultra-violet
V	volt

Amino acids

D	aspartic acid
T	threonine
S	serine
E	glutamic acid
P	proline
G	glycine
A	alanine
C	cysteine
V	valine
M	methionine
I	isoleucine
L	leucine
Y	tyrosine
F	phenylalanine

H	histidine
K	lysine
R	arginine
W	tryptophan
Q	glutamine
N	asparagine

Websites

NCBI	www.ncbi.nlm.nih.gov
JGI	www.mycocosm.jgi.doe.gov/mycocosm/home
NetPrimer	www.premierbiosoft.com/NetPrimer/AnalyzePrimer.jsp
IDT-Oligoanalyzer	www.idtdna.com/pages/tools/oligoanalyzer

INTRODUCTION

1.1 Fungal secondary metabolites (SMs): when secondary actors become the main characters

One of the reasons why fungal secondary metabolites (SMs) generate such a great fascination is, perhaps, because they can be conceived as collateral metabolic products that, however, have a great impact in organisms' lifestyle. They are not indispensable for survival in optimal conditions, but they define the way by which organisms interact with their environment, thus determining their ecological identity. SMs could be defined as small accessories that decorate the core toolkit for survival, conferring abilities that allow organisms to better adapt to their niches and to colonize new ones. The biological sense of SMs origin is not clear, but one hypothesis could be that they originated as the result of the recycling of primary metabolism residues, a process where they underwent enzymatic modifications. These modifications conferred them new bioactive properties, resulting in advantageous skills which evolution has been selecting and shaping for over 500 million years (Heitman, 2011).

Humans feel attracted towards the study and discovery of new SMs because most of them are powerful bioactive and allelopathic molecules, useful for industry and biomedical applications. These low-molecular-weight compounds, sometimes smaller than 3 kDa (Keswani et al., 2017), encompass a large number of animal and plant toxins, as well as carcinogenic, cytotoxic, immunosuppressive or mutagenic molecules; others instead, exert antibacterial, antifungal, antiviral and antitumoral effects or are used as growth hormones, food colorants and preservative agents (Keller et al., 2005; Schor and Cox, 2018; Linnakoski et al., 2018; Enespa and Chandra, 2019).

In this context, fungi are well known to be the largest source of SMs (Enespa and Chandra, 2019). Fungi constitute a hugely diverse group, highly adapted to colonize and survive in any environment, and their evolutionary success might be, at least in part, due to the extensive and versatile repertoire of SMs they possess (Sarrocco, 2016).

1.1.1. Broad spectrum of functions

Production of SMs is highly susceptible to ambiental changes, and different biosynthetic replies can occur to overcome these challenges. Inside the cell, SMs are able to interact with proteins, nucleic acids and cellular membranes (Wink and Schimmer, 2018),

triggering molecular responses that enable fungi to cover their physiological demands. Nutritional status, mostly nitrogen, carbon and metals availability, temperature, pH, light and redox balance are known to induce or repress SMs synthesis in fungi (Keller, 2018).

SMs production is dependent on the life stage of the fungus and it is intimately associated to the transitions occurring between development and differentiation programs. Formation of reproductive, resistance and other specialized structures from hyphae involves reprogramming in SMs production, like sporogenic factors, pigments and mycotoxins (Calvo et al., 2002; Keller, 2018). In addition, certain SMs (such as melanin) accumulate on the external layers surrounding these structures, enabling their protection from UV and oxidative damage or long-term conservation, and suggesting a possible role as chemical shields for escaping from fungivores (Bayram and Braus, 2012; Rohlfs, 2015).

Nevertheless, the most sophisticated feature of SMs is their ability to act as signalling elements, giving rise to molecular dialogues with the environment, establishing competition or cooperation interactions between fungi that produce them and the surrounding living organisms.

In order to guarantee the survival of the own colony, an exquisite cell-to-cell communication has evolved in fungi to improve the adaptation to local environmental changes. This communication is also mediated by SMs, which are responsible of triggering reproduction, conidiation, competence or secretion of virulence factors (Mehmood et al., 2019). For example, oxylipins are fatty acid-derived molecules that respond to cell density, regulating sexual-asexual reproduction switches, mycotoxin release and plant host colonization in Aspergilli (Tsitsigiannis et al., 2006; Brown et al., 2009).

Fungi have to maintain their niches, facing with competitors and predators and ensuring enough space to grow and suitable nutrient uptake. SMs with antimicrobial activity enable fungi to succeed by impairing or inhibiting the growth and development of other fungi, bacteria, nematodes, insects or fungivores, or even killing them (Künzler, 2018). For example, production of the cyclic depsipeptides enniatins A1 and B1 from the endophyte *Fusarium tricinctum* are induced in presence of *Bacillus subtilis* and are effective antibiotics against several bacterial species (Ola et al., 2013). An intrinsic characteristic of SMs is that their effects are often gradient-dependent, being able to act as molecular

messengers that activate/repress certain physiological processes or instead, as mere molecules that kill or constrain the growth of other organisms at higher concentrations, as shown for phenazine in *A. fumigatus* (Zheng et al., 2015). In the same way, SMs with antimicrobial activity can act as chemo-attractants or promote the growth of other microbes, depending on the range of species present in the environment (Macheleidt et al., 2016). SMs are key mediators in modulating symbiotic associations between fungi and other organisms. SM-mediated signalling enables, for example, nutrient exchange, joint host-colonization or the development of pathogenic alliances that improve the ecological fitness of both partners (Schelarch and Hertweck, 2018).

A vast group of SMs of special interest are mycotoxins, compounds produced by fungi that are harmful for humans and their livestock causing enormous harvest loses. In some producer fungi, these toxic compounds are synthesized as biocides, and play a crucial role in fungus-plant/animal and fungus-microbe interactions. Some mycotoxins are able to disrupt quorum sensing signals in competing bacteria, preventing the release of bacterial-derived antifungal compounds and, in contrast, they can promote the formation of mixed biofilms leading to beneficial associations with bacteria (Venkatesh and Keller, 2019). In addition, mycotoxins from one species can modulate the expression of genes involved in the biosynthesis of mycotoxins from other species, leading to a delicate crosstalk that determines the antagonistic relationships between fungi (Malmierca et al., 2016). Nonetheless, the most remarkable feature of mycotoxins is that they contribute to pathogenicity and virulence of fungi, being able to subvert or suppress host defence responses, thus promoting the successful colonization and the establishment of infection in both animals and plant hosts (Susca et al., 2017).

Probably, one of the most complex language in which fungal SMs are important players is that involved in communication with plants. Fungi have evolved the ability to secrete certain SMs that enhance plant growth and/or elicit its defense responses, including hormones. Some fungal SMs are really excellent mimickers of phytohormones (Pusztahelyi et al., 2015), boosting the plant vigour or protecting it from pathogens or abiotic stresses, while the fungus takes advantage of the nutrients the plant solubilizes in the rhizosphere or that it can uptake within plant tissues (Tejesvi and Pirttilä, 2018). Mycorrhizal and endophytic fungi are able to share SMs with similar or higher activity than those of their host plant, reducing plant colonization from pathogens and parasites,

even herbivores (Tanaka et al., 2005), and improving plant nutrient uptake from soil (Alurappa et al., 2018).

All those properties make fungal SMs bioproducts with high potential to be employed in agricultural practices, that could improve yield in crop plants without releasing the producer organism in field (Pusztahelyi et al., 2015; Enespa and Chandra, 2019).

1.1.2 Genes involved in SMs biosynthesis and gene clusters

The high diversity of SMs is originated from molecules derived from few primary metabolic pathways. Alkaloids, polyketides, non-ribosomal peptides and terpenes constitute the major classes of SMs in fungi (Hoffmeister and Keller, 2007).

Enzymes responsible of building these secondary compounds can be divided in core enzymes and additional or tailoring enzymes. The core enzymes transform simple precursors in backbone molecules that are further remodelled by tailoring enzymes (P450 monooxygenases, hydrolases or methyltransferases, among others), conferring different bioactive properties and contributing to the vast variety of SM (Keller, 2018).

In alkaloid biosynthesis, the first committed step is catalyzed by the dimethyl-allyl tryptophan synthases (DMATSs), aromatic prenyl transferases isolated for the first time in the fungus *Claviceps purpurea*, the causal agent of ergot in rye (Heinstein et al., 1971). Alkaloid production was extensively studied in *C. purpurea*, which accumulates alkaloids such as lysergic acid in developing sclerotia after plant infection (Tudzynski et al., 1999; Mahmood et al., 2010). During the last years, a great number of alkaloids have been isolated from other Ascomycota, showing a broad range of structures and bioactive properties (Xu et al., 2014).

Polyketides are the most abundant SMs isolated from fungi (Keller, 2005). They are synthesized by polyketide synthases (PKSs), large multi-domain proteins that condense acyl-CoA thioesters in carbon skeletons varying in both chain length and reduction level. While the ketoacyl synthase (KS), acyl transferase (AT) and acyl carrier protein (ACP) domains are indispensable for polyketide biosynthesis, the dehydratase (DH), ketoreductase (KR) and enoyl reductase (ER) domains can be absent in fungal PKSs (Keller et al., 2005; Schümann et al., 2006). Most filamentous fungi can harbour 10-50 PKSs in their genomes, giving rise to hundreds of compounds showing an enormous

variety of bioactive properties (Schor and Cox, 2018). One of the best characterized fungal polyketides due to its economic impact are aflatoxins, produced by some species of *Aspergillus* as pathogenicity factors, that lead to important harvest losses, as they contaminate a broad spectrum of crop plants (Taniwaki et al., 2018). In contrast, strobilurins have inspired the creation of the β-methoxyacrylate class of commercial fungicides for agricultural use (Zubrod et al., 2019). Another well studied compound derived from the polyketide biosynthetic pathway is melanin, one of the most stable and resistant pigment that protects producer fungi from a broad range of abiotic stressors, and also acts as a virulence factor (Belozerskaya et al., 2015).

Non-ribosomal peptides (NRPs) derive from condensation of mostly amino acids and are synthesized by the non-ribosomal peptide synthases (NRPSs). NRPSs are multi-modular enzymes: each module contains the adenylation (A), the pantothenylation/peptidyl carrier (P) and the condensation/peptide bond formation (C) as core domains, along with several specialized C-terminal domains responsible of chain termination, while additional modifying domains such as epimerization (E) and methyltransferase (M), among others, can be present as well (Bushley et al., 2010). Fungal genomes are less enriched in NPRSs than in PKSs, hosting an average of ca. 10 of those signature genes (Keller, 2015). In the same way of polyketides, the natural functions of NRPs are as diverse as their structures. The most famous serendipitous compound belonging to this class is penicillin, which in 1928 triggered an unstoppable exploration of the SMs universe in all the living organisms and enabled to discover other NRP-antibiotics like cyclosporine. Siderophores, another class of NRPs of great relevance in fungal survival, facilitate iron uptake and also act as virulence factors in many fungi (Anke et al., 2018). From the mycotoxin side, gliotoxin was originally isolated from *Myrothecium verrucaria* and constitutes an important virulence factor for some *Aspergillus* species (Dolan et al., 2015), it being currently used in medical therapy for its powerful anti-tumor and anti-metastasis activities (Comas et al., 2019).

Modules of PKSs and NRPSs constitute functionally independent units that can be exchangeable, leading to the emergence of hybrid PKS-NRPS enzymes during evolution (Guzmán-Chávez et al., 2018). Hybrid SMs are very common in fungal genomes and they are also originated in hybrid clusters containing both core enzymes (Schor and Cox, 2018). These two types of synthetases are also found embedded in terpene biosynthetic gene clusters (BGCs), leading to the formation of hybrid polyketide-terpenoid

compounds, like the terpenophenolic metabolites known as meroterpenoids (Keller, 2015), as well as hybrid NRP-terpenes.

Terpenoid compounds, and the aspects concerning their biosynthesis and ecological roles in fungi, will be discussed in the following section (1.2).

The availability of fungal genomes and the development of computational genomic tools have given a burst in the study of the genetic structure as well as of the organization of genes involved in SMs biosynthesis. In most cases, genes encoding enzymes involved in the biosynthesis of the same SMs are arranged in cluster (Keller et al., 2005). Clustering genes have enormous advantages, including co-inheritance, co-transcriptional regulation or coordinated management of post-transcriptional processes, such as exportation for protein synthesis or compartmentalization (Chavali and Rhee, 2017).

Architecture of BGCs is polymorphic, and in addition to genes strictly required for carry out all enzymatic steps that configure the final compound, genes encoding transcription factors (TFs) and efflux transporters can also be present (Rokas et al., 2018). In some cases, BGCs encode their own specific regulatory proteins, but approximately half of the known fungal clusters are governed by global TFs (Macheleidt et al., 2016). The presence of transporter-encoding genes ensures not only the secretion of the SMs, but also protects producer fungi from their toxic activity; in those cases, BGCs can also harbour duplicated or resistant target-proteins, as well as genes encoding enzymes that chemically modifies the SMs, reducing or suppressing their toxicity (Keller, 2015).

1.2 Terpenes and terpene synthases (TSs) as key players in the interaction between fungi and the environment

Isoprenoids are a large superfamily of compounds, fundamentally encompassing terpenes, their precursors (polyprenyl-pyrophosphates) and derived compounds (terpenoids), which take this name as they can be broken away yielding isoprene units (Pérez-Gil et al., 2019). It is estimated that 60% of isolated natural compounds belong to this group (Battineni et al., 2018).

In spite of their huge variety, all fungal terpenes are synthesized from few precursors. Isopentenyl-pyrophosphate (IPP) and its isomer dimethylallyl-pyrophosphate (DMAPP), both synthetized from acetyl-coA, are the 5 carbons (C) isoprene building blocks for the

biosynthesis of linear polyprenyl-pyrophosphates: 10C geranyl-pyrophosphate (GPP), 15C farnesyl-pyrophosphate (FPP) and 20C geranylgeranyl-pyrophosphate (GGPP) (Quin et al., 2014). These are synthesized by the isoprenyl-pyrophosphate synthases (IPSs) (also known as polyprenyl synthases), and constitute the precursors that undergo further modifications by terpene synthases (TSs) and prenyl transferases (PTs), the core enzymes in terpenoid biosynthesis, both mediating the committed steps (Guzmán-Chávez et al., 2018).

According to the origin of their scaffolds, terpenes can be distinguished in those exclusively formed by isoprenyl units (monoterpenes, sesquiterpenes, diterpenes, sesterterpenes and triterpenes), and those with mixed origin (meroterpenoids, indole terpenoids and indole alkaloids).

Monoterpenes are 10 C compounds derived from the condensation of DAMPP with IPP. Although production of monoterpenes in fungi seems to be uncommon (Shmidt-Dannert 2014), monoterpene 1,8-cineole has been recently purified from *Hypoxylon* sp. (Shaw et al., 2015), and some others have been detected in the volatilome of *Trichoderma virens* (Inayati et al., 2019).

A significant part of the numerous terpenes produced by fungi are sesquiterpenes (Kramer et al., 2011). These 15 C compounds are produced by the cyclization of FPP by Class I sesquiterpene synthases (Guzmán-Chávez et al., 2018). As observed for other phytohormones, abscisic acid (ABA) is also produced by certain phytopathogenic fungi, such as *Cercospora rosicola*, *Botrytis cinerea*, and *Rhizopus nigricans*, where it is considered as a tool to increase their pathogenicity (Takino et al., 2018). However, fungal sesquiterpenes that have attracted researchers' interest, for their impact in yield and quality of crop plants, are trichothecenes. Nivalenol (NIV) and deoxynivalenol (DON) are potent mycotoxins mainly produced by *Fusarium* spp. and specifically induced during spike colonization of wheat, acting as virulence factors in the establishment of infection (Venkatesh and Keller, 2019). DON production becomes indispensable for the fungus to spread across spikelets, but it also provides competitive advantages against other organisms during its saprotrophic phase, for example by inhibiting the release of chitinases by mycoparasites (Audenaert et al., 2013). Similarly, phytotoxin botrydial enhances virulence of *B. cinerea* during plant infection facilitating colonization and triggering the plant phosphatidic acid production, that positively regulates the oxidative burst caused by reactive oxygen species (ROS) required for *B. cinerea* infection

(D'Ambrosio et al., 2018). Is worth to mention the striking role of volatile sesquiterpenes as excellent carriers of information in long-distance communications, acting as pheromones that attract insects involved in spore dissemination, and nematodes, which prey on fungivore-insect larvae (Kramer et al., 2011).

Diterpenes are a well-described class of SMs with a demonstrated role in fungal ecology. Cyclization of GPP by either monofunctional Class I or bifunctional class II diterpene synthases are required to build the 20 C diterpene scaffold (Farhat et al., 2018). Fascinating fungal diterpenes are gibberellins, hormones that are synthetized not only by those phytopathogenic fungi causing the so-called "foolish-seedling" disease, but also by fungal endophytes and rhizosphere competent fungi (Bömke and Tudzynski, 2009), suggesting a beneficial role on plant/fungal interactions. Similarly, some non-hormonal diterpenes are able to intercept plant hormonal signalling pathways, acting like protein effectors that subvert plant immune responses. Higginsianin B of *Colletotrichum higginsianum* suppress jasmonate (JA) signalling in *Arabidopsis* by preventing degradation of JAZ proteins, repressors of JA defence responses, and also affecting the plant auxin (indol-acetic acid, IAA) signalling (Dallery et al., 2019). Some fungal phytopathogens have evolved to cause extreme damage just by using a molecule. Brassicicenes from *Alternaria brassicicola* cause leaf spot by activating permanently the plasma membrane H^+-ATPase in *Brassica* plants (De Boer and Leeuwen, 2012). In addition, diterpene production in fungi is spread in almost all ecological scenarios and constitutes a growing source of compounds important for human uses. For example, asperolides, altrosides and wentilactones are diterpenoids isolated from marine fungi that show herbicidal, antifungal and anti-tumor activities (Sun et al., 2012; Afiyatullov et al., 2000; Xu et al., 2015). In addition, they can also be found in endophytes not only of higher plants (Feng et al., 2014), but also in those of bryophytes (Wijeratne at al., 2012) and in lichenous fungi (Wang et al., 2011).

Geranylfarnesyl-pyrophosphate (GFPP) are the 25 C polyprenyl precursor of sesterterpenes, which are rare among terpenoid compounds and mostly produced by marine fungi, especially those present in mangroves (Yan et al., 2018). However, an increasing number of these compounds are being discovered in other ecological contexts. The best known sesterterpenes are probably ophiobolins – mainly produced by *Aspergillus* spp. attacking crop plants– that show phytotoxic, antimicrobial and nematocidal activities (Tian et al., 2017). Recently, novel sesterterpenes have been

isolated from *Colletotrichum orbiculare*, and genes involved in their biosynthesis have shown to be induced during infection of *Nicotiana benthamiana*, suggesting a possible role of these metabolites in modulating host plant physiology (Gao et al., 2018).

Although some triterpenoid compounds have been identified in ascomycete fungi, the vast majority have been isolated in basidiomycetes (Schmidt-Dannert, 2014). In eukaryotes, condensation of two FPP by PT squalene synthase and posterior oxidization step leads to the common 30 C precursor oxidosqualene, which is cyclized by Class II oxidosqualene cyclases/lanosterol-protostadienol synthases in either lanosterol or protostadienol, providing the two-principal precursors of all triterpenes (Mitsuguchi et al., 2009; Quin et al., 2014). While lanosterol is the precursor of sterols and bioactive SMs compounds, protostadienol constitutes the backbone for helvolic acid biosynthesis, a compound isolated from *Cephalosporium caerulens* and the human pathogenic fungus *A. fumigatus* that shows antibiotic activity and could act as virulence factor during the infection process (Abe et al., 2001; Lodoiro et al., 2009).

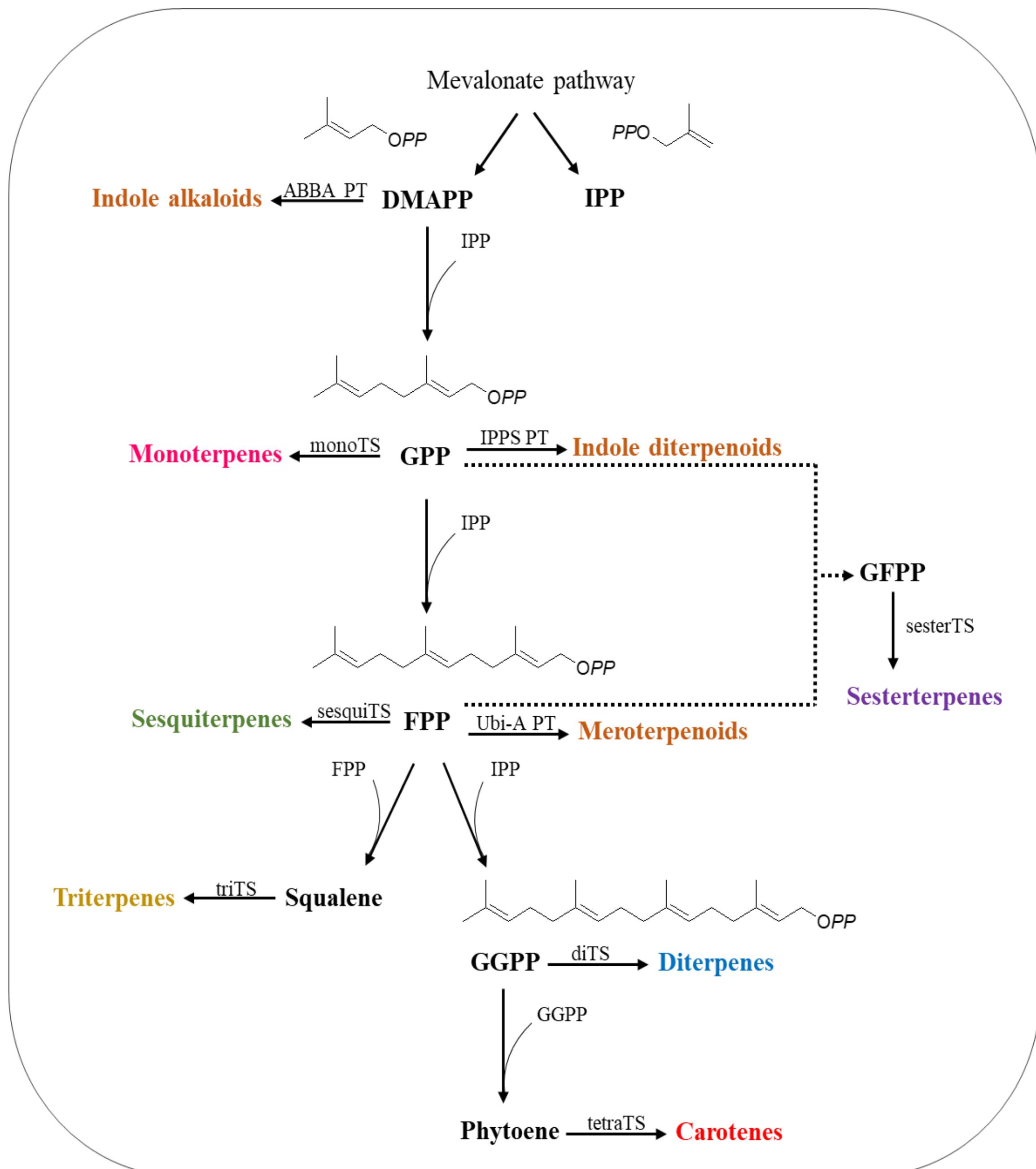
It is worth to mention that many fungi have the ability to produce carotenes, tetraterpenoids of 40 C that are synthesized by the condensation of two molecules of GGPP by PT phytoene synthase, thus originating phytoene, the primary precursor of all carotenoid compounds (Avalos and Limón, 2014). Lycopene, which results from oxidization of phytoene is, in turn, the precursor that undergoes cyclization by Class I lycopene cyclase leading to β-carotene. Carotenes encompass important pigments protecting fungi from oxidizing and light stressors, although its role as pheromones in sexual mating has also been showed (Avalos et al., 2017).

Thereby, enzymes involved in canonical terpene biosynthesis (Fig. 1) can be grouped as: IPSs, prenyl transferases (PTs) that transfer isoprene units to allylic substrates, yielding polyprenyl-pyrophosphates (GPP, FPP, GGPP and GFPP) through head-to-tail condensation of these units; PTs, that yield long chain linear terpenes through head-to-head condensation of polyprenyl-pyrophosphates (e.g. squalene synthase and phytoene synthase); and terpene cyclases (TCs), TS that yield terpenes by cyclizing polyprenyl-pyrophosphates (Shmidt-Dannert, 2014).

Enzymes responsible of the biosynthesis of hybrid terpenoids are known as aromatic PTs, since they catalyze the transference of polyprenyl-phosphates to aromatic compounds,

Fig. 1. Terpenoid biosynthetic pathway

Dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP), originated from the Mevalonate pathway, are the 5C building blocks for the biosynthesis of all the terpenic compounds. A family of isoprenyl pyrophosphate synthases (IPSs) are responsible for chain elongation and biosynthesis of geranyl-pyrophosphate (GPP), farnesyl-pyrophosphate (FPP), geranylgeranyl-pyrophosphate (GGPP) and geranylfarnesyl-pyrophosphate (GFPP). These intermediates constitute the substrates that are modified by terpene cyclases (TCs) and prenyltransferases (PTs), leading to the biosynthesis of monoterpenes (monoTS), sesquiterpenes (sesquiTS), diterpenes (diTS), triterpenes (triTS), sesterterpenes (sesterTS), carotenes (tetraTS) and hybrid terpenoids as indole alkaloids (ABBA-PTs), meroterpenoids (UbiA-PTs) and indole diterpenoids (IPPS-PTs).



which originate outside of the terpene biosynthesis pathways. Aromatic PTs can be divided in three different types, each one responsible of the biosynthesis of one type of mixed terpene compounds (Shmidt-Dannert, 2014).

Meroterpenoids are synthetized by Ubi-A PTs that transfer one FPP to a polyketide-derived aromatic ring. These less studied terpenoids exhibit interesting bioactive properties ranging from cytotoxic, antibiotic or anti-inflammatory effects that are being exploited in medical therapies (Shaaban et al., 2017). For example, terretonin produced by *Aspergillus terreus* is a meroterpenoid mycotoxin (Hamed et al., 2019) with potential to treat Alzheimer's disease as it irreversibly inhibits the acetylcholinesterase (Lo et al., 2012).

ABBA-type PTs transfer DAMPP to indoles, polyketides, phenols, sugars, etc., to synthetize indole alkaloids. Fusicoccin A of *Diaporthe amygdali* (syn. *Phomopsis amygdali)* causes wilting disease in trees and is able to modulate plant physiology through a broad range of mechanisms, like stomata opening/closure control, auxin-like growth stimulation, antagonism with ABA or seed dormancy induction, among others (De Boer et al., 2012).

Hybrid terpenes synthesized by IPPS-type PTs by transferring GPP to indole groups are indole-diterpenoids. One of its peculiarities is that a large part of these compounds isolated from fungi have tremorgenic effects, what makes them potential insecticides (Farhat et al., 2018). This feature could explain the ecological role of lolitrem B, produced by the grass-endophytic fungi *Neotyphodium lolii* (Young et al., 2006) and *Epichloë festucae*, which confers protection to their host plants from insects and herbivorous mammals Saikia et al., 2012).

1.2.1 Fungal TSs

Biosynthesis of terpenes by TSs begins with a Mg^{2+}-mediated binding of the prenyl/polyprenyl-pyrophosphate substrate to the active centre of the enzyme. Subsequent substrate dephosphorylation or protonation step, leads to the carbocation that triggers the formation of new carbon-carbon bonds producing cyclic (TCs), linear (PTs) or hybrid terpenes (aromatic PTs). Cyclization reactions are carried out by high-affinity terpene cyclase enzymes, which generate a single product, or by promiscuous enzymes

that can generate terpenoid blends containing up to 52 different products (Christianson, 2008).

TSs and PTs proteins are usually classified based on the mechanism triggering the carbocation (Class I, Class II or ABBA), which derives from differences in protein sequence and structure, but they are also classified based on their substrate specificity , which rely on the length of the prenyl pyrophosphate they accept (monoTS, sesquiTS, diTS, triTS, tetraTS, ecc) (Pérez-Gil et al., 2019).

All the TSs and PTs proteins share one of the three structural TS-folds (Class I, Class II or ABBA), each one associated to a specific Mg^{2+}-binding conserved motif. In this context, these enzymes share clear structural homology, but scarce sequence similarity regardless the metal-binding motifs. It has been suggested that the lack of sequence homology is due to these enzymes have evolved rapidly, and it has been proven that mutations in the active-site residues confer enormous plasticity to these enzymes, resulting in an exquisite diversity of reactions and a wide variety of products (Shaw et al., 2015).

The TS-fold of Class I enzymes are characterized by an α-helical bound that forms an hydrophobic active-site cavity flanked by two highly conserved aspartate-rich motifs, the D[D/E]xx[D/E] and the [N/D]Dxx[S/T]xx[K/R)][D/E] (NSD/DTE triad), that cooperate to bind the prenylated pyrophosphate via Mg^{2+} (Schmidt-Dannert, 2014). Substrate dephosphorylation generates a carbocation, which either reacts with a double C-C bond, triggering the cyclization of the substrate (TCs), or forms a new C-C bond with the prenyl chain (PTs). The NSD/DTE sequence determines whether one or various products will be synthesized in the same reaction, as shown for aristolochene synthase of *Penicillium roqueforti* and *A. terreus* (Quin et al., 2014). IPSs, PTs, monoTSs, sesquiTSs, sesterTSs, and tetraTSs belong to this class.

In contrast, the TS-fold of Class II enzymes shares a double α-helical barrel bound that also leaves an hydrophobic cavity, in which carbocation formation is initiated by the addition of a proton from the aspartate-rich motif to a double C-C (aspartate-rich motif DxDD), or to an epoxide-ring (aspartate-rich motif DCTAE) via Mg^{2+} (Schmidt-Dannert, 2014). Carbocation formation via protonation triggers the cyclization cascade in triTSs and diTSs. In turn, triTSs such as oxidosqualene cyclase can be identified by the presence

of the QW repeats, though to be responsible of the stabilization of the protein structure, enchaining the outer helices of the barrels (Racolta et al., 2012).

Bifunctional and chimeric enzymes, as well as aromatic PTs, constitute an example where evolution of TSs reaches its maximum, promoting the appearance of combinations that give rise to an enormous wealth of products. Bifunctional Class II diTSs are constituted by both Class I C-terminal and Class II N-terminal domains, performing Class II cyclization and a subsequent Class I cyclization of GPP (e.g. copalyl diphosphate/ent-kaurene synthase). In addition, the so far little studied sesterTSs are chimeric bifunctional enzymes, probably originated from a gene fusion event (Matsuda et al., 2015; Quin et al., 2014), that display both Class I cyclase and PT domains, being able to synthetize their own polyprenyl chain (GFPP) as substrate for the subsequent cyclization. A particular case of gene fusion is that of tetraTS PT phytoene synthase and Class I lycopene cyclase, which are translated from a unique gene as a single polypeptide containing both proteins, that undergoes subsequent cleavage releasing two independent enzymes (Breitenbach et al., 2012). Evolution of Ubi-A and IPPS PTs is enigmatic. Both share the Class I fold but unlike IPPSs, which contain the typical D[D/E]xx[D/E], Ubi-A PTs does not (Schmidt-Dannert, 2014). Integral membrane domains of Ubi-A PTs enable these enzymes to embed prenyl chains into the membrane, and it is hypothesized that they could be the ancestor of all the Class I proteins, or instead, they could be the result of a convergent evolution (Shmidt-Dannert, 2014). Stranger and striking is the case of ABBA PTs, which have a completely different fold (ABBA fold) showing αββα-secondary structure and no aspartate-rich motifs involved in the Mg^{2+} binding of the prenyl diphosphate substrates (Saleh et al., 2009).

Based on the knowledge of these conserved structures, it is possible to find out putative TS-encoding genes in fungal genomes. *In silico* prediction of TSs sequences has boosted the identification of TS proteins functionally characterized in filamentous fungi (Table 1).

Table 1. TS proteins functionally characterized in filamentous fungi

Strain	Accession	Function	Classification	Reference
Fusarium fujikuroi m567	CAA75244.1	copalyl pyrophosphate synthase/ent-kaurene synthase	Bifunctional Class II Cyclase - diTS	Tudzynski et al., 1998
Fusarium fujikuroi m567	CAD19988.1	phytoene synthase-lycopene cyclase polypeptide	Class I PT GFPP synthase - Class I Cyclase - tetraTS	Linnemannstöns et al., 2002
Fusarium sporotrichioides NRRL 3299	AAN05035.1	trichodiene synthase	Class I Cyclase - sesquiTS	Rynkiewicz et al., 2001
Fusarium graminearum GZ3639	ACY69978.1	longiborneol synthase	Class I Cyclase - sesquiTS	McCormick et al., 2009
Fusarium graminearum H3	ABR87941.1	trichodiene synthase	Class I Cyclase - sesquiTS	Jiao et al., 2008
Fusarium sambucinum R-6380	AAB02038.1	trichodiene synthase *tox5*	Class I Cyclase - sesquiTS	Hon et al., 1992
Sphaceloma maniticola Lu949	CAP07655.1	copalyl pyrophosphate synthase/ent-kaurene synthase	Bifunctional Class II Cyclase - diTS	Bomke et al., 2008
Phoma betae Frank PS-16	BAD29971.1	aphidicolan-16β-ol synthase	Bifunctional Class II Cyclase - diTS	Oikawa et al., 2001
Phoma betae N2	BAF45924.1	fusicoccadiene synthase *PaFs*	Bifunctional Chimeric Class I Cyclase-PT - diTS	Toyomasu et al., 2007
Aspergillus clavatus NRRL 1	A1C8C3.1	ophiobolin synthase *AcOS*	Bifunctional Chimeric Class I Cyclase-PT - sesterTS	Chiba et al., 2013
Aspergillus variecolor NBRC 32302	BAU98235.1	astellifadiene synthase	Bifunctional Chimeric Class I Cyclase-PT - sesterTS	Matsuda et al., 2016
Aspergillus variecolor NBRC 32302	BAX76657.1	quiannulatene synthase	Bifunctional Chimeric Class I Cyclase-PT - sesterTS	Okada et al., 2016
Aspergillus variecolor NBRC 32302	BAT32888.1	variediene synthase	Bifunctional Chimeric Class I Cyclase-PT - sesterTS	Qin et al., 2015
Aspergillus terreus ATCC 20516	AAF13263.1	aristolochene synthase	Class I Cyclase - sesquiTS	Sishova et al., 2007
Aspergillus fumigatus Af293	XP_747936.1	oxidosqualene cyclase/protosta17(20)Z,24-dien-3b-ol synthase	Class II Cyclase - triTS	Lodeiro et al., 2009
Aspergillus flavus NRRL6541	AAT65718.1	biosynthesis of aflatrem *atmC*	IPPS PT aromatic - indolediTS	Nicholson et al., 2009
Aspergillus flavus NRRL3357	XP_002378991.1	pentalenene synthase putative	Class I Cyclase - sesquiTS	Nierman et al., 2015
Penicillium brasilianum NBRC 6234	BBD05404.1	sesterbrasiliatriene synthase	Bifunctional Chimeric Class I Cyclase-PT - sesterTS	Mitsuhashi et al., 2017
Penicillium verruculosus TPU1311	BBD05405.1	preasperterpenoid synthase	Bifunctional Chimeric Class I Cyclase-PT - sesterTS	Mitsuhashi et al., 2017
Penicillium roqueforti NRRL 849	AAA33694.1	aristolochene synthase as	Class I Cyclase - sesquiTS	Calvert et al., 2002
Penicillium paxilli PN2013	AAK11531.1	biosynthesis of paxilline (indolediterpene) *paxG*	IPPS PT aromatic - indolediTS	Young et al., 2001
Botrytis cinerea T4	AAQ16575.1	presilphiperfolan-8β-ol synthase *BcBOT2*	Class I Cyclase - sesquiTS	Pinedo et al., 2008
Botrytis cinerea B05.10	XP_001550978.1	*Bcsct5* ABA biosynthesis	Class I Cyclase - sesquiTS	Izquierdo-Bueno et al., 2018
Cephalosporium caerulens	AAL56020.1	oxidosqualene cyclase/lanosterol synthase	Class II Cyclase - triTS	Abe et al., 2001
Neurospora crassa 15300	AAA19428.1	phytoene synthase-lycopene cyclase polypeptide	Class I PT GFPP synthase - Class I Cyclase - tetraTS	Schmidhauser et al., 1994
Neotyphodium lolii Lp19	ABF20225.1	biosynthesis of lolitrem B	IPPS PT aromatic - indolediTS	Young et al., 2006
Hypoxylon sp. E7406B	AHY23922.1	1,8 cineole synthase	Class I Cyclase - monoTS	Shaw et al., 2015
Ganoderma lucidum CGMCC 5.616	AHN91949.1	squalene synthase sqs	Class I PT - triTS	Zhou et al., 2014
Trichoderma brevicompactum IBT40841	CAX94841.1	trichodiene synthase	Class I Cyclase - sesquiTS	Tijerino et al., 2011
Trichoderma arundinaceum IBT40837	CBX36793.1	trichodiene synthase	Class I Cyclase - sesquiTS	Malmierca et al., 2013
Trichoderma reesei QM6a	AFX82678.1	FPP synthase *erg20*	Class I PT - sesquiTS	Pilsyk et al., 2014
Trichoderma virens Gv29-8	ABE60722.1	volatile sesquiTS	Class I PT - sesquiTS	Crutcher et al., 2013

1.3 *Trichoderma*: a genus with great SMs diversity

1.3.1 A widespread fungus, a wide range of lifestyles

Trichoderma is a genus of ubiquitous filamentous fungi belonging to the ascomycetous *Hypocreaceae* family, described for the first time by Persoon (1794). They are frequently found in soil or decaying wood as well as in many other substrates, demonstrating a high opportunistic potential and adaptability to the changing ecological conditions (Druzhinina et al., 2011). The genus comprises species showing a broad spectrum of lifestyles, including mycoparasites, saprotrophs, opportunistic human pathogens and plant symbionts, with members able to act as Biocontrol Agents (BCAs) against crop-plant pathogens, as well as to promote plant growth and/or to elicit the plant defence responses (Harman et al., 2004; Rai et al., 2019).

Mycoparasitism, wherein a fungus takes nutrients from another fungus, has been revealed as the ancestral lifestyle of *Trichoderma* (Kubicek et al., 2011). It has been speculated that ancestors of *Trichoderma* were mycoparasites on wood-degrading fungi, and evolved saprotrophic features to chase their prey in their substrate (Rossman et al., 2013). *Trichoderma* spp. secrete hydrolytic enzymes (i.e. chitinases, glucanases, proteases) that digest fungal (and oomycete) cell walls (Howell 2003). Other species of this genus (i.e. *T. reesei*) developed important arsenals of cellulose and xylan degrading enzymes that enabled them to evolve a strong biomass-degrading activity, becoming important saprotrophs with high value in industry (Druzhinina et al., 2011). The ability to produce enzymes, combined with the release of antimicrobial molecules, makes these fungi efficient competitors for space and nutrients, able to rapidly colonize ecological niches as it is the case of plant roots (Lorito et al., 1996; Howell et al., 2006; Lorito et al., 2010; Hermosa et al., 2012; Saravanakumar et al., 2016). Many *Trichoderma* spp. established themselves in the rhizosphere, where the plant-root exudates, mycorrhizae and phytopathogenic fungi constitute a great feeding-resource (Druzhinina et al., 2011). This probably boosted the development of beneficial interactions with plants and the ability of *Trichoderma* to colonize endophytically the external layers of the root tissues (Druzhinina et al., 2011). In contrast, few species, such as some strains of *T. longibrachiatum* and *T. citrinoviride*, have been proven to opportunistically infect immune-compromised human patients (Kuhls et al., 1999).

1.3.2 Biological roles of SMs in *Trichoderma*

Trichoderma produces a wide variety of SMs in a strain-dependent manner (Yu and Keller, 2005) being peptaibols, polyketides and terpenes the most relevant (Reino et al., 2008).

The biological roles associated to SMs of *Trichoderma* has been extensively reviewed, and their central role as chemical communicators involved in the interactions with plants, insects, animals and microorganisms has been highlighted (Hermosa et al., 2014; Contreras-Cornejo et al., 2016, 2018; Patil et al., 2016; Salwan et al., 2019; Rai et al., 2019). In the rhizosphere, SMs released by *Trichoderma* act as effector molecules triggering significant morphological and physiological changes in the host plant (Ramírez-Valdespino et al., 2019), affecting plant growth and nutrition and enhancing tolerance against biotic and abiotic stresses (Contreras-Cornejo et al., 2016). IAA is a phytohormone produced by *Trichoderma*, that not only promotes plant growth and development (Contreras-Cornejo et al., 2009), but also confers saline stress adaptation in host plants (Waqas et al., 2012). In addition, *Trichoderma* siderophores enhance iron sequestration facilitating its uptake by plants and by *Trichoderma* itself, demonstrating a key role in competition for this metal in the rhizosphere (Anke et al., 1991; Kubicek et al., 2011). Harzianolide, 6-pentyl-pyrone (6-PP), harzianic acid and aspinolides are only few examples of SMs from *Trichoderma* that elicit the plant-defence responses, providing them protection against phytopathogens such as *B. cinerea*, *Leptosphaeria maculans* and *R. solani* (Vinale et al., 2008; Malmierca et al., 2015; Manganiello et al., 2018). Interestingly, Harzianic acid is a siderophore involved in iron-chelation also showing antifungal activity (Vinale et al., 2013). In the same way, it has been reported that *T. atroviride* associated with maize-roots infected with the lepidopteran *Spodoptera frugiperda* in leaves, releases 6-PP with an attracting effect towards female wasps of *Campoletis sonorensis*, the natural enemy of *S. frugiperda* (Contreras-Cornejo et al., 2018). These are examples illustrating that a single metabolite can trigger multiple effects both on the producer organisms and in the species interacting with them. Nevertheless, SMs from *Trichoderma* are well known for their antimicrobial effects, which directly inhibit or supress the growth of competitors, enabling *Trichoderma* to protect itself and its resources (Vey et al., 2001). For example, a recent study showed that volatile SMs of *Trichoderma* inhibit the growth, germination of macroconidia and perithecial development in *Fusarium graminearum* (El-Hasan et al., 2017). Recently, metabolites

from 19 *Trichoderma* spp. and its biological properties have been recently reviewed (Li et al., 2019).

In addition to siderophores, peptaibols constitute the most representative NRPs of *Trichoderma*, showing a strong antimicrobial activity against a wide range of organisms, as well as the ability to induce the plant defence responses (Rai et al., 2019). Some of these peptides form voltage-dependent ion channels in lipidic membranes, modifying the membrane permeability and inducing cell death (Molle et al., 1987). Another relevant group within NRPs are epipolythiodioxopiperazines (ETPs), which can inactivate proteins by binding thiol groups and by generating ROS (Gardiner et al., 2005). Among these small peptides, gliotoxin – the first SM isolated in *Trichoderma* (Brian and Hemming, 1945) – has attracted great attention, since it showed potent antifungal effects against *R. solani* (Howell et al., 1993).

Polyketides produced by *Trichoderma* display toxic effects against plant pathogens such as *F. oxysporum* and *Rhizoctonia solani*, *Phytophthora cinnamomi* and *Phytium middletonii* (Patil et al., 2016), and are responsible of the characteristic green/yellow pigmentation (Baker et al., 2012). In addition, some polyketides are involved in defence, mechanical stability and stress resistance in *Trichoderma* (Atanasova et al., 2013). Examples of *Trichoderma* polyketides are trichoharzins, trichodimerols and koningins (Kobayashi et al., 1993; Zhang et al., 2014; Almassi et al., 1991; Ghisalberti et al., 1993). These last have been shown to act also as plant growth regulators (Cutler et al., 1989; Cardoza et al., 2005). Nevertheless, the best-known polyketide from *Trichoderma* is 6-PP, which was the first volatile antifungal compound isolated from these fungi, also responsible of the "coconut aroma" associated to these fungi (Bisby et al., 1939).

1.3.2.1 Relevance of terpenoids in *Trichoderma*

Trichoderma spp. are reported to produce all types of terpenoids, including volatile compounds (Pachauri et al., 2019). These metabolites have shown to play important roles in the physiology of *Trichoderma* and in its interactions with other organisms, acting as mycotoxins, chemical messengers, structural components in membranes, regulators of genes related to stress and inducers of plant defence responses (Zeilinger et al., 2016; Pachauri et al., 2019).

Although *Trichoderma* spp. were thought to not produce monoterpenes, some have been recently detected in the volatilome of *T. virens* (Inayati et al., 2019). For example, *β*-myrcene regulates the expression of *Trichoderma* genes related to biotic and abiotic stresses, while *cis*- and *trans-β*-ocimene are able to induce the JA-dependent defence response in *Arabidopsis thaliana* (Pachauri et al., 2019).

Harziandione is claimed to be the first diterpene isolated in *Trichoderma* (Ghisalberti et al., 1993). It was firstly detected in *T. harzianum* and although it lacks on antifungal activity, a compound with identical structure showing antifungal potential was isolated from *T. viride* (Sivasithamparam and Ghisalberti, 1998).

A number of triterpenoid compounds have been reported in *Trichoderma*. In addition to ergosterol, which is essential to stabilize cell membranes, other triterpenes showing antifungal properties have been isolated (Hermosa et al., 2014). Ergokonins A and B are steroid antibiotics, which also inhibit the growth of *A. fumigatus* by suppressing the 1,3-β-D-glucano synthase activity (Onishi et al., 2000; Vicente et al., 2001). *T. virens* produces the fungistatic steroid viridin, which also shows plant-growth inhibitory effects (Howell et al., 1993), and whose reduced form viridiol has herbicidal properties (Jones and Hancock, 1987).

Trichoderma sesquiterpenoids have shown both antibacterial and antifungal activities, as in the case of the 3,4-dihidroxicarotene isolated from *T. virens* and *T. viride* and lignoren isolated from *T. viride* (Berg et al., 2004). A number of volatile sesquiterpenes have been also reported in *T. atroviride*, including *β*-bisabolene and *α*-bergamotene (Stoppacher et al., 2010). A comparison of the metabolic profiles and the ability to enhance the plant growth of nine *Trichoderma* spp., suggested a positive correlation between the emission of volatile terpenes and the bio-stimulation ability of these strains (Lee et al., 2016). Some sesquiterpenes are involved in regulating developmental stage switches, as it has shown the carot-4-en9,10-diol, which has a stress-related conidiation-inducing role in *T. virens* (Wang et al., 2013). Nevertheless, sesquiterpenoid compounds that have attracted more attention due to their ecological importance are trichothecenes, whose production has been reported in *T. arundinaceum* and *T. brevicompactum*. Trichodermin produced by *T. brevicompatum* is a mycotoxin with antifungal and phytotoxic effects (Tijerino et al., 2011b), as it inhibits protein synthesis in eukaryotic cells (Gilly et al., 1985). In contrast, Harzianum A (HA) produced by *T. arundinaceum* promotes plant defence responses and inhibits the growth of phytopathogenic fungi (Malmierca et al., 2013). Volatile

sesquiterpene trichodiene, which is the product of the first enzymatic step in the biosynthesis of trichothecenes, have been shown to elicit the tomato defence responses and the activation of virulence genes in *B. cinerea* (Malmierca et al., 2015).

1.3.2.2 <u>TSs in *Trichoderma*</u>

Although many terpenes have been isolated from *Trichoderma* species, there is no extensive information about the TS-encoding genes involved in their biosynthesis, and only few members of the TS family have been experimentally characterized (Bansal and Mukherjee, 2016). Functional characterization of TS genes in *Trichoderma* has been mainly focused on the trichodiene synthase (TRI5)-encoding gene, which catalyses the first committed step in the biosynthesis of trichothecenes in *T. arundinaceum* and *T. brevicompactum*. Overexpression of *tri5* enhances trichodermin production and antifungal activity in *T. brevicompactum* (Tijerino et al., 2011a). Disruption of *tri5* in *T. arundinaceum* alters the expression of other genes involved in terpenoid biosynthesis and reduces the antagonistic activity against *R. solani* (Malmierca et al., 2013). In addition, Δ*tri5* mutants of *T. arundinaceum* unable to produce HA synthesize, the polyketides aspinolides, which are able to inhibit the growth of *B. cinerea* and induce the plant defence responses (Malmierca et al., 2015). Interestingly, heterologous expression of *tri5* in *T. harzianum* led to trichodiene production, which induced defence genes and promotes lateral root growth in tomato (Malmierca et a., 2015). Furthermore, trichodiene induces an upregulation of BOT genes (involved in botrydial biosynthesis) in *B. cinerea*, thus suggesting a signalling role of this volatile organic compound (VOC) in the interactions of *Trichoderma* with both plants and microorganisms. Outside from the trichothecene biosynthesis pathway, TS genes experimentally characterized in *Trichoderma* are *erg-20* and *vir4*. Overexpression of the farnesyl pyrophosphate synthase (*erg-20*) in *T. reesei* affects the activities of enzymes of the dolichol and sterol biosynthetic pathways, modifying the ergosterol levels (Pilsyk et al., 2013). On the other hand, disruption of *vir4* gene showed this terpene cyclase is required for the biosynthesis of mono- and sesquiterpenes in *T. virens* (Crutcher et al., 2013).

1.4 Genome mining for discovering SMs-related genes in *Trichoderma*

1.4.1 General aspects of genome mining

Recent advances in genome sequencing technologies over the past decade, combined with the development of efficient bioinformatic tools, have boosted the discovery of the genetic potential of a growing number of fungal species (Palazzotto and Weber, 2018). Genome mining has been established as a powerful tool to estimate the metabolic potential of a given strain by scanning the genome of interest and identifying secondary metabolite BGCs (Ziemert et al., 2016). Despite the great diversity of SMs, the core enzymes involved in their biosynthesis show highly conserved domains, which enabled the development of a plethora of software to mine them from genomic data (Ziemert et al., 2016; Chavali and Rhee, 2017).

Since genes involved in SMs biosynthesis are organized in clusters, mining of neighbouring genes encoding tailoring enzymes, transporters and TFs enables the identification of the complete structure of a given BGC. Identification of BGCs involves the assumption of pre-defined rules regarding cluster size and cluster composition, which will determine the accuracy and fidelity of the prediction (Chavali and Rhee, 2017). This often constitutes a limitation, since these rules are based on the knowledge derived from experimentally characterized BGCs and, therefore, the approach is supported on sequence databases of homologous enzymes or clusters, frequently leading to the miss-prediction of unknown BGCs (Ziemert et al., 2016).

Another interesting strategy is based on comparative phylogenomic analysis, which couple genomics and phylogenetics to predict the function of a given gene (Hautbergue et al., 2018). If the target protein clades with proteins of known function, this function could be hypothesized for such target protein. Inversely, if the target protein does not clade with known proteins, it suggests novel or unknown functions.

1.4.2 Advances on SMs-gene detection driven by genome mining in *Trichoderma*

The growing availability of the complete genome sequences of *Trichoderma* spp. and the development of genomic tools has revealed a vast repertoire of genes putatively involved in SMs biosynthesis in these fungi. Mukherjee et al. (2011) compared the gene content of NRPSs, PKSs and TSs in the mycoparasites *T. atroviride*, *T. virens* and in the biomass-

degrading *T. reesei*. Results showed that mycoparasitic species have an expansion on PKS and NRPS genes (18 PKSs, 16 and 26 NRPSs, respectively) comparing to *T. reesei* (11 PKSs and 10 NRPSs). Another study carried out on these three species, showed that *T. virens* is enriched in NRPS-PKS hybrids (4) comparing to *T. atroviride* (1) and *T. reesei* (2) (Kubicek et al., 2011). A full phylogeny of NRPS-encoding genes suggested that expansion on these genes in *T. virens* is due to recent duplications of genes encoding cyclodipeptide synthases, cyclosporin/enniatin synthases and NRPS-hybrid proteins. In addition, they found that the three genomes share the ferricrocin BGC, and two NRPSs responsible of the biosynthesis of short- (7 modules) and long- (18-20 modules) chain peptaibols. Later, 14-module peptaibol synthases were identified in the genomes of these three species (Degenkolb et al., 2012). *In silico* analysis of their active residues and conserved domains revealed the structural diversity of these enzymes and enabled to predict their putative derived products. In addition, module skipping was proposed as the mechanism by which a single NRPS gene can lead to different peptaibolic products. Based on a similar approach, Marik et al., (2019) mined the peptaibol synthases present in the genomes of species belonging to the clade *Longibrachiatum*, and some of the genes located in their respective BGCs.

Similarly, phylogenomic analysis of PKS genes showed that most of them occurred as orthologous in all the three species mentioned, and PKS genes putatively involved in pigment biosynthesis were also identified (Baker et al., 2012). In addition of the PKS gene clusters involved in conidial pigment, 20 new putative PKS gene clusters have been identified in these species (Bansal and Mukherjee, 2016). These authors also indicated that 6 TS proteins are encoded in the genome of *T. reesei*, 7 in *T. atroviride* and 11 in *T. virens*.

More recently, Guzmán-Guzmán et al. (2019), mined the genome of *T. atroviride* for the detection of genes putatively involved in phytohormone biosynthesis. These authors found candidate genes for the biosynthesis of salicylic acid (SA), JA, ethylene (ET), gibberellins (GAs) and cytokinins (CKs), suggesting the fungus have the potential to produce these compounds. In any case, the production of SA by *T. parareesei* and GAs by *T. asperellum* and *Trichoderma* sp. has been respectively described by Pérez et al. (2015), Zhao and Zhang (2015) and Jaroszuk-Sciseł et al. (2019).

In *T. viride*, a combination of genome mining, heterologous expression and metabolomic approaches enabled the detection of a novel sesquiterpene synthase and its associated products (Sun et al., 2019).

1.5 An overview of *Trichoderma gamsii*

T. gamsii has a relatively recent history in applied biosciences comparing to other species of the genus. The biological and ecological behaviour of this fungus have enabled de development of commercial biopesticides. Remedier® is a preventive biopesticide whose bioactive ingredients comprise the isolate ICC080 of *T. gamsii* together with the isolate ICC012 of *T. asperellum*. This product is used to control different plant pathogenic fungi, mostly soil-borne, such as *Verticillium* spp., *Sclerotinia* spp., *Rhizoctonia* spp., *Sclerotium* spp., *Phytophthora* spp. and *Phytium* spp. On the other hand, different isolates of *T. gamsii* have shown the ability to endophytically colonize plant roots. An endophytic isolate of *T. gamsii* is a promising BCA against *F. oxysporum* f. sp *cubense*, the causal agent of Fusarium wilt of banana (Taribuka et al., 2017). Furthermore, *T. gamsii* NFCCI 2177, isolated from lentil roots, produces diffusible and volatile compounds with inhibitory effects against a wide range of phytopathogenic fungi (Rinu et al., 2014).
T. gamsii is also being applied for bioremediation purposes. The isolate FCR16 is effective in removing hexavalent chromium ions from acidic electroplating effluent contaminated with high levels of this and other metals (Kavita and Keharia, 2012). Noteworthy, the application of *T. gamsii* is not restricted to the agricultural sciences. In addition, *T. gamsii* could be applied in clinical microbiology. *T. gamsii* IPT853, isolated from a sugar cane plantation soil, has shown the biogenic capacity of producing silver nanoparticles, which are active against *Streptococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Ottoni et al., 2017).

1.5.1 *Trichoderma gamsii* T6085

T. gamsii T6085 has been isolated from uncultivated soil in Crimea (Ukraine). During the last ten years, it attracted our attention due to its ability to antagonize *F. graminearum*, one of the most aggressive causal agents of Fusarium Head Blight (FHB) on wheat (Parry et al., 1995), although around 20 *Fusarium* spp. contribute to this disease (Aoki et al., 2014). FHB is a destructive disease that causes enormous economic losses worldwide. In

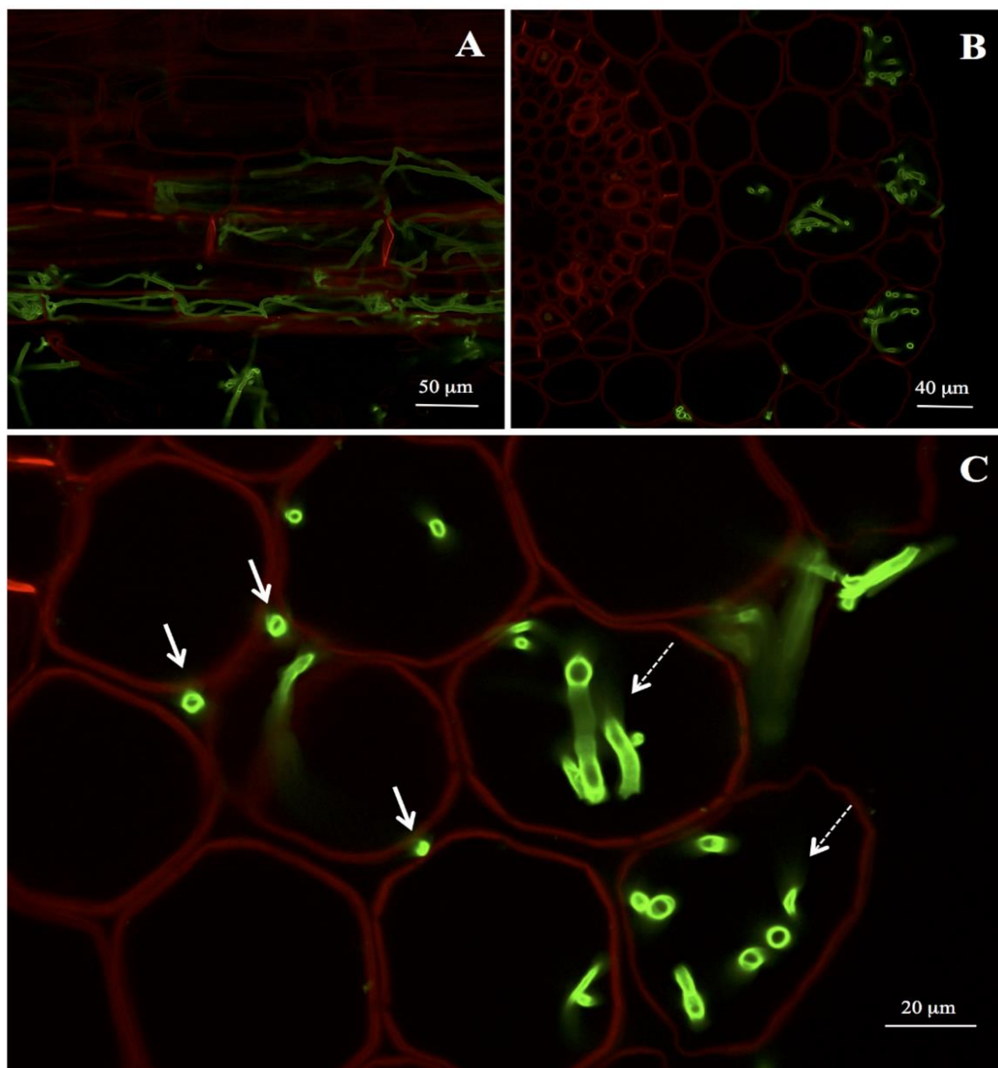
FHB, yield loss derives from sterility of infected florets, while grain quality reduction is mainly due to the accumulation of trichothecenes, highly toxic for humans and their livestock. During the disease cycle, crop residues and spikes at anthesis are considered as the most critical points: crop debris are used by pathogens to overwinter and to produce ascospores that will become the initial inoculum at anthesis, which represents the most susceptible phase for fungal infection. To the date, different strategies may be effective in controlling FHB, but none of these is effective alone. These practices include adequate crop rotations and usage of a combination of fungicides and tillage practices aimed to manage crop residues (Wegulo et al., 2011). In this scenario, integrated pest management involving the use of BCAs, such as *T. gamsii* T6085, during these two critical steps is a promising approach to reduce the disease incidence and prevent the risk of mycotoxin accumulation.

T. gamsii T6085 is able to reduce the growth of the pathogen, as well as the production of DON, the major mycotoxin produced by *Fusarium* spp. (Sarrocco et al., 2013a). In addition, *T. gamsii* T6085 is able to tolerate high DON concentration (50 ppm, while the limit of DON accumulation on durum wheat in Europe is 1.75 ppm) in *in vitro* conditions, a quite not widespread character within the *Trichoderma* genus. When grown in presence of DON, T6085 does not modify mycotoxin concentration, and no DON derivates have been found in the culture substrate (Matarese et al., 2012). An involvement of ABC transporters with specificity to DON in T6085 was hypothesized (Sarrocco et al., 2013b), but further analyses are needed to support this. *T. gamsii* T6085 is able to antagonize *F. graminearum* not only by reducing its growth, by also by parasitizing its hyphae by forming short loops and coilings. *T. gamsii* T6085 has been successfully applied in field on wheat crops at anthesis during two following seasons. This resulted in a reduction of the incidence and the severity of FHB, with the corresponding reduction of the risk of mycotoxin accumulation in kernels (Matarese et al., 2010, 2012; Sarrocco et al., 2013b). T6085 is able to compete with *F. graminearum* for substrate possession, such as wheat and rice kernels, where a reduction of trichothecenes production has been registered (Sarrocco et al., 2019). The application of *T. gamsii* T6085 on wheat straw reduces the ability of *F. graminearum* to develop perithecia on crop residues, thus reducing the primary inoculum of the pathogen (Sarrocco et al., 2020). Furthermore, *T. gamsii* T6085 is also able to colonize the rhizosphere, behaving as an endophyte within wheat roots (Fig. 2) and inducing the plant defence responses (Sarrocco et al., 2020).

The importance of *T. gamsii* as a promising BCA against FHB and its biological traits make this fungus an interesting target for the study of SMs potential and its implications in different ecologic scenarios. The complete genome sequence of *T. gamsii* T6085 has been publicly released, consisting of 38 Mbp and 10,944 predicted protein-coding genes (Baroncelli et al., 2016), which significantly facilitates to get an overview of the SMs potential of this fungus by genome mining strategies.

Fig. 2. Endophytic colonization of *T. gamsii* T6085 on wheat roots

Colonization of *T. gamsii* T6085 in wheat root seven days after inoculation: A) close up of *T. gamsii* T6085 hyphae inhabiting epidermal cells of wheat root; B) Transversal section of wheat root showing internal colonization of *T. gamsii* T6085 in epidermal and cortical layers near to vascular system; C) arrows indicate intracellular (dashed line) and intercellular (continuous line) colonization by *T. gamsii* T6085 hyphae. Fungal cells were detected with WGA-Alexa Fluor 488 (green channel): the plant cell wall was detected with FM4-64 dye (red channel). Sarrocco et al. (2020).



OBJECTIVES

1. Mining genes and BGCs involved in SMs biosynthesis within the genomes of 21 isolates belonging to 17 *Trichoderma* spp. and comparative genomic study aimed to provide an overview of the SMs potential in the genus *Trichoderma*.
2. Genomic characterization of genes involved in terpenoid biosynthesis (TSs) in the selected *Trichoderma* spp. in order to assess the diversity within the gene family and provide an overview of the terpenoid inventory in the genus.
3. Assessment of expression patterns of TS genes in *T. gamsii* T6085 as to provide a preliminary picture of the regulation of these genes in different environmental contexts.

MATERIALS & METHODS

2.1 Computational analyses

2.1.1 Genomic platform

Genomes including gene annotation, available in public databases (National Center for Biotechnology Information – NCBI-, www.ncbi.nlm.nih.gov; Joint Genome Institute-JGI-, www.mycocosm.jgi.doe.gov/mycocosm/home) and comprising 21 isolates belonging to 17 *Trichoderma* species, were used for computational analyses. Model organism *Beauveria bassiana* was used as outgroup due to its close phylogenetic relation with *Trichoderma* spp. Fungal strains, genomes, accession numbers and information concerning each isolate are shown in Table 2.

2.1.2 Evaluation of the SMs potential in *Trichoderma* spp.

2.1.2.1 Multi-locus sequence analysis and phylogenetic relations of *Trichoderma* spp.

Full nucleotide sequences from the actin (*act*), calmoduline (*cal*) and transcription-elongation factor 1 (*tef-1*) genes were extracted from *Trichoderma* genomes and aligned by MAFFT v7.450 (Rozewicki et al., 2019). Multi-locus alignment was used as input for phylogenetic analysis, using the FasTree v2.1.11 of Geneious 10.0.9 (www.geneious.com).

2.1.2.2 Prediction of total SMs backbone genes

InterPro (IPR) Scan v5.44-79.0 (Jones et al., 2014) was used to identify genes encoding enzymes that constitute the signature proteins in SMs biosynthetic pathways in *Trichoderma* and *B. bassiana* proteomes, based on the following IPR terms associated to their conserved domains:

- Non-Ribosomal Peptide Synthases (NRPSs; AMP-binding domain: IPR000873, PP-binding domain: IPR009081, Condensation domain: IPR001242; NRPS-like: AMP-binding domain: IPR000873, PP-binding domain: IPR009081, NAD-binding domain: IPR013120);
- Polyketide Synthases (PKSs; β-ketoacyl synthase N-terminal domain: IPR014030, β-ketoacyl synthase C-terminal domain: IPR014031);

Table 2. *Trichoderma* spp. and *B. bassiana* genomic platform.

Complete genome sequences of *Trichoderma* spp. and *B. bassiana* were obtained from public databases of NCBI and *JGI*. Whole Genome Sequencing (WGS) Project accessions of each species are listed in the second column. Phylogeny according to Druzhinina et al. (2018) and Kubicek et al. (2019) and information about the isolation of each strain are also shown.

Strain	WGS Project	Phylogeny	Isolate information	Reference
Beauveria bassiana ARSEF 2860	ADAH00000000.1	Outgroup	Isolated from aphid cadavers (Idaho, USA)	Xiao et al., 2012
Trichoderma asperellum CBS 433.97	MBGH00000000.1	Clade Viride	Isolated from soil (Maryland, USA)	Druzhinina et al., 2018
Trichoderma asperellum TR356 v1.0	Unreleased	Clade Viride	Isolated from soil (Brazil)	Kubicek et al., 2019
Trichoderma hamatum GD12	ANCB00000000.2	Clade Viride	Isolated from potato field (UK)	Studholme et al., 2013
Trichoderma gamsii A5MH	MTYH00000000.1	Clade Viride	Isolated from wheat cropping soil (Australia)	Unpublished
Trichoderma gamsii T6085	JPDN00000000.2	Clade Viride	Isolated from soil (Ukraine)	Baroncelli et al., 2016
Trichoderma atroviride IMI 206040	ABDG00000000.2	Clade Viride	Isolated from a plum tree (Sweden)	Kubicek et al., 2011
Trichoderma arundinaceum IBT 40837	PXOA00000000.1	Clade Brevicompactum	Isolated from soil (Iran)	Proctor et al., 2018
Trichoderma brevicompactum IBT 40841	PXNZ00000000.1	Clade Brevicompactum	Isolated from soil (Iran)	Proctor et al., 2018
Trichoderma citrinoviride TUCIM 6016	MBDI00000000.1	Clade Longibrachiatum	Unknown	Druzhinina et al., 2018
Trichoderma longibrachiatum ATCC 18648	MBDJ00000000.1	Clade Longibrachiatum	Isolated from mud (Ohio, USA)	Druzhinina et al., 2018
Trichoderma parareesei CBS 125925	LFMI00000000.1	Clade Longibrachiatum	Isolated from subtropical rain forest (Argentina)	Yang et al., 2015
Trichoderma reesei QM6a	AAIL00000000.2	Clade Longibrachiatum	Isolated from Salomon Islands	Li et al., 2017
Trichoderma reesei RUT C-30	JABP00000000.1	Clade Longibrachiatum	Obtained from three rounds of random mutagenesis of QM6a strain	Jourdier et al., 2017
Trichoderma virens Gv29-8	ABDF00000000.2	Clade Virens	Isolated from rhizosphere	Kubicek et al., 2011
Trichoderma pleuroticola Tr1	MTYI00000000.1	Clade Harzianum	Submitted as *T. harzianum* - Isolated from carbendazim contaminated vineyard soil (China)	Unpublished
Trichoderma pleuroti TPhu1	MDJU00000000.1	Clade Harzianum	Isolated from oyster mushroom (Hungary)	Marik et al., 2017
Trichoderma harzianum CBS 226.95	MBGI00000000.1	Clade Harzianum	Isolated from soil (England, UK)	Druzhinina et al., 2018
Trichoderma harzianum TR274	NQLC00000000.1	Clade Harzianum	Isolated from soil (Brazil)	Kubicek et al., 2019
Trichoderma guizhouense NJAU 4742	LVVK00000000.1	Clade Harzianum	Isolated from soil with compost of traditional Chinese medicine (China)	Druzhinina et al., 2018
Trichoderma afroharzianum T6776	JOKZ00000000.1	Clade Harzianum	Submitted as *T. harzianum* - Isolated from soil (Pisa, Italy)	Baroncelli et al., 2015
Trichoderma atrobrunneum ITEM 908	PNRQ00000000.1	Clade Harzianum	Isolated from *Olea europaea* (Puglia, Italy)	Fanelli et al., 2018

- Dimethylallyl Transferases (DMATSs; Aromatic prenyl transferase domain: IPR017795);
- Ribosomally synthesized and post-translationally modified peptide-encoding genes (Fungal RiPPs: IPR021765);

- Terpene Synthases (TSs; Isoprenoid synthase domain: IPR008949, terpene cyclase/prenyl transferase domain: IPR008930).

2.1.2.3 Prediction of SMs BGCs

SMs backbone genes embedded in gene clusters in *Trichoderma* spp. and *B. bassiana* genomes were predicted by antiSMASH 5.0 (Blin et al., 2019). Briefly, the software searches for profile Hidden Markov Models (pHMM) within the amino acid sequence translations of all protein-encoding genes, based on multiple sequence alignments of experimentally characterized signature proteins or protein-conserved domains. Based on available information of the minimal components of each gene cluster type taken from the scientific literature, gene clusters are defined by locating signature gene pHMM hits spaced within <10 kb mutual distance. To include flanking accessory genes, gene clusters are extended by 5 to 20 kb on each side of the last signature gene pHMM hit, depending on the type of gene cluster detected. The software refers to "hybrid clusters" when two separated gene clusters are spaced very closely each other or, instead, when a single gene cluster combines two or more signature genes and produces a hybrid compound. Recorded hits are then compared to the MiBiG repository, a database of experimentally characterized BGCs which enables to assign a putative function to the predicted clusters. According to this methodology, the software predicts backbone genes including PKSs, NRPSs, RiPPs-encoding genes, DMATSs and TSs, either belonging to single gene clusters or hybrid gene clusters.

2.1.3 Phylogenetic characterization of TSs in *Trichoderma* spp.

Algorithms included in the IPR interface, such as Pfam, PIRSF, Prosite and Panther, were used to identify motifs relatives to sites, superfamily membership, variants of prenyl transferase domains, and trans-membrane (TM) regions associated to TS proteins.

In order to characterize TS enzymes in *Trichoderma* spp. and *B. bassiana* we used a combination of different approaches: 1) Prenyl transferases (PTs) were identified based on Pfam and Panther motifs, which in turn enabled differentially identification of Terpene Cyclases (TCs) proteins; 2) Conserved aspartate-rich metal-binding motifs associated to Class I (D[D/E)xx[D/E]) and Class II (DxDD) TS-folds (Gao et al., 2012) of mono- and bifunctional enzymes were identified searching for pHMM in the amino-acidic sequences; 3) Substrate-specificity and putative functions were assigned by phylogenetic analysis. Firstly, *Trichoderma* spp. and *B. bassiana* TS proteins were aligned by MAFFT v7.450 (Katoh et al., 2013) along with TS proteins of known functions characterized and described in literature (Table 1). Phylogenetic tree was built with: MrBayes (Ronquist & Huelsenbeck, 2003), FasTree v2.1.11 (Price et al., 2010) and PhyML v3.3.2 (Guindon et al., 2010). The best substitution model was obtained using ProtTest (Abascal et al., 2005). Phylogenetic tree was reconstructed using the WAG + I evolutionary model (Whelan and Goldman 2001). The posterior probabilities and bootstrap values threshold were 50%. Phylogenetic trees were visually checked and topology conservation evaluated.

Sequences used for alignments and corresponding to each phylogenetic cluster identified were individually screened for conserved domains through InterProScan as described above.

2.2. Biologic assays

2.2.1 Substrates

Substrates used throughout the work are listed below:

- Potato Dextrose Agar (PDA): 24 g/L
- Potato Dextrose Broth (PDB): 24 g/L
- ½ PDB: 12 g/L
- FRIES: 9 g/L sucrose (321.3 g/mol), 1 g/L NH_4NO_3 (80.04 g/mol), 5 g/L $C_4H_{12}N_2O_6$ (184.15 g/mol), 1 g/L K_2HPO_4 (174.18 g/mol), 0.5 g/L $MgSO_4$, 0.13 g/L $CaCl_2$ (111 g/mol), 0.1 g/L NaCl (58.44 g/mol), 0.0183 g/L $FeSO_4$ (278.02 g/mol), 0.0035 g/L $ZnSO_4$ (161.47 g/mol), 0.002 g/L M_nCl_2 (197.91 g/mol)
- ½ FRIES: 4.5 g/L sucrose (321.3 g/mol), 0.5 g/L NH_4NO_3 (80.04 g/mol), 2.5 g/L $C_4H_{12}N_2O_6$ (184.15 g/mol), 0.5 g/L K_2HPO_4 (174.18 g/mol), 0.25 g/L $MgSO_4$, 0.065

g/L $CaCl_2$ (111 g/mol), 0.05 g/L NaCl (58.44 g/mol), 0.00915 g/L $FeSO_4$ (278.02 g/mol), 0.00175 g/L $ZnSO_4$ (161.47 g/mol), 0.001 g/L M_nCl_2 (197.91 g/mol)

- FRIESM: FRIES without sucrose
- FRIESM H_2O_2: FRIESM with 0.5 mM H_2O_2
- FRIESM N: FRIESM with a hundred times less concentration of NH_4NO_3 (0.01 g/L) and g/L $C_4H_{12}N_2O_6$ (0.05 g/L)
- FRIESM 200 salt: FRIESM with 200 mM of NaCl
- Malt Extract Agar (MEA): 20 g/L D-glucose (198.17 g/mol), 1 g/L mycological peptone, 20 g/L malt extract, 15 g/L agar, pH 5.6
- Malt Extract Broth (ME): 20g/L D-glucose (198.17 g/mol), 1g/L mycological peptone, 20 g/L malt extract, pH 5.6
- ½ ME: 10 g/L D-glucose, 0.5 g/L mycological peptone, 10 g/L malt extract, pH 5.6

Commercial suppliers of media and media components were Sigma-Aldrich, Panreac, Carlo Erba, J.T. Baker, Riedel-de Haën and Difco.

2.2.2 Fungal isolates and wheat plants.

Trichoderma gamsii T6085 (*Tgam*) was isolated from soil in Ukraine and *Fusarium graminearum* ITEM 124 (*Fgra*), isolated from rice harvested in Italy, were maintained on PDA under mineral oil at 4°C. When actively growing, or sporulated, colonies were needed, fungi were grown on PDA plates at 25°C, 12h/12h light/darkness. Seeds from *Triticum aestivum* cv. *Apogee* (wheat) were sown in pots with commercial peat-based potting mix (Esselunga) and perlite (2:1) and incubated in a growth chamber with photoperiod 16h light/8 h dark, at 20°-22°C. Before all experiments, wheat seeds were surface sterilized with a solution of NaClO (0.6% active chlorine), for 3 minutes on shaking, followed by three washing steps of 10 minutes each with sterile distilled water. Seeds were stored in sterile distilled water at 4°C for 3 days for vernalization.

2.2.3 Liquid cultures of *Tgam* on different stress conditions

Mycelium of *Tgam* was obtained following a two-step liquid culture procedure. The first step consisted in a pre-culture of the fungus in order to generate actively growing mycelium. Spores of *Tgam* were collected from 1-week-old PDA plates with 7 mL of sterile distilled water and inoculated in 50 mL flasks containing 25 mL FRIES, at a final

concentration of 10^6 spores mL^{-1}. Flasks were incubated at 28°C on a rotatory shaker at 180 rpm for 60 h. Pre-cultures were collected by centrifugation at 10000 rpm for 10 minutes and supernatants were discarded. Pellets were resuspended in sterile distilled water and centrifugated at 10000 rpm for 10 minutes in order to wash it. For the second culture step, 25 mL of different liquid substrates were inoculated in 50 mL flasks containing pellets from washed pre-cultures. These substrates consisted in FRIES, FRIESM, and FRIESM modified by adding different stressors, such as 0.5 mM H_2O_2, only 0.01% N, and 200 mM NaCl. FRIESM was used as reference control. Flasks were incubated at 28°C on a rotatory shaker at 180 rpm for 4 days. Mycelium was collected by filtration using Miracloth (475855-1R, Millipore), frozen in liquid N_2, and stored at -80°C until RNA extraction. Three independent biological replicates were included for each condition.

2.2.4 *Tgam* interactions in FHB scenario

Tests with wheat plants included four conditions: control (uninoculated) wheat, wheat inoculated with *Tgam* alone, wheat inoculated with *Fgra* alone, and wheat inoculated with *Tgam* and *Fgra*.

Wheat seeds were sown in potting mix and incubated in a growth chamber with photoperiod 16 h light/8 h dark, at 20°-22°C respectively, until plants reached the anthesis stage (5 weeks). Three biological replicates of three plants each were included per each condition. For *Tgam* inoculation, spores were collected by washing 1-week-old PDA plates with 20 mL of sterile 0.01% Tween-80 solution. The spore suspension, at a final concentration of 10^7 spores mL^{-1}, was sprayed on spikes of *Tgam* alone and *Tgam* + *Fgra* plants, while 0.01% Tween-80 solution was sprayed in control plants. Plants were covered with a white bag, previously moistened inside with sterile 0.01% Tween-80 solution to maintain humidity, and with a black bag to facilitate penetration by the fungus (Dufresne M., personal communication). Plants were incubated in a growth chamber for 48 h in the same conditions as described above. For inoculation of the pathogen, *Fgra* conidia were collected by washing 2-week-old PDA plates with 20 mL of sterile 0.01% Tween-80 solution, and the suspension, at a final concentration of 10^5 spores mL^{-1}, was sprayed on spikes of *Fgra* alone and *Tgam*+*Fgra* plants. Plants were covered again with a white bag, previously moistened inside with 0.01% Tween-80 solution, and a black bag

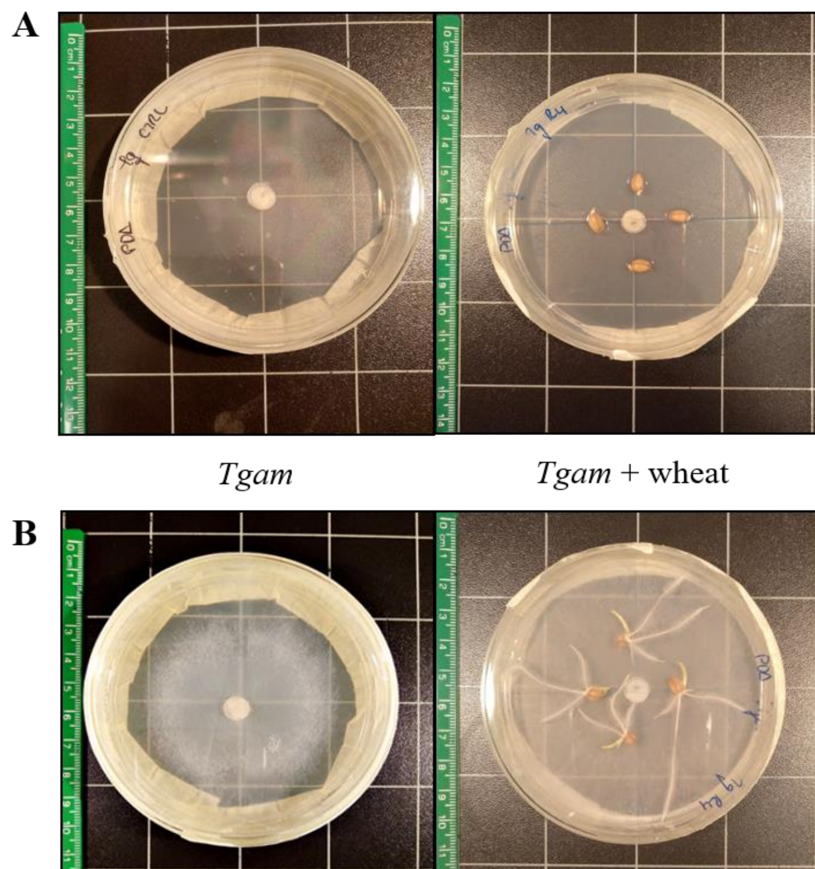
was also placed above to facilitate the infection by the pathogen. Plants were incubated in a growth chamber in the same conditions described above for 24 h. Bags were removed and after 1h, the white bags were placed back for 24 h. Six days after *Fgra* inoculation, reduction of FHB symptoms was evaluated by calculating the percentage of healthy and symptomatic spikelets in *Fgra* alone and *Tgam* + *Fgra* plants, on ten spikes per condition. Spikes were collected, frozen liquid N_2 and stored at -80°C until RNA extraction.

2.2.5 *Tgam* - wheat roots interaction

Four wheat seeds were placed on each PDA plate at 1.5 cm of distance from the centre of the petri dish (Fig. 3). Plates were closed with tape and protected from light with an aluminium sheet, then incubated for 24 h in a growth chamber with photoperiod 16 h light/8 h dark, at 20°-22°C respectively.

Fig. 3. Interaction *Tgam*-wheat roots.

Experimental set up **A)** Inoculation of agar plug colonized by *Tgam* in PDA plates containing germinated wheat seeds and in PDA plates without plants as control, 24 h after seed sowing. **B)** Plates 72 h after inoculation of *Tgam*.



After 24 h, an agar plug of 1 cm^2 colonized by 1-week-old culture of *Tgam* was placed at the centre of the plates containing wheat seedlings, as well as in PDA plates without plants as controls. Plates were closed with tape and protected from light with aluminium sheet and incubated for 3 days in a growth chamber according to the conditions described above. Mycelium from *Tgam* control plates and wheat roots colonized by *Tgam* were collected, frozen in liquid N_2, and stored at -80°C until RNA extraction. Three biological replicates were included per each condition.

2.2.6 Gene expression analyses

2.2.6.1 Primer design and efficiency

Primers used for analysis of expression of TS genes in *Tgam* are listed in Table 3. Primer properties, such as melting temperature, dimmers and hairpins, were analyzed *in silico* by NetPrimer (www.premierbiosoft.com/NetPrimer/AnalyzePrimer.jsp) and IDT-Oligoanalyzer (www.idtdna.com/pages/tools/oligoanalyzer). Primers were tested by PCR and agarose electrophoresis, and qPCR using gDNA of *Tgam*, while DNA of *Fgra* and wheat were used as templates in order to validate the presence/absence of amplification products, according to the methodology described in section 2.2.6.3. and 2.2.6.4. Full gene sequences, including primer location, are listed as supplementary material.

The $2^{-\Delta\Delta Ct}$ method for calculating relative gene expression assumes that the amplification efficiency of the target gene and the endogenous reference gene is optimal (100%) and identical. In order to validate this, a standard curve (Amplification Efficiency Curve) was constructed for each couple of primers using serial dilutions of gDNA of *Tgam* using 100 ng, 10 ng, 1 ng, 0.1 ng and 0.01 ng per reaction, respectively. Efficiency was calculated using the following formulas:

$$y = mx + b \quad ; \quad E = 10^{[-\frac{1}{m}]}$$

y = dependent variable

m = slope

x = independent variable

b = intersection

E = efficiency

The amplification efficiency ranges from 0 to 1, being 1 the 100% of the efficiency, which means that the amount of qPCR product duplicates on each cycle of amplification. To achieve this, the slope value must be between -3.2 and -3.5 being the optimal m = -3.32, which means 95-100% of amplification efficiency. Primers were also checked for dimmer formation and non-specific amplification, by checking the Melting Curves of each primer pair.

Table 3. Primer sequences used for gene expression analysis (5'to 3')

Name	Sequence	Gene	Amplicon size
TUB-F TUB-R	GCTACCTGACCTGCTGCTCTAT AGTCTGGATGTTGTTGGGGAT	*β-tubulin*	128 bp
TRI5s-F TRI5s-R	TTCTGCGTCATTATGGAGGC AAGGAAGCCAGGATAGTCGC	*tri5*	137 bp
TS1-F TS1-R	TCAACGCCCACTACCCAGA ACCGTGTCGTCCCAGCAG	*ts1*	205 bp
TS3-F TS3-R	GATTTTACATCATCCCACCCC CAGTTGGCGGAGGACTTCAG	*ts3*	113 bp
TS4-F TS4-R	ATCACAAATGGCGGCAAGA CAGCGTGGAAGAAGAAAATAGTC	*ts4*	235 bp
TS5-F TS5-R	TGCTTAGTGTTACCGTCCTTCTG TAATCTTCTTCTTCATCTTGGGC	*ts5*	153 bp
TS6-F TS6-R	CCTTTTCTTTGCTGTCGTGG GCAGTTTCTCGGCTGTCATTC	*ts6*	198 bp
TS7-F TS7-R	GGTCATCTCTCCGCATTTCCC CTCACTACTCCATTCATCGCTGTT	*ts7*	183 bp
TS9-F TS9-R	AAACCATAAACTCAGCCAACTACG CAGTCTTGTTCCCCACCATCTC	*ts9*	198 bp
TS11-F TS11-R	CAACTCGGGCAGGCGGAC TCGGCGATACTGTTTGAAGCA	*ts11*	139 bp

2.2.6.2 RNA extraction and cDNA synthesis

2.2.6.2.1 RNA extraction from *Tgam* - roots interaction and liquid cultures

Fungal biomass from liquid cultures, wheat roots and fungal mycelium from the *Tgam* - roots interaction were ground in liquid N_2 using pre-chilled mortar and pestle. One hundred mg of powder were used for total RNA extraction using the RNeasy® Plant Mini Kit (Qiagen), followed by DNase I treatment (DNase I Amplification Grade, AMPD1 Sigma-Aldrich), according to the manufacturer's instructions.

2.2.6.2.2 RNA extraction from *Tgam* interactions in FHB scenario

Wheat spikes were ground in liquid N using pre-chilled mortar and pestle. Three hundred mg of powder were used for total RNA extraction according to the method described by Logemann (1987), adapted for RNA extraction from tissues with high polysaccharide content. The powder was placed in a pre-chilled mortar and re-grinded with 800 µL of Extraction Buffer (8 M guanidine-HCl, 20 mM MES, 20 mM EDTA, 50 mM β-mercaptoethanol). Half volume of Tris-saturated Phenol and half volume of Tris-saturated 24:1 Chloroform/IA were added to the mixture and used to re-grind the tissues. The mixture was centrifugated 15 min at 10000 rpm and 4°C. The upper phase was recovered and 1 volume of 1:1 buffer (Phenol: Chloroform/IA) was added. The upper phase was recovered after 15 min of centrifugation at 10000 rpm and 4°C. A volume of 0.7 of 100% ethanol and a volume of 0.2 of 1M acetic acid were added, and the mixture was incubated at -80°C for 1 h for RNA precipitation. After 30 min of centrifugation at 12000 rpm and 4°C supernatant was discarded, and the pellet was washed twice with 3M sodium acetate and 70% ethanol by centrifuging 5 min at 12000 rpm and 4°C. The pellet was dried at 37°C for few minutes, then resuspended in 50 µL of DEPC-H_2O. RNA solubilization was aided by heating the tube 10 min at 65°C, following by incubation in ice for 2 h. RNA was centrifugated for 2 min at 12000 rpm and 4°C, in order to eliminate polysaccharide residues. Supernatant containing RNA was recovered and stored at -80°C.

2.2.6.2.3 RNA integrity check and cDNA synthesis

RNA integrity (3 µL of each sample) was checked by agarose electrophoresis in TBE 0.5X (1% agarose; 90 V: 400 mA). RNA samples were quantified and quality checked

by Qubit™ fluorometer (#K1642, Thermo Fisher Scientific) and NanoDrop™ (Thermo Fisher Scientific). Four hundred ng of RNA were used for cDNA synthesis using Maxima First Strand cDNA synthesis kit (K1642 Applied biosystems) according to the manufacturer's instructions. cDNA samples were stored at -80°C.

2.2.6.3 PCR setting

The reaction was set up with 1 µL of gDNA (10 ng), 6.25 µL of GoTaq® (Promega), 1 µL of primers forward and reverse (0.5 µM) and Nuclease-Free water up to 12.5 µL of final volume. Nuclease-Free water was used as Non-Template Control and β-tubulin as endogenous gene. All PCR reactions were performed using a Q-Cycler 24 (HAIN-Lifescince) thermocycler under the following conditions: initial heat activation, 95°C, 2 min; 35 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension 72°C for 30 seconds; final extension at 72°C for 5 min. Amplicon size or absence of amplicons was checked by running PCR products on agarose electrophoresis using TBE 0.5X as running buffer (1% agarose; 90 V: 400 mA) and loading 3 µL of sample.

2.2.6.4 Quantitative Real-time-PCR (qPCR) setting

The reaction was set up with 1 µL of cDNA (20 ng), 10 µL of QuantiNova SYBER® Green PCR Master Mix 2X (Qiagen), 1.4 µL of primers forward and reverse (0.7 µM) and Nuclease-Free water up to 20 µL of final volume. Nuclease-Free water was used as NTC, and Threshold Cycles (Ct) were calculated using β-tubulin gene as endogenous control. All qPCR reactions were performed in triplicate using Rotor-Gene Q cycler (Qiagen) under the following conditions: initial heat activation, 95°C, 2 min; 40 cycles of denaturation, 95°C for 5 sec and combined annealing/extension, 60°C, 10 sec. Relative gene expression was obtained using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), which expresses the differences between *Ct* values of the sample and those of the control as shown below:

$$\Delta CT = 2^{-(\Delta Ct\ sample\ -\ \Delta Ct\ control)}$$

2.2.7 Metabolic profiles of *Tgam* on different substrates

2.2.7.1 Metabolites produced on 21-day static cultures

Spores of *Tgam* were collected from 2-week-old PDA plates with 7 mL of sterile distilled water and inoculated in 50 mL flasks containing 25 mL of PDB, ½ PDB, ME, ½ ME, FRIES or ½. FRIES substrates. Flasks were incubated statically at 28°C for 21 day on dark. Mycelium was recovered by filtration using Miracloth (475855-1R, Millipore). Fermentation broth was filtered through 0.22 µm (VWR International) filter, frozen in liquid N_2 and stored at -80°C.

LC-MS method for detection of fungal metabolites was developed using an HPLC 1260 Infinity Series (Agilent Technologies) coupled to a Q-TOF mass spectrometer model G6540B (Agilent Technologies) with a Dual Electrospray Ionization (ESI) source and equipped with a DAD system (Agilent Technologies). An Ascentis® Express C18 column (2.7 µm, 50 mm x 3.0 mm i.d., Supelco©) was used for separations. Flow rate was set at 0.500 mL min^{-1}. Elution was done at constant temperature of 40°C, using a linear gradient composed by A: 0.1% (v/v) formic acid (FA) in water and B: 0.1% (v/v) FA in acetonitrile (ACN). The gradient was as follows: starting condition 5% B, ramping to 95% B in 6 min, holding at 100% B for 2 min, lowering to 5% B in 2 min and equilibration at 5% B for 2 min. UV spectra were collected by DAD every 0.4 sec from 190 to 750 nm with a resolution of 2 nm. MS parameters were set with Agilent MassHunter Data Acquisition Software, rev. B.05.01. The instrument operated in both positive and negative mode; MS spectra were recorded in centroid mode, with an *m/z* 50-1700 mass range and with a speed of 3.3 spectra sec^{-1}. Capillary voltage was set at 2000 V, fragmentor at 180 V, cone 1 (skimmer 1) at 45 V, Oct RFV at 750 V. Drying gas flow was set at 11L min^{-1} at a temperature of 350°C, and the nebulizer was set at 45 psig. The injected sample volume was 7 µL.

In order to perform real-time lock mass correction, an Isocratic pump (1260 Infinity Series, Agilent Technologies) was used to infuse a standard solution consisting of two reference mass compounds: purine ($C_5H_4N_4$, *m/z* 121.050873, 10 µmol/L) and hexakis (1H,1H,3H-tetrafluoropentoxy)-phosphazene ($C_{18}H_{18}O_6N_3P_3F_{24}$, *m/z* 922.009798, 2 µmol L^{-1}). Flow rate was set at 0.06 mL min^{-1} while the detection window and the

minimum height were set at 1000 ppm and 10000 counts, respectively, for reference mass correction.

Solvents were LC-MS grade, and all other chemicals were analytical grade. All were from Sigma-Aldrich unless otherwise stated. ESI-TOF tune mix was purchased from Agilent Technologies.
The chromatograms were analyzed by Mass Hunter software (Agilent Technologies) matching their mass data with known compounds registered in an in-house database including over 4000 fungal SMs.

2.2.7.2 Metabolites produced in 12-day and 15-day cultures: major fractions

For induction of SM production on liquid culture, spores of *Tgam* were collected from 2-week-old PDA plates with 10 mL of sterile distilled water and inoculated in 14 flasks of 1L containing 300 mL of PDB, at a final concentration of 10^6 spores mL^{-1}. Flasks were incubated at 28°C on a rotatory shaker for 12 days. Fungal biomass was recovered by filtration and fermented broth was filtered through 0.22 µm (VWR® Bottle Top Filtration) filter under sterile conditions. Fermentation was stopped adding 500 mL of HPLC-ethyl acetate (Fisher) per each liter of filtrate, to reach 6 L of mixture. The mixture was filtered again and saturated with NaCl, then was extracted twice with 500 mL of ethyl acetate and washed twice with 250 mL of water.

For induction of SM production on solid cultures, 60 plates of MEA were inoculated with two mycelium plugs (1 cm^2) made from PDA colonized by *Tgam* and incubated at 28°C for 15 days. Then, both mycelium and solid agar MEA medium were extracted with ethyl acetate (2 x 500 mL) using an ultrasonic bath.

Purification by semipreparative and analytical HPLC was performed with a Hitachi/Merck L-7100 apparatus equipped with a differential refractometer detector (RI-5450). Two LiChrospher 60 (5 µm) and LiChrospher 60 (10 µm) columns were used in isolation experiments. Silica gel (Merck) was used for column chromatography. Thin layer chromatography (TLC) was performed on Merck Kiesegel 60 F_{254} (thickness, 0.2 mm). Optical rotation was determined with a digital polarimeter. Infrared spectra were recorded on a Fourier transform infrared spectroscopy (FTIR) spectrophotometer and reported as wave number (cm^{-1}). ^1H-NMR and ^{13}C-NMR measurements were recorded on Agilent NMR 400 and Agilent 500 NMR spectrometers. HRMS was recorded with a

double-focusing magnetic sector mass spectrometer in positive ion mode or with a quadrupole time of flight (Q-TOF) mass spectrometer in positive ion electrospray mode at 20 V cone voltage. High resolution electrospray ionisation mass/ mass spectrometry (HRESIMS/MS) experiments were performed with a Q-TOF mass spectrometer at 20 V cone voltage and 10 eV collision energy. Chemicals were by Aldrich or PanReac. All solvents were freshly distilled. Organic extracts from both liquid and solid cultures were dried over Na_2SO_4. Evaporation of solvent was made under reduced pressure afforded dense brown oil (517 mg, and 915 mg, respectively) that was separated by column chromatography on silica gel eluted with mixtures containing increasing percentages of ethyl acetate/hexane (10 -100%) as solvent, and finally the column was washed with methanol. Final purification of selected fractions was carried out by HPLC. Isolated metabolites were characterized by extensive spectrometric and spectroscopic analysis by HRMS, ^{1}H-NMR and ^{13}C-NMR.

2.3. Statistical analysis

Differences in PKSs, NRPSs, TSs, RiPPs-encoding genes, PKS-NRPS synthases and DMATSs family-size between phylogenetic clades of *Trichoderma* were statistically analyzed by Wilcoxon Test. P value (P) < 0.05 was used as confidence interval. Results were expressed as the average of gene content of each family in each clade.

For gene expression, statistical analysis was carried out using SYSTAT©v.13.2. Analysis of variance (ANOVA) was performed on $2^{-\Delta\Delta Ct}$ values obtained from the *Tgam*-roots interaction (section 2.2.5) and *Tgam* in FHB (section 2.2.4). $2^{-\Delta\Delta Ct}$ data obtained from liquid cultures of *Tgam* (section 2.2.3) was analyzed by ANOVA and Tukey test. Disease Severity values of FHB on wheat spikes treated or not with *Tgam* were analyzed by ANOVA after angular transformation. P value (P) < 0.05 was used as confidence interval

Data from metabolic profiles obtained by LC-MS (section 2.2.7.2.), was statistically analysed using the Mass Profiler Professional Software 13.0 (G3835AA, Agilent Technologies. Significant statistical differences among treatments ($P < 0.05$) were assessed by one-way ANOVA and principal component analysis (PCA).

RESULTS & DISCUSSION

Chapter 1:

Insights into the diversity of Secondary Metabolites of *Trichoderma* spp. using a combined genomic approach

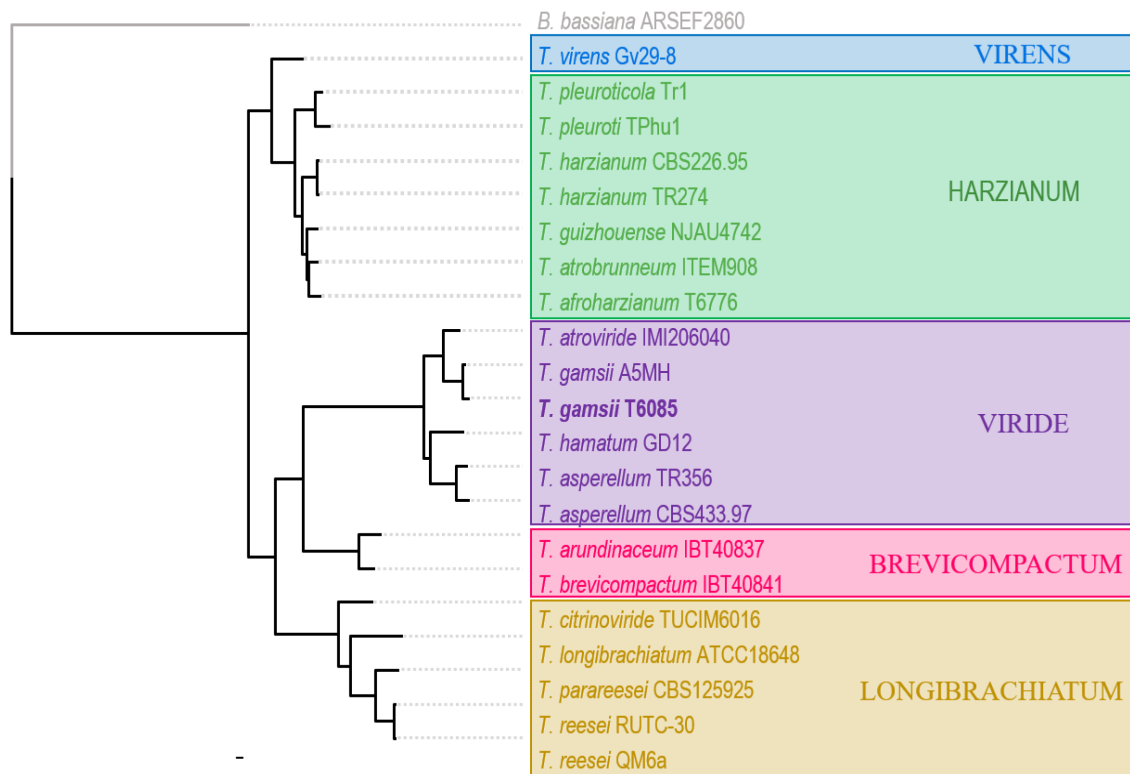
During the last decade, the study of *Trichoderma* genomes has shed some light towards the understanding of the biology of these fungi. Several studies have provided important findings, elucidating mycoparasitism as the ancestral lifestyle of *Trichoderma* and how it evolved leading to more complex interactions with other organisms, such as animals and plants (Kubicek et al., 2011; Druzhinina et al., 2011, 2013). They have highlighted the most relevant gene families, mainly involved in the primary metabolism of *Trichoderma*, and those that have undergone expansions or contractions, contributing to lifestyle diversification (Druzhinina et al., 2016; Druzhinina and Kubicek 2016; Kubicek et al., 2019). In addition, they have proposed Horizontal Gene Transference (HGT) events from *Trichoderma*'s hosts and prays as key processes in the evolution of these fungi (Druzhinina et al., 2018).

Trichoderma spp. are prolific producers of SMs (Pachauri et al., 2019), and understanding the gene arsenal involved in their biosynthesis is crucial for assessing their harmless effects as well as their role in *Trichoderma* peculiar lifestyles. Furthermore, understanding the genetic basis of SMs biosynthesis would enable a money-saving approach for selection of isolates to be used as BCAs, prior to their metabolic screening. However, there are not many reports addressing the study of the diversity within the gene families involved in SMs biosynthesis in *Trichoderma*, and in those cases, the diversity of the genus has been mainly limited to three species – *T. virens*, *T. atroviride* and *T. reesei*– or has been focused on few gene families (Kubicek et al., 2011; Mukherjee et al., 2012, 2013; Baker et al., 2012; Degenkolb et al., 2012; Bansal and Mukherjee 2016; Zeilinger et al., 2016; Guzmán-Guzmán et al., 2019; Marik et al., 2019). According to the taxonomy described by Druzhinina et al. (2018) and Kubicek et al. (2019), *Trichoderma* spp. used in the present work fall into five different clades (Fig. 4). This diversity enabled to discuss the impact of the phylogenetic distribution of the species on their SMs potential.

The present study was carried out using the complete genomes of 21 isolates belonging to 17 *Trichoderma* spp., and *B. bassiana* ARSEF2860 as outgroup. Multi-locus phylogenetic analysis of the *act*, *cal* and *tef-1* genes concatenated alignment showed the evolutionary relationships of *Trichoderma* spp. here analysed (Fig. 4).

Fig. 4. Phylogenetic relationships among *Trichoderma* spp.

Phylogenetic tree showing evolutionary relationships between 21 isolates belonging to 17 *Trichoderma* spp. and *B. bassiana* was constructed using MAFFT v7.450 and FasTree v2.1.11, using concatenated alignment of *act*, *cal* and *tef-1* genes. Phylogenetic clades, according to Druzhinina et al. (2018) and Kubicek et al. (2019) are highlighted in different colours.



3.1. Mining the SMs backbone genes in *Trichoderma* genomes

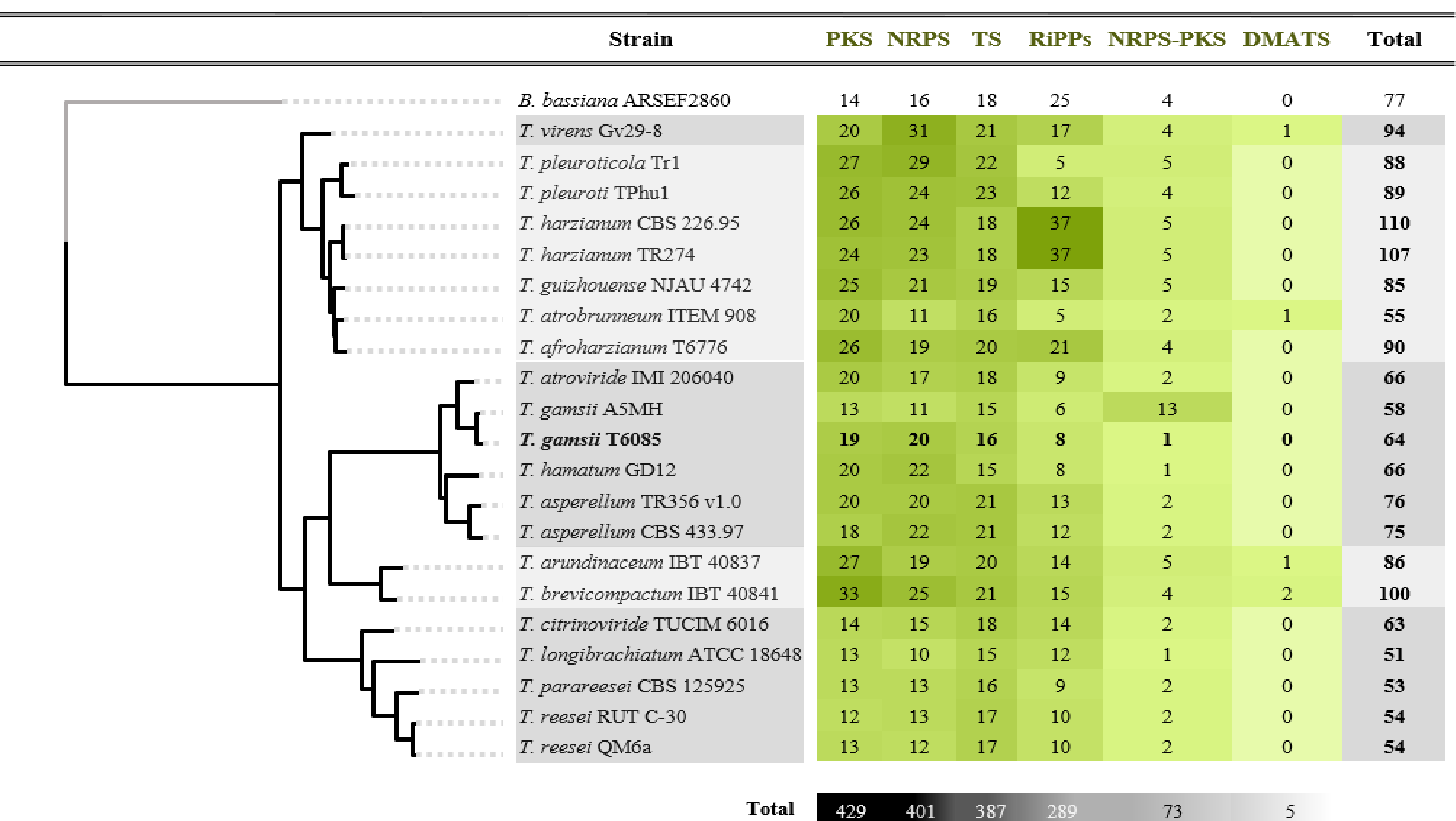
Prediction of total SMs backbone genes showed that *Trichoderma* spp. have the potential to produce a wide repertory of SMs (Fig. 5). Species within the same clade tend to have similar SMs arsenals, as it has been also observed in other *Hypocreales* fungi such as *Fusarium* (Stępień et al., 2019). One exception is *T. atrobrunneum*, which shows a smaller number of genes (55 genes) comparing to its related species of the *Harzianum* clade (85-100). This clearly demonstrates that most of the genomic repertory-size for SMs biosynthesis in *Trichoderma* is affected by the phylogenetic distribution of the species. *T. harzianum* and *T. brevicompactum* constitute the species with the highest SMs potential (100-110 genes), whereas the smaller gene inventory was found in *T. longibrachiatum* (55 genes). Still, intraspecific variability can be observed as well, indicating the SMs potential is, at least partially, affected by the niches occupied by each

isolate. Differences found within the clades reflect the specificity of the SMs arsenal, which are mainly due to discordances in the abundance of RiPPs and NRPS genes. According to this, bioactive peptide biosynthesis, either ribosomally (RiPPs) and non-ribosomally (NRPs), would significantly contributes in generating species/isolates-specific SMs arsenals in phylogenetically close *Trichoderma* species. Although the ecological roles of RiPPs have not been described so far in *Trichoderma*, non-ribosomal peptides, such as peptaibols, are indispensable for successfully competition and constitute the SMs weaponry hallmark of these fungi against other microorganisms (Mukherjee et al., 2013). In this sense, the need of rapidly evolve their SMs inventories to overcome different ecological challenges probably places RiPPs and NRPS-encoding genes under a strong environmental pressure, which ultimately leads to new gene variants and specific products.

Furthermore, the divergence found between taxonomic clades enables including *Trichoderma* spp. in different categories according to their SMs biosynthetic potential. Species belonging to *Harzianum* and *Brevicompactum* are potential super-producers of SMs within the genus as characterized by a wide arsenal of SMs backbone genes (85-110 and 86-100 genes, respectively), followed by those species belonging to the *Virens* clade (94 genes). Species belonging to *Viride* can be considered as moderates (58-76 genes), while those from *Longibrachiatum* have the smaller SMs gene-repertory (51-63 genes), therefore constituting limited producers among *Trichoderma* spp. These differences could be the result of the gene loss reported in species of the clade *Longibrachiatum* and the gene expansion that took place in the other clades during the evolutionary process (Kubicek et al., 2019). Nevertheless, the diversity of lifestyles found on these species could explain, at least partially, quantitative differences in their SMs repertories as well, as suggested by Mukherjee et al. (2013). These authors hypothesized that the differences found on the SMs arsenal of *T. reesei* compared to those of *T. virens* and *T. atroviride* could be related to different demands for attacking or interacting with other organisms. Species belonging to the clade *Longibrachiatum* are, in general, not-opportunistic and strong biomass-degrading fungi, including opportunistic human pathogens, such as some strains of *T. citrinoviride* and *T. longibrachiatum* (Kubicek et al., 2019). In contrast, species belonging to clades *Virens*, *Harzianum*, *Viride* and *Brevicompactum* are known for their ability to stablish complex interactions with microorganisms and plants, which

Fig. 5. Distribution of signature genes involved in SM biosynthesis in *Trichoderma* spp.

Distribution of Poliketyde synthases (PKSs), Non-Ribosomal Peptide synthases (NRPSs), Terpene synthases (TSs), Ribosomal Peptides-encoding genes (RiPPs), hybrid PKS-NRPS and Dimethylallyl Tryptophane synthases (DMATSs) among the genomes analysed are highlighted in different green shades depending on their amount. Total number of genes belonging to each family predicted in *Trichoderma* spp. are shown in the bottom highlighted with different grey shades. Total number of genes predicted per each species are shown in the right. Two different grey shades were used to group species belonging to the same phylogenetic clade.



Strain	PKS	NRPS	TS	RiPPs	NRPS-PKS	DMATS	Total
B. bassiana ARSEF2860	14	16	18	25	4	0	77
T. virens Gv29-8	20	31	21	17	4	1	**94**
T. pleuroticola Tr1	27	29	22	5	5	0	**88**
T. pleuroti TPhu1	26	24	23	12	4	0	**89**
T. harzianum CBS 226.95	26	24	18	37	5	0	**110**
T. harzianum TR274	24	23	18	37	5	0	**107**
T. guizhouense NJAU 4742	25	21	19	15	5	0	**85**
T. atrobrunneum ITEM 908	20	11	16	5	2	1	**55**
T. afroharzianum T6776	26	19	20	21	4	0	**90**
T. atroviride IMI 206040	20	17	18	9	2	0	**66**
T. gamsii A5MH	13	11	15	6	13	0	**58**
***T. gamsii* T6085**	**19**	**20**	**16**	**8**	**1**	**0**	**64**
T. hamatum GD12	20	22	15	8	1	0	**66**
T. asperellum TR356 v1.0	20	20	21	13	2	0	**76**
T. asperellum CBS 433.97	18	22	21	12	2	0	**75**
T. arundinaceum IBT 40837	27	19	20	14	5	1	**86**
T. brevicompactum IBT 40841	33	25	21	15	4	2	**100**
T. citrinoviride TUCIM 6016	14	15	18	14	2	0	**63**
T. longibrachiatum ATCC 18648	13	10	15	12	1	0	**51**
T. parareesei CBS 125925	13	13	16	9	2	0	**53**
T. reesei RUT C-30	12	13	17	10	2	0	**54**
T. reesei QM6a	13	12	17	10	2	0	**54**
Total	429	401	387	289	73	5	

in most cases, and with the exception of phytotoxic *T. brevicompactum* (Tijerino et al., 2011b), has enabled their use as BCAs of crop plant diseases (Kubicek et al., 2019). In this context, a more complex lifestyle, in terms of inter-organism communication, could require a more extensive and flexible SMs repertoire.

PKSs (429 genes) and NRPSs (401 genes) are the most represented SMs gene-families in *Trichoderma*, followed by TSs (387 genes) and RiPPs (289 genes), while NRPS-PKS hybrids (73 genes) and DMATSs (5 genes) are less abundant. Comparison of gene-family size between the clades revealed statistical differences in the composition of their respective SMs inventories.

PKS-family size varies significantly depending on the clade: species of *Brevicompactum* have the higher number of PKSs (average= 30; P= 0.029), followed by *Harzianum* species (average= 24.86; P= 0.015), while species of *Longibrachiatum* have the smaller PKS arsenal (average= 13; P= 0.002) and those of *Viride* did not show differences (average= 18.33; P= 0.27). According to this, polyketide biosynthesis would significantly contribute in generating species-specific SMs arsenals in phylogenetically distant *Trichoderma* species, leading to different clade-specific polyketide catalogues. On the other hand, NRPS-family size is homogenic among the clades (*Viride* average= 18.67; P= 0.84 – *Brevicompactum* average= 22; P= 0.47 – *Harzianum* average= 21.29; P= 0.05), with the exception of species belonging to *Longibrachiatum* which NRPSs content is significantly lower (average= 12.6; P= 0.011). *Trichoderma* spp. are able to produce more than 1000 different SMs compounds (Hermosa et al., 2014), and results show that PKSs and NRPSs have a great contribution to its production. Most polyketides have antifungal properties (Cardoza et al., 2005), facilitate competition for substrates and communication (Khosla 2009), and PKS are the most likely genes being responsible of the green-yellow pigmentation of *Trichoderma* conidia (Baker et al., 2012). On the other hand, non-ribosomal peptides are indispensable for successfully competition, being peptaibols and siderophores among the most remarkably SMs traits of *Trichoderma* (Mukherjee et al., 2013).

Ribosomal peptides, which are encoded by structural genes, are synthesized as part of a larger precursor peptide, which is post-translationally modified and subsequently proteolytically released (Luo and Dong, 2019). Usually, the precursor peptide consists of a N-terminal conserved leader sequence and a hypervariable core sequence, but many precursor peptides have a C-terminal recognition sequence that is important for excision

and cyclization (Arnison et al., 2013). RiPPs-family size is very heterogenous in species within the same clade, but no statistically differences were found between phylogenetic clades (*Viride* average= 9.33; P= 0.07 – *Brevicompactum* average= 14.50; P= 0.30 – *Harzianum* average= 18.86; P= 0.14 – *Longibrachiatum* average= 11; P= 0.64). It is worth to mention the high number of these genes found in both isolates of *T. harzianum* (37 genes), comparing to the lower numbers (5-21 genes) found in the other genomes here analysed. Since this gene family contributes significantly to the biosynthesis of species/isolate-specific SMs in *Trichoderma*, it is necessary to broaden the isolation of these metabolites and characterize of the RiPPs genomic arsenal. Fungal RiPPs have a very recent history, the first family was described only in 2007 (Hallen et al., 2007). To date, only four families of fungal RiPPs have been discovered (Umemura et al., 2014; Johnson et al., 2015; Nagano et al., 2016; Ding et al., 2016; Van der Velden et al., 2017), and the genes responsible of their biosynthesis have been little described (Vogt et al., 2019). This evidence the need to provide genomic tools that enable the identification of structurally differences in the hypervariable peptide sequences in RiPPs-encoding genes, aimed to correlate them to their final biosynthetic products.

Previous works have reported the presence of genes encoding NRPS-PKS hybrids in *Trichoderma* genomes (Mukherjee et al., 2013), providing evidences that some of them are involved in *Trichoderma*-plant roots interaction (Mukherjee et al., 2012). These enzymes are more abundant in species of *Harzianum* (average= 4.50; P= 0.01), while no statistical differences were found in the gene-content among the other clades (*Viride* average=3.50; P= 0.14 – *Brevicompactum* average= 4.29; P= 0.26 – *Longibrachiatum* average= 1.8; P= 0.05). Thus, NRPS-PKS hybrids contribute in generating specific SMs in species of *Harzianum*. Interestingly, *T. gamsii* A5MH lacks 15 PKS and NRPS genes comparing with *T. gamsii* T6085, which led to hypothesize whether the presence of 12 additional PKS-NRPS genes in A5MH could be the result of gene fusions between PKSs and NRPSs.

Although the absence of genes involved in alkaloid biosynthesis has been previously reported in *Trichoderma* (Kubicek et al., 2019), the analysis performed in this work revealed the presence of several signature genes. DMATS-encoding genes were found in four phylogenetically distant *Trichoderma* spp.: *T. virens*, *T. atrobrunneum* and *T. arundinaceum* genomes contain 1 gene each, while the genome of *T. brevicompactum* have 2 of these genes. No DMATSs were found in species of clades *Viride* or

Longibrachiatum. Given that *T. brevicompactum* contains 2 DMATSs, differences were statistically significant in the gene family-size of *Brevicompactum* comparing to other clades (average= 1.5; P= 0.002). Comparison of the sequences of the encoded DMATS proteins with those of DMATSs from other fungi by BLASTp analyses showed the most similar hits corresponded to species of genus *Monosporascus*, *Tolypocladium*, and *Scytalidium*. It suggests that DMATSs arose in *Trichoderma* through different HGT events from phylogenetically distant *Pezizomycotina* species. This is in agreement with a recent study showing that HGT events to *Trichoderma* from its fungi-associated hosts are not uncommon (Druzhinina et al., 2018). Indeed, DMATS of *T. arundinaceum* and *T. brevicompactum* likely arose in their common ancestor from *Monosporascus* spp, and was then vertically transferred to both species. In *T. atrobrunneum*, it could have been acquired from *Tolypocladium* spp. On the other hand, the second DMATS found in *T. brevicompactum* could have been acquired from *Scytalidium* spp. either after the divergence between *T. brevicompactum* and *T. arundinaceum*, or before it, with a subsequent gene loss in *T. arundinaceum*. Furthermore, *Scytalidium* spp. seems to be the donors of DMATS to *T. virens* as well. According to these results, some *Trichoderma* spp. would have acquired the ability to synthesize alkaloids or their derivates under certain conditions that are still unknown.

The striking number of TSs found in the genomes (387 genes) demonstrates that terpenoid biosynthesis significantly contributes to the SMs complexity in *Trichoderma*. Indeed, the TS-gene inventory found on these species (15-23 genes) clearly outnumbers those found in other fungi considered as rich productors of SMs, such as *Aspergillus* spp. (2-10 genes) (de Vries et al., 2017; Kubicek et al., 2019). This probably reflects the importance of TSs and terpenoids in the ecology of these fungi. TS-family size is fairly uniform among the phylogenetically close species, and no statistical significances were found in the family-size between clades (*Viride* average= 17.67; P= 0.38 – *Brevicompactum* average= 20.50; P= 0.25 – *Longibrachiatum* average= 16.60; P= 0.06 – *Harzianum* average= 19.43; P= 0.08). This indicates that terpenoid biosynthetic potential is less correlated to lifestyle or evolutionary history in *Trichoderma*. Although *Trichoderma* spp. are rich in TSs, most of these genes have not been characterized (Kubicek et al., 2019) comparing to PKSs and NRPSs, and a more complete description of the variability within the TS family in *Trichoderma* is needed. Therefore, part of this work was focused on providing an overview of the terpene biosynthetic potential within *Trichoderma* genus (Section 3.3.).

3.2 Mining the SMs BGCs in *Trichoderma* genomes

Analyses revealed that in almost all of the genomes, approximately half of the SM backbone genes are included in BGCs in *Trichoderma*, ranging from 42% in *T. harzianum* CBS 226.95 to 59% in *T. hamatum*; one exception is *T. atrobrunneum* that shows a higher number of clustered SMs genes (72%) (Fig. 6). This suggests that in *Trichoderma*, approximately half of the SMs genes are involved in specific biosynthetic pathways as they require co-expression with tailoring enzymes, whereas genes not included in clusters may participate as donors of SMs precursors in more than one metabolic pathway. While the quality of genome most likely does not affect gene identification, cluster identification could be affected by fragmentation of the genome assemblies. Considering as example that the genome of *T. atrobrunneum* has been assembled into 804 scaffolds while *T. reesei* is a complete genome, the conserved percentage of gene clustering indicate that differences in fragmentation of the assembly did not affect prediction of total BGCs.

While the abundance of clustered NRPSs and TSs is in agreement with the trend described above, the number of clusters associated with PKSs is significantly higher, ranging from 48 % in *T. arundinaceum* to 92 % in *T. gamsii* A5MH. Results showed a high variability within taxonomic clades, suggesting that the number of clustered PKSs in *Trichoderma* is less affected by evolutionary distances. However, it’s important to consider that the numbers of hybrids PKS-NRPS BGCs exceed the total PKS-NRPS backbone genes predicted, as these clusters can harbour either hybrid PKS-NRPS-encoding genes or two or more genes with PKS or NRPS function. Most of PKS-encoding genes occurs as orthologs in *Trichoderma* spp., and the diversity of polyketides could rely on the diversity of tailoring enzymes accompanying PKSs (Baker et al., 2011). In this sense, the need to compensate the scarce variability of PKSs may generates a greater tendency of clustering these with a wide variety of accessory enzymes, leading to many genomic combinations and expanding the catalogue of *Trichoderma* polyketides.

RiPPs clusters were only detected in *T. harzianum* CBS 226.95 and *T. guizhouense*. Considering the high number of total RiPPs-encoding genes found here across the species, using our approach their associated clusters have been probably underpredicted.

Fig. 6. Distribution of gene clusters of genes involved in SMs biosynthesis in *Trichoderma* spp.

Distribution of clusters containing Poliketyde synthases (PKSs), Non-Ribosomal Peptide synthases (NRPSs), Terpene synthases (TSs), Ribosomal Peptides-encoding genes (RiPPs), Dimethylallyl Tryptophane synthases (DMATSs) and hybrid clusters among the genomes analysed are highlighted in different orange shades depending on their amount. Total number of gene clusters belonging to each family predicted in *Trichoderma* spp. are shown in the bottom highlighted with different grey shades. Total number of gene clusters predicted per each species are shown in the right. Two different grey shades were used to group species belonging to the same phylogenetic clade.

Strain	PKS	NRPS	TS	NRPS-PKS	Other hybrids	Alkaloid b.	RiPPs	Total
B. bassiana ARSEF2860	9	15	8	5	2	0	0	39
T. virens Gv29-8	14	16	10	6	2	1	0	**48**
T. pleuroticola Tr1	14	16	10	6	2	0	0	**48**
T. pleuroti TPhu1	19	13	10	4	2	0	0	**48**
T. harzianum CBS 226.95	20	9	9	7	1	0	1	**47**
T. harzianum TR274	20	13	9	6	0	0	0	**48**
T. guizhouense NJAU 4742	18	12	10	7	1	0	1	**49**
T. atrobrunneum ITEM 908	18	10	7	3	2	1	0	**40**
T. afroharzianum T6776	18	11	9	5	3	0	0	**46**
T. atroviride IMI 206040	12	10	8	3	1	0	0	**34**
T. gamsii A5MH	12	8	8	2	1	0	0	**31**
***T. gamsii* T6085**	**12**	**9**	7	**3**	**1**	**0**	**0**	**32**
T. hamatum GD12	14	14	7	4	0	0	0	**39**
T. asperellum TR356 v1.0	11	10	11	5	1	0	0	**38**
T. asperellum CBS 433.97	11	12	11	5	1	0	0	**40**
T. arundinaceum IBT 40837	13	9	8	9	3	1	0	**42**
T. brevicompactum IBT 40841	22	15	8	8	4	2	0	**58**
T. citrinoviride TUCIM 6016	8	8	7	4	1	0	0	**28**
T. longibrachiatum ATCC 18648	9	6	6	3	0	0	0	**24**
T. parareesei CBS 125925	9	6	7	4	1	0	0	**27**
T. reesei RUT C-30	8	7	8	3	0	0	0	**26**
T. reesei QM6a	9	6	8	4	0	0	0	**27**
Total	294	216	178	102	29	5	2	

We should consider that RiPPs prediction by antiSMASH 5.0 is based on the ustiloxin B cluster from *A. flavus*, and that characterization of fungal RiPPs clusters is still in a state of flux, therefore more precise algorithms are required to correlate the mature RiPP with its corresponding BGC (Luo and Dong, 2019; Vignolle et al., 2020).

As observed for total SMs backbone genes, closest phylogenetically species tend to harbour similar number of SMs clusters in *Trichoderma*. Number of clusters is uniform in species within the same clade, but not in clade *Brevicompactum*, in which *T. arundinaceum* lacks 14 SMs clusters comparing to *T. brevicompactum,* despite being related species. Species from clades *Brevicompactum* (42-58 clusters), *Harzianum* (40-49 clusters) and *Virens* (48 clusters) have the highest number of SMs clusters, followed by species from *Viride* (31-40 clusters). Species from the clade *Longibrachiatum* contain the lowest number of clustered SMs genes (24-28 clusters).

Trichoderma spp. have a great number of hybrid clusters (131), most of them of T1PKS-NRPS type (102), while only 29 correspond to other hybrid types. More detailed information of other hybrid clusters is shown in Supplementary Table 1. The number of hybrid clusters is uniform between species belonging to the same clade, with species of clade *Brevicompactum* showing the highest hybrid cluster content. Interestingly, of 13 PKS-NRPS backbone genes predicted in *T. gamsii* A5MH, only 2 are predicted to belong to BGCs. Probably those genes not included in clusters may encode proteins acting as substrate-donors in several biosynthetic pathways, and for this reason, are not included in specific clusters.

The analysis revealed the 5 DMATSs found in *Trichoderma* genomes are included in alkaloid biosynthetic related clusters. In *T. arundinaceum*, *T. brevicompactum*, and *T. atrobrunneum*, DMATSs belong to hybrid NRPS-indole clusters harbouring all the 6 genes belonging to the dihydrolysergic acid (DHLA) BGC of *Claviceps africana*. These clusters also contain some tailoring enzymes that are absent in the original DHLA BGC, suggesting they probably biosynthesize DHLA derivates. In *T. virens*, the hybrid cluster resembles the clapurines BGC, but the scarce similarity found (45%) suggests it is more likely to be involved in the biosynthesis of a different kind of alkaloid. The genome of *T. brevicompactum* contains another DMATS embedded in an indole BGC, which is absent in other *Trichoderma* genomes. Our results suggest that DMATSs included in DHLA-like clusters would have been acquired by *Trichoderma* from distant *Sordariomycetes*

species, whereas *Leotiomycetes* species would constitute donors of DMATSs for the *Brevicompactum* and the *T. virens* clades. As mentioned, we propose independent HGT events from distant *Pezizomycotina* species as the processes responsible of the origin of DMATSs in *Trichoderma*. Diversification of alkaloid BGCs across *Sordariomycetes* likely occurred through separate HGT events from distant *Pezizomycotina* species; however, it's not clear whether they originated by direct transference from *Eurotiomycetes*, or instead, *Leotiomycetes* could have acted as intermediaries (Marcet-Houben et al., 2016). Fungal alkaloids can act as potent antimicrobials against other microorganisms (Mahmood et al., 2010), and also protect their associated host plants from grazers (Schardl et al., 2004; Wäli et al., 2013). In this sense, and since production of alkaloids has not been previously reported in *Trichoderma*, their biosynthesis would confer adaptive advantages to certain *Trichoderma* spp. under still unknown conditions.

TS-containing clusters (178) constitutes the third more abundant family of total SMs clusters. As observed in total SMs backbone genes, the number of TSs clusters (7-11) is more uniform among the species analysed comparing to PKSs and NRPSs clusters (8-22 and 6-16). The analysis enabled to assign putative functions to 268 clusters, of which 9 were TS-containing or TS-hybrid clusters. Three clusters with 100% similarity to the sesquiterpene Harzianum B BGC were identified in *T. guizhouense* and in both isolates of *T. asperellum*. Two clusters with 100% similarity to diterpenes gibberellin BGC were identified in both isolates of *T. asperellum*. Clusters with lower percentages of similarity were predicted to contain only the signature gene or were partially conserved BGCs. For example, in *T. pleuroticola* and *T. afroharzianum* clusters with 32% similarity with the diterpene sordarin BGC were found. Similarly, two clusters with 33% and 8% similarity with the trichothecene biosynthetic pathway were found in *T. gamsii* T6085 and *T. arundinaceum*, respectively, both containing only the TRI5-encoding gene.

3.3 Genomic characterization of TSs in *Trichoderma*

The 387 TS proteins found in the genomes of *Trichoderma* spp. were characterized *in-silico* following a three-level identification approach. Firstly, we identified PT and TC proteins according to their conserved domains. Subsequent detection of the metal-binding motifs enabled protein classification as Class I, Class II or Bifunctional enzymes. Finally, clustering-based phylogenetic analysis using biologically characterized fungal TSs

enabled determining their substrate-specificity, as well as assigning putative functions of 15 groups of TSs. TSs sharing conserved domains and metal-binding motifs clustered in the same phylogenetic group, each one highlighted in different colour (Fig. 7). More detailed information about TS-content per each specie showing specific portions of the TS inventory in *Trichoderma* is available in Supplementary Table 2.

Although the TS-family size is very homogeneous within *Trichoderma*, we were able to identify clade-specific TSs, which reflect specific portions of the terpenoid inventory shared by phylogenetically close species. Thus, despite their similar terpenoid biosynthetic potential, the species of *Trichoderma* have adapted their terpene production according to different environmental demands. Species of clade *Viride* constitute an example, as they lack some groups of TSs that are widely distributed in most of the clades, but have evolved specific TSs which are absent in these clades.

Light blue colour represents the HAD-like proteins, containing two conserved domains: the HAD-like domain (Pfam 13419) is present in the N-terminal, while the C-terminal contain a TS domain (IPR008930).

Dark-green colour represents proteins sharing a TRI5 domain (Pfam 06330), thus, were identified as sesquiTS of the TRI5 superfamily. An overlapping TRI5 motif (PIRSF 001388) was also predicted for 7 proteins forming one subcluster. Phylogenomic analysis revealed that these 7 TSs corresponded to **Trichodiene synthases** (TRI5), while another subcluster of 15 proteins was identified as **Longiborneol synthases**. Other sesquiTSs of the TRI5 superfamily, which clustered in two independent groups, were named as "**Uncharacterized group 1"** and **"2"**, respectively, as they did not cluster with any known protein. The sister clade of the TRI5 superfamily (**Light-green colour)** includes proteins containing the Terpene synthase C domain (Pfam 03936), and it comprises mainly sesquiTSs. **Presilphiperfolan-8β-ol synthases** and **Pentalenene synthases** were identified within this group, in addition to two groups of proteins that did not cluster with any known protein, and were therefore named as "**Uncharacterized group 3"** and **"4"**, respectively.

Orange colour represents proteins containing a Squalene synthase-phytoene synthase domain (Pfam 00494), in which a trans-membrane helix region was also predicted in the C-terminal. These proteins also contain overlapping Squalene synthase

(PTHR11626:SF2) and Farnesyl diphosphate-farnesyl transferase (PTHR11626) motifs, and phylogenetic analysis confirmed them as **Squalene synthases (SQSs)**. **Red colour** proteins share the prenyl transferase domain (Pfam 00432), and also contain overlapping motifs that enabled to identify them as **Type I Geranylgeranyl transferases** (PTHR11774:SF4) (GGTases 1), **Type II Geranylgeranyl transferases** (PTHR11774:SF11) (GGTases 2) and **Farnesyl transferases** (PTHR11774:SF6) (FTases).

Light-brown proteins share both N-terminal (Pfam 13249) and C-terminal (Pfam 13243) of the Squalene-hopene cyclase conserved domain, and phylogenetic analysis identified them as **Oxidosqualene cyclases (OSCs)**. **Dark-brown** contains another group of proteins sharing a Squalene synthase-phytoene synthase domain (Pfam 00494), but no phylogenetic relation was found with SQSs, although both share the squalene synthase-phytoene synthase conserved domain. In addition, they did not clustered either with the lycopene-phytoene synthases characterized from fungi included in the analysis, and they were therefore named as "**Uncharacterized group 5"**.

Grey highlights bifunctional **diTSs**, some of them containing Kaurene-synthase (PIRSF 036498) and Ent-copalyl diphosphate synthase (PTHR31739:SF4) domains. **Dark-blue colour** represents proteins sharing the polyprenyl synthase domain (Pfam 00348), while some of them also contain a N-terminal Terpene synthase C domain (Pfam 03936). Within this group, **GGPP synthases** (PTHR12001) and **FPP synthases** (PTHR11525) were identified. Phylogenetic analysis also revealed a set of highly conserved **indole diTS**. Finally, proteins containing both prenyltransferase and Teperne synthase C domain, or one of them, were named as **Chimeric-like**, as some of them clustered with known sesterTS.

According to our results, *Trichoderma* spp. have a huge potential for sesquiterpene biosynthesis. We identified 8 groups of sesquiTSs, which constitute almost the third of the total number of TSs found in this work. Species of *Viride* are particularly rich in sesquiTS belonging to the TRI5-superfamily, and they also contain HAD-like TSs which are absent in species of other clades. Although HAD-like proteins did not cluster with known TSs from fungi, the presence of both Class I DDxxE and Class II DxDTT motifs indicates they are bifunctional enzymes. The DxDTT motif is a variant of the DxDD motif

Fig. 7. Phylogenomic characterization of TS proteins in *Trichoderma* spp.

TS proteins sharing conserved domains are highlighted in different colours: HAD-like (light blue), TRI5 (dark green), Terpene synthase C (light green), Squalene synthase-phytoene synthase (orange), prenyl transferase (red), Squalene/Hopene cyclase (light and dark brown), Kaurene synthase and/or Ent-copalyl diphosphate synthase (grey), polyprenyl synthase and/or Terpene synthase C (dark blue). Putative functions of terpene cyclases (TCs) and prenyl transferases (PTs) were assigned based on phylogenetic analysis performed with TS proteins with known function from filamentous fungi (Table 1). Proteins which did not clustered with any known protein were designed as Uncharacterized TSs. Aspartate-rich motifs of Class I, Class II and Bifunctional enzymes were identified in the amino-acidic sequences of each group of proteins. Bootstrap values > 50 are shown in the correspondent branches of the tree.

Phylogeny	Predicted function	Enzyme type	Aspartate-rich motif
	HAD-like	Bifunctional	DDXXE and DXDTT
	Uncharacterized group 1	Class I – TS	DDXXX
	Trichodiene synthases	Class I – TC	DDSRE / DDSIE
	Uncharacterized group 2	Class I – TS	[D/E]XX[D/E]
	Longiborneol synthases	Class I – TC	DDHFD / DEHFD / NDHFD
	Uncharacterized group 3	Class I – TS	DDIRE
	Presilphiperfolan-8β-ol synthases	Class I – TC	DDQFD / DDPAA
	Pentalenene synthases	Class I – TC	DD(M/V/L)FD
	Uncharacterized group 4	Class I – TS	DDXXD
	Squalene synthases	Class I – PT	DDMTI / DDMTH
	GGTases 1	Class I – PT	DEXXV / DAXXV
	GGTases 2	Class I – PT	DEWGE
	FTases	Class I – PT	D[D/E]XX[D/E]
	Uncharacterized group 5	Class I – TS	DXXXE
	Oxidosqualene cyclases	Class II – TC	DCTSE / DCISE
	Diterpene synthases	Bifunctional	DEXXE and DXDD
	GGPP synthases	Class I – PT (IPS)	DDVID and DDMLD
	FPP synthases	Class I – PT (IPS)	DDIMD and DDXXD
	Indole-diterpene synthases	Class I – PT	DDYLD and DDVQD
	Chimeric-like	Chimeric PT-TC PT/TC	D[D/E]XXD and/or D[D/E]XXD

found in Class II diTS (Nakano et al., 2005 and 2009). Shinohara et al. (2016), reported that some sesquiTS can contain HAD-like domains and DxDTT motifs, leading to FPP cyclization through a protonation step, instead of by an ionization step. We hypothesized

that HAD-like TSs found in species of *Viride* might be particular bifunctional sesquiTS synthesizing specific metabolites of this clade.

TRI5 are sesquiTSs that catalyze the cyclization of FPP to trichodiene, opening the trichothecene biosynthesis pathway (Desjardins et al., 1996). They all show a highly conserved Class I DDSRE motif at position 109-114, but that from *T. guizhouense* showing DDSIE, and a highly conserved NSD/DTE triad (NDLFSFYKE), 119-120 residues downstream from the metal-binding motif. The analysis showed they are present in species from the clade *Brevicompactum*, *T. gamsii*, *T. asperellum* and also in *T. guizhouense* from the clade *Harzianum*. This phylogenetic distribution indicates that TRI5 is not a monophyletic trait in *Trichoderma*, opening questions about its origin in the genus.

Previous studies have reported that some *Trichoderma* spp. have the potential to synthesize longiborneol (Bansal and Mukherjee 2016), an intermediate in the culmorin biosynthetic pathway (McCormick et al., 2010; Bansal and Mukherjee 2016). Species of clade *Viride* were the only lacking Longiborneol synthases, which were found highly conserved in among the other species. According to this, longiborneol biosynthesis is very widespread in *Trichoderma*, but is absent in species of clade *Viride*. Since culmorin production has not been reported in *Trichoderma*, longiborneol could be synthesized as a solely compound or as an intermediate participating in unknown biosynthetic pathways in these species. Most of these proteins show a conserved D(D/E)HFD motif, which is partially conserved (NDHFD) in proteins of *T. arundinaceum* and *T. brevicompactum*. Site-directed mutagenesis and crystallography studies on TSs have revealed that the first aspartate residue (D) of the metal-binding motif interacts directly with Mg^{2+} (Starks 1997), and its replacement can lead to aberrantly metal-binding and anomalous cyclization products or a product mixture (Cane et al., 1996). According to this, Longiborneol synthases of *Brevicompactum* species could be actually enzymes involved in terpenoid blend formation, which can even contain new terpenoids.

SesquiTSs included in “Uncharacterized group 1”, showing Class I DDxxX and NDV(L/I)SFYKE motifs, were only present in *Viride* and some species of the *Longibrachiatum* clade. In species of *Viride*, there are present two sesquiTS of “Uncharacterized group 2”, while *T. arundinaceum* and *T. virens* have only one. These contain Class I [D/E]xx[D/E] and NDILSFYKE motifs. These synthases are mainly

present in *Viride* species, indicating they probably contributed to adapt their terpenoid arsenal to particular environmental demands.

According to the results, most *Trichoderma* spp. can potentially produce presilphiperfolan-8β-ol. This compound is thought to play a central role in the biosynthesis of a wide range of polycyclic sesquiterpenes in fungi (Pinedo et al., 2008), thus, these synthases may contribute in generating a variety of structurally complex terpenoids in *Trichoderma.* Presilphiperfolan-8β-ol synthases are absent in *Viride* species, *T. arundinaceum* and *T. atrobrunneum*. They contain a conserved Class I DDQFD motif, DDPAA in both isolates of *T. reesei*, and a highly conserved ND(L/I)LSYKKE triad located 102-150 residues downstream from the metal-binding motif.

Pentalenene synthases were found in all the *Trichoderma* isolates analysed. These enzymes cyclize FPP to form the sesquiterpenic compound pentalenene, the parent hydrocarbon of the pentalenolactone family of fungal antibiotics (Kim et al., 1998). A conserved Class I DD(M/V/L)FD motif was found in all the proteins, excepting in those from *T. pleuroti* (one of them), *T. gamsii* A5MH and *T. brevicompactum*. These last two proteins show a DDxxD motif 18 residues upstream the NSD/DTE triad, that is also present in all the other proteins. The NSD/DTE triad is conserved as NDILSYRKE in all the proteins, but lacks in *T. citrinoviride*, *T. parareesei*, *T. longibrachiatum*, *T. asperellum* CBS433.97 and in *T. pleuroti*. Results derived from studies of plant sesquiTS reported that the presence of an additional DDxxD motif supplies the absence of the NSD/DTE triad, enabling the protein to be functional (Back and Chappell, 1996; Steele et al., 1998). This suggests that pentalenene synthases from these species could be functional, while presilphiperfolan-8β-ol synthases from both isolates of *T. harzianum* could be not.

TSs of “Uncharacterized group 4” were found widely distributed across the species, but are particularly represented in *T. virens* and *T. pleuroticola*. They show Class I DDxxD motif and a N(D/E)xxSxxKE. Differently, proteins of “Uncharacterized group 3” seems to be exclusive of species belonging to the *Harzianum* clade, and their phylogenetic proximity to both groups of sesquiTS suggests it is also composed by this type of TSs. They show Class I DDIRE motif and a NDILSYNKE triad 18 residues downstream.

Analyses revealed a large group of PTs, comprised by SQSs and enzymes involved in protein prenylation (Jeong et al., 2018), such as GGTases 1, GGTases 2 and FTases. SQSs

catalyse the condensation of two FPP to form squalene, the central precursor in the biosynthesis of all the triterpenic compounds (Quin et al., 2014). They show a Class I DDMTI motif (DDMTH in *T. pleuroti*), at position 82-86. In addition, they also present a C-terminal TM helix region of 23 residues, which is universally conserved in all the eukaryotic SQSs and is responsible to bind the protein to the endoplasmic reticulum (Linscott et al., 2016). SQSs are present as unique copy in all the *Trichoderma* genomes analysed here, indicating that single-copy SQS provide the squalene precursor required for triterpene biosynthesis in these fungi. However, *T. pleuroti* has two copies of these genes, suggesting that one of them might be involved in a specific triterpene biosynthetic pathway.

TSs involved in protein prenylation GGTases 1, GGTases 2 and FTases are present in single-copy in the genomes of *Trichoderma*. These enzymes are responsible of transferring prenyl groups to target proteins containing CaaX motifs (Kamiya et al., 1979). This process, called prenylation, enables proteins to become membrane-associated due to the hydrophobic nature of the prenyl group, enabling them being involved in cellular signalling functions (Jeong et al., 2018). GGTases 1 show Class I DExxV motif, DAxxV in proteins from *T. arundinaceum* and *T. brevicompactum*. In the same way, type GGTases 2 show a Class I DEWGE motif, while FTases show the Class I D[D/E]xx[D/E] motif, which is partially conserved in some cases.

Similar to SQSs, OSCs were found in single copy in all the *Trichoderma* isolates analysed. These proteins are exclusive of eukaryotes and are responsible of the conversion of oxidosqualene to either lanosterol or protostadienol, precursors of triterpenes (Quin et al., 2014). A DCTSE aspartate-rich motif was found in 7 of these proteins, while DCISE was found in the other 14 proteins, both variants of the characteristic DCTAE motif associated to OSCs (Abe et al., 2001). Furthermore, these OSCs contain 5 conserved QW motifs, which are thought to be responsible of strengthening the structure of the enzyme and stabilize the carbocation intermediates (Kushiro et al., 2000). Proteins belonging to "Uncharacterized group 5" are also present in all the *Trichoderma* spp, analysed one per each isolate, showing a partially Class I conserved motif DxxxE.

Copalyl-pyrophosphate/Ent-kaurene synthases (CPS/KS) were found in *T. asperellum*, known for its ability for gibberellin biosynthesis (Zhao and Zhang 2015). Bifunctional enzymes clustering with CPS/KS were found in *T. citrinoviride*, *T. parareesei*, *T. reesei*

and species of clade *Brevicompactum* (PTHR31739:SF4), but their low sequence similarity with CPS/KS indicates these are diTS not involved in ent-kaurene biosynthesis. Harziandione was the first diterpene isolated from *Trichoderma* spp. (Ghisalberti et al., 1992), and a number of these compounds have been recently reported in these species, mainly comprising endophyte and marine isolates (Miao et al., 2012; Adelin et al., 2014; Song et al., 2018; Chen et al., 2019; Zhao et al., 2019). Nevertheless, the low number of diTS found here suggests the ability of *Trichoderma* spp. for diterpene biosynthesis is scarce and not very widespread within the genus.

IPSs, responsible of the biosynthesis of universal terpene precursors GGPP and FPP, were found in all the *Trichoderma* spp. GGPP synthases show two Class I aspartate-rich motifs separated by 129 aa, DDVID and DDMLD. FPP synthases show two Class I DDxxD metal-binding motifs, excepting one in *T. arundinaceum,* which is shorter and contains only one motif. The presence of two conserved aspartate-rich motifs characterize GGPP and FPP synthases, in which the first motif is responsible of the accommodation of the allylic substrates and Mg binding, while the second motif triggers the chain extension with IPP units (Wend et al., 1998; Gao et al., 2012). Some *Harzianum* and *Brevicompactum* species have 2/3 copies of these PTs class, suggesting that at least some of them could be actually pathway-specific. Additional GGPP and FPP synthases may act as donors of terpenoid precursors in specific biosynthetic pathways in these species. This probably reflects specific portions of the terpenoid inventory of these species, and guarantees an efficient distribution of terpenoid precursors between primary and secondary metabolism.

Analysis also revealed a set of highly conserved indole diTS, of which some species have more than one. They have two Class I motifs, DDYLD and DDVQD, excepting that of *T. longibrachiatum*, which has no conserved motifs suggesting this protein might be not functional. Considering that production of indole diterpenes has not been reported in *Trichoderma*, our results reveal that these species have at least the potential to produce these compounds. Production of indole diterpenes has been reported in some Sordariomycetes being involved in protecting their reproductive structures from fungivores (Saikia et al., 2008). Furthermore, many of indole diterpene-producer fungi stablish symbiotic relationships with plants, thus, biosynthesis of these compounds may confer ecological advantages on *Trichoderma*-host associations as well (Parker and Scott 2004).

The last group contains Class I TSs clustering with known chimeric TSs from fungi, which were absent in species of the *Viride* clade. Most of these proteins contain only polyprenyl synthase or terpene synthase C domains. Nevertheless, we found one protein in *T. asperellum* TR456 containing both domains and DDIED and DDIVD motifs, which is highly similar to ophiobolin F synthase from *Aspergillus clavatus*, suggesting this specie is able to produce sesterterpenes. Proteins showing only polyprenyl synthase domain and Class I motifs, DDLDE or DDIQD, were found in *T. gamsii* A5MH, *T. pleuroti*, *T. afroharzianum* and in both isolates of *T. asperellum*. Proteins showing only terpene cyclase domain and Class I DDEME motif, are present in *T. virens*, *T. pleuroti*, *T. harzianum*, *T. guizhouense*, *T. atrobrunneum*, *T. afroharzianum, T. gamsii* A5MH, and *T. arundinaceum* and in all the members of the clades *Brevicompactum* and *Longibrachiatum*.

No monoTS were found among the genomes analysed in this work, although production of monoterpenes has been reported in *T. virens* (Crutcher et al., 2013; Inayati et al., 2019). No *bona fide* monoTS have been identified in fungi (Schmidt-Dannert, 2014), and the scarce availability of sequences of these enzymes probably leaded to miss-predict them. Biochemical studies have shown that fungal sesquiTS are able to cyclize GPP (Lopez-Gallego et al., 2010), thus, we hypothesize that enzymes involved in monoterpene biosynthesis in *Trichoderma* could be actually included within "Uncharacterized group 4", since they are phylogenetically close to sesquiTS, and other uncharacterized proteins were found restricted to some clades not including *T. virens* or fell into the TRI5-superfamily.

3.4 Assessment of the genomic context of *tri5* genes in *Trichoderma*

Trichothecenes are a family of sesquiterpenes produced by some species from multiple fungal genera, including *Fusarium*, *Isaria*, *Microcyclospora*, *Myrothecium*, *Peltaster*, *Spicellum*, *Stachybotrys*, *Trichoderma*, and *Trichothecium* (Cole et al., 2003; Kikuchi et al., 2004; McCormick et al., 2011; Surup et al., 2014; Venkatasubbaiah et al., 1995). The first committed step in the biosynthesis of trichothecenes is catalysed by TRI5, which cyclize the precursor FPP to form trichodiene (Desjardins et al., 1996). Trichodiene undergoes subsequently chemical modifications by oxygenases, acetyltransferases, and some other tailoring enzymes, leading to different types of trichothecenes (Proctor et al.,

2018). Genes encoding these enzymes are arranged in the trichothecene (*TRI*) BGC, whose gene-content varies depending on the final product (Proctor et al., 2018). In *Trichoderma*, the *TRI* cluster and the production of trichothecenes have only been reported in species of the clade *Brevicompactum*, *T. arundinaceum* and *T. brevicompactum* (Cardoza et al., 2011).

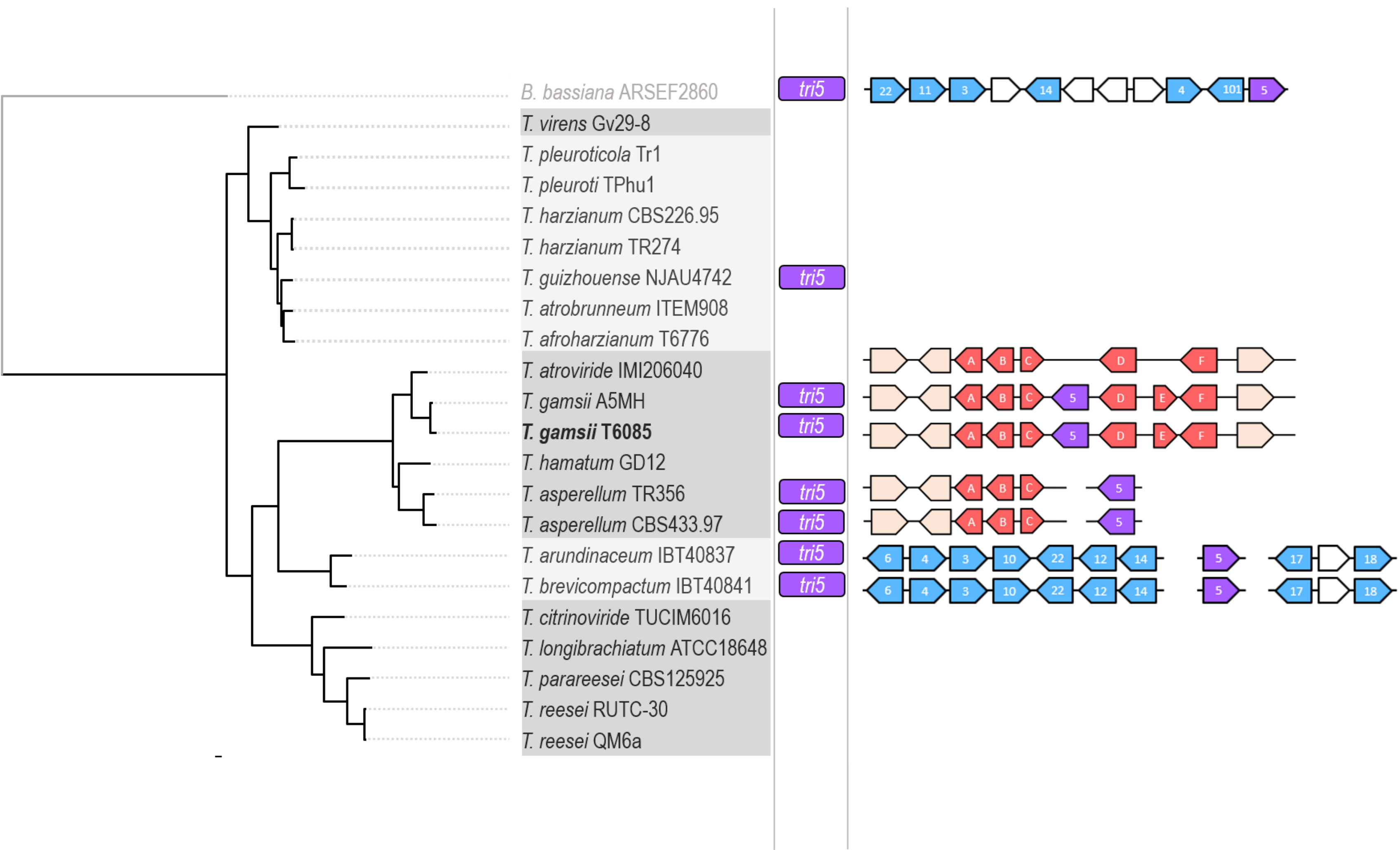
Analysis of the TS-gene content in *Trichoderma* showed that *tri5* is also present in *T. guizhouense*, and in both isolates of *T. gamsii* and *T. asperellum*. Pairwise alignments of each TRI5 protein with that of *T. arundinaceum* and *T. brevicompactum* showed 77% similarity in *T. guizhouense*, 80-82% identity in *T. asperellum* isolates and 87% similarity in *T. gamsii*.

Assessment of the TS-associated clusters based on antiSMASH showed that unlike *T. asperellum*, *T. guizhouense,* and *Brevicompactum* species, *tri5* is included in a BGC in *T. gamsii*. The 21.3 Kb cluster found in *T. gamsii*, encloses 6 other genes, which were named as *A*, *B*, *C*, *D*, *E* and *F* (Fig. 8). Manual characterization based on conserved domains and similarity with characterized proteins in other systems enabled to identify four tailoring enzymes, one efflux transporter and one regulatory protein. The three genes located upstream of *tri5* encode a Zn2-C6 transcription factor (TF) (*A*), oxygenase (*B*), and alpha-beta hydrolase (*C*), while the three located downstream were identified as oxygenase (*D*), Major Super-facilitator Family (MSF) transporter (*E*) and carbonic anhydrase (*F*). The presence of a TF-encoding gene within the cluster suggests a pathway-specific regulation, as it has been observed in approximately half of the SM BGCs studied to date (Macheleidt et al., 2016). Furthermore, the presence of an MSF transporter suggests this cluster is involved in the biosynthesis of a sesquiterpenoid with extra-cellular functions.

Comparison of the proteins found in *T. gamsii* with the TRI proteins described in the trichothecene-producer species showed no sequence neither function similarities, excepting on TRI5. Since *tri5* is not included in the *TRI* cluster in *T. arundinaceum* and *T. brevicompactum*, as found in other fungal species (Proctor et al., 2018), a search of the TRI proteins was carried out in the genomes containing *tri5* by BLASTp analysis, using the TRI protein sequences from both species of the clade *Brevicompactum* and those of *F. graminearum*, available in public databases.

Fig. 8. Phylogenetic distribution of *tri5* and tri5-associated clusters in *Trichoderma*.

Genes belonging to variants of the TRI loci reported in species of the clade *Brevicompactum* and *B. bassiana*, respectively, are shown in light blue. Genes found associated to *tri5* (in purple) in *Viride* species are shown in red. *Trichoderma* clades are highlighted in different grey shadows.



Results showed that these species lack on the entire set of TRI proteins excepting TRI5, and a distantly related homolog of *tri101* was found in the genome of *T. gamsii*, which has been already reported (Proctor et al., 2018). In the trichothecene biosynthetic pathway, the first two enzymatic steps, catalysed by genes *tri5* and *tri4* (trichodiene oxygenase), are conserved among the producer species and are strictly required for the formation of the 12,13-epoxytrichothec-9-ene ring (EPT) needed for trichothecene production (Alexander et al., 2009; Kimura et al., 2007; Izquierdo-Bueno et al., 2018; Proctor et al., 2018). Together with *tri3* (C-15 acetyltransferase), they constitute the minimal *tri*-kit which is present in all the producer species. The absence of *tri4* could be countervailed by the existence of another trichodiene oxygenase, as suggested to explain trichothecene production on *Spicellum roseum*, which lacks this gene (Proctor et al., 2018). However, and unlike *S. roseum*, no other *tri* core genes were found in the genomes of *Viride* species, suggesting these fungi are unable to produce trichothecenes. In the other hand, the presence of a *tri101* homolog in non-trichothecene producer fungi is not uncommon. It has been proposed that some *tri101* homologs have a function(s) other than trichothecene biosynthesis, and paralogs of this gene evolved the *tri101* C-3 acetyltransferase function required for trichothecene production (Proctor et al., 2018).

We used the protein sequences encoded in the cluster found in *T. gamsii* as queries in BLASTp analyses to search for homologous proteins in the *Trichoderma* genomes used here. Genes *A*, *B* and *C* were also found in all the other *Trichoderma* spp. belonging to the *Viride* clade, with conserved synteny ; preliminary BLAST analyses suggest that these genes may be originated by HGT from a donor belonging to the Eurotiomycetes; further analyses are needed in order to better understand the evolutionary origins of these genes. Instead, genes *D* and *F* are present in some of the genomes analysed in closely related species; while gene *E* seems to be specific to *T. gamsii*. Polymorphism in variable loci containing BGCs between phylogenetically close species have been observed, even between isolates of the same species, in which the synteny is conserved but the gene-composition is variable (Zhang et al., 2012; Lind et al., 2017; Proctor et al., 2018). Differences observed in the *tri5*-associated cluster may stems to gene gain/loss and chromosomic rearrangements probably occurred in this genomic region during the evolution of *Viride* species and contributing to metabolic diversity and host adaptation, as suggested by Zhang et al. (2012) for genomic regions containing genes involved in SM biosynthesis.

These findings suggest the origin of a novel *tri5*-associated gene cluster in the genus *Trichoderma*. In addition of HGT events, gene gain/loss, relocations and/or duplications and posterior neofunctionalization of native genes have been proposed as mechanisms driving the birth of new BGCs (Rokas et al., 2018; Proctor et al., 2018; Ramdass et al., 2019; Brown et al., 2019). Further analyses are needed to find evidences for processes that have contributed to the formation of this novel cluster. Since *tri5* orthologues seems to be functionally associated to two different SM clusters in *Trichoderma*, it could be hypothesized that this gene is involved in different metabolic pathways within this genus. This novel *tri5*-associated cluster could lead to an uncharted trichodiene-derived sesquiterpenic biosynthetic pathway in *T. gamsii*, which could synthetize novel metabolites with agronomic interest. Thus, we propose the involvement of *tri5* in other metabolic pathways different from the biosynthesis of trichothecenes. The availability of new complete genome sequences from other *Trichoderma* spp. will enable to check whether this cluster could be present in other species or instead, if it is specific of *T. gamsii*. Expression studies on these genes are required to decipher their expression patterns and whether they are co-regulated. In addition, functional analysis combined with metabolic profiles of the fungus will enable determining the metabolite/s produced by this BGC. Furthermore, analysis of *tri5* from *T. asperellum* and *T. guizhouense* and metabolic profiles of these fungi will determine whether this gene cooperates in other metabolic pathways different from that of *T. gamsii* and species from *Brevicompactum*, or instead, it is only involved in the production of trichodiene.

Chapter 2:

Expression studies on TS genes: *Tgam* in different ecologic contexts.

Metabolic profiles of *Tgam*.

Relationships occurring between the many terpenes reported in *Trichoderma* and the genes responsible for their synthesis are still not well known, which outlines an intriguing area of research. The striking genomic potential for terpenoid production found in *Trichoderma* spp. in this work suggests that functional differentiation of gene family members is the driver for the high TS gene numbers of these species. Assessing changes in the relative expression of TS when the fungus grows under different environmental conditions, or when interacts with other organisms, enables individuating genes that could play a role in these frameworks.

Tgam strain T6085 constitutes an interesting model for this purpose, due its versatile lifestyle. As reported in section 1.3.4.1, *Tgam* competes with *F. graminearum* for space and nutrients on wheat, reduces its growth and mycotoxin production, and the ability of the pathogen to develop reproductive structures (i.e. perithecia) on wheat straw, thus resulting in a reduction of incidence and severity of FHB. In addition, this fungus shows endophytic skills, being able to colonize the external layers of epidermis and cortex of wheat roots and induce the plant defence responses, indicating the existence of a symbiotic interaction between both partners.

Computational analysis based on IPR terms enabled to identify 16 TS-encoding genes in the genome of *Tgam* (Table 4). Proteins identified with IPR008949 were named as TS 1, TS 3-11 and TRI5, while proteins identified with IPR008930 were named as TC 1-5. Phylogenetic analysis revealed these genes putatively correspond to unknown sesquiTS (TS3, TS4 and TS7), “uncharacterized group 4” (TS1), squalene synthase (TS6), pentalenene synthase (TS5), indole diTS (TS9), “uncharacterized group 5” (TS11), trichodiene synthase (TRI5), HAD-like (TC4), oxidosqualene cyclase (TC2), GGTase 1 (TC3), GGTase 2 (TC5), FTase (TC1), GGPP synthase (TS8) and FPP synthase (TS10).

Analysis of BGCs revealed 7 TS genes included in clusters in *Tgam* – *tri5*, *ts1*, *ts3*, *ts4*, *ts5*, *ts6* and *tc2*–. In addition to the *tri5*-containing cluster, antiSMASH 5.0 founds similar hits when using the oxidosqualene cyclase (TC2)-containing cluster as query, which was predicted to encode a lanosterol synthase.

For gene expression studies, we focused on 9 genes encoding TS belonging to Class I – TS1, TS3, TS4, TS5, TS6, TS7, TS9, TS11 and TRI5 – which represented a high diverse functional group according to our analyses. Thus, we excluded genes encoding Class II

proteins and those involved in the biosynthesis of terpene precursors and protein prenylation.

Table 4. TS proteins found in *Tgam*.

JGI accession numbers of each protein are shown in the first column. Putative protein functions derived from phylogenomic analysis (section 3.3) are shown in the second column. Proteins belonging to BGCs are shown in the fourth column, highlighted in pink. Proteins included in gene expression analyses are shown in the third column, highlighted in blue.

JGI ID	Putative function	Name	Cluster
Trigam1|5596	bifunctional HAD-like	TC4	No
Trigam1|4742	**TRI5**	**TRI5**	Yes
Trigam1|162	**pentalenene synthase**	**TS5**	Yes
Trigam1|9843	**sesquiTS**	**TS7**	No
Trigam1|1824	**sesquiTS**	**TS4**	Yes
Trigam1|4947	**sesquiTS**	**TS3**	Yes
Trigam1|5367	**uncharacterized group 4**	**TS1**	Yes
Trigam1|340	**squalene synthase**	**TS6**	Yes
Trigam1|3208	GGTase 1	TC3	No
Trigam1|5139	GGTase 2	TC5	No
Trigam1|3927	FTase	TC1	No
Trigam1|4065	GGPP synthase	TS8	No
Trigam1|2917	FPP synthase	TS10	No
Trigam1|9898	**indole diTS**	**TS9**	No
Trigam1|8345	oxidosqualene cyclase	TC2	Yes
Trigam1|6072	**uncharacterized group 5**	**TS11**	No

In order to assess the environmental signals regulating the expression of TS genes selected in *Tgam*, the fungus was grown i) under different stress conditions and in presence/absence of C source, ii) in FHB scenario, and iii) in interaction with wheat roots.

4.1 Regulation of TS genes in *Tgam* in liquid substrates under different stress conditions

Genome mining data available in literature indicate that the ability of fungi to produce SMs has been substantially underestimated. The discordance found between the high number of genes and clusters involved in SMs biosynthesis, in comparison with the amount of SMs detected in fungal metabolomes, suggests that many of these genes are silent under standard laboratory conditions (Hertweck 2009). Since the early days of the fermentation science, it has been known that the choice of cultivation parameters is critical for the number and the nature of the SMs produced by microorganisms. Thus, manipulating nutritional or environmental factors, as well as inducing stress conditions, can promote differential SMs biosynthesis (Scherlach and Hertweck, 2009). This facilitates determining the conditions triggering their production and enables to hypothesize about the processes they could be involved in.

In this context, and since there is no extensive information about the regulation of TS genes in *Trichoderma*, changes in TSs expression were investigated in *Tgam* by growing the fungus in minimal medium (FRIESM), minimal medium amended with 0.9% sucrose (FRIES), or FRIESM supplemented with different stressors, such as 0.5 mM H_2O_2 (FRIESM H_2O_2) (oxidative stress), low nitrogen concentration (FRIEM N) (N starvation), or 200 mM NaCl (FRIESM 200 salt) (saline stress), using FRIESM as reference control.

Spores of *Tgam* were firstly incubated for 60 h in the minimal medium with sucrose (FRIES) as sole C source. This enabled the fungus to grow and develop a biomass in optimal conditions. Mycelium was transferred to fresh media of FRIES, FRIESM and FRIESM with different stressors. Since SM production usually begins late in the growth of the fungus, often upon the stationary phase (Bu'Lock 1961), second step-cultures of *Tgam* were incubated for 4 days, in agreement with previous experiments performed in this laboratory, in which growth curves of the fungus were determined by measuring its dry weight when growing in different liquid substrates (data not shown).

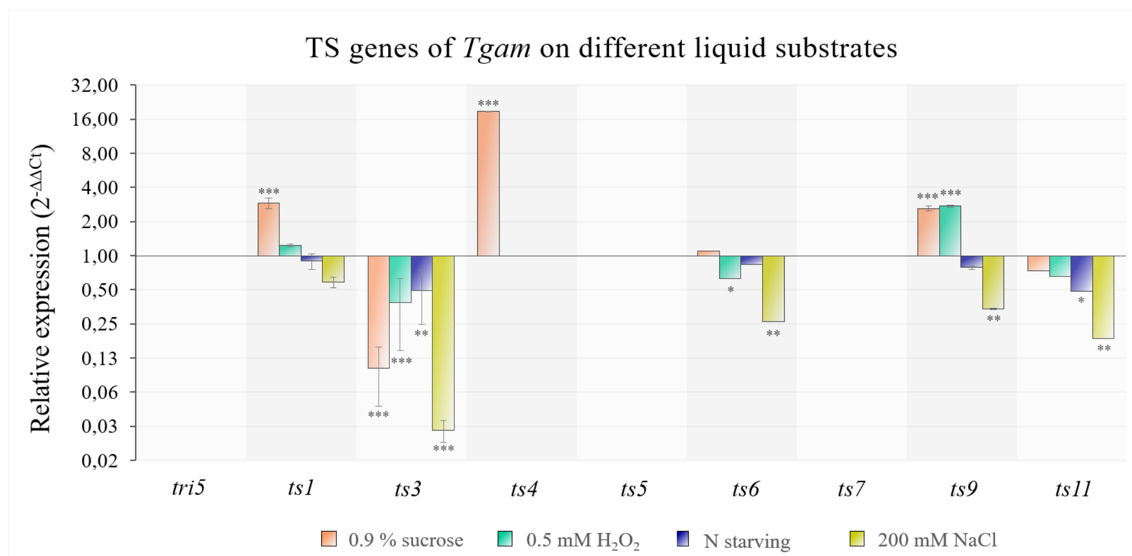
Analysis of expression (Fig. 9) revealed the absence of *tri5* transcripts in all the conditions tested. Addition of 0.9% of sucrose to the culture media did not induce *tri5* expression in *Tgam*, differently from what observed in *T. brevicompactum* when grown in presence of 1% or 2% sucrose (Tijerino et al., 2011a). Furthermore, these authors found the highest

tri5 expression levels in 3-day-old cultures of *T. brevicompactum* grown in minimal medium with 0.5 mM H_2O_2, and an increased expression of this gene by H_2O_2 has been also reported in *F. graminearum* (Ponts et al., 2007). In contrast, addition of 0.5 mM H_2O_2 to the culture media did not induce *tri5* expression in 4-day-old cultures of *Tgam*, suggesting different types of *tri5* regulation in *Tgam* and *T. brevicompactum*. In *T. brevicompactum* and *T. arundinaceum*, *tri5* is expressed by growing the fungi both in conventional rich and minimal substrates, leading to trichothecene accumulation (Cardoza et al., 2011; Malmierca et al., 2013). Anyway, it is conceivable that microbial

cultures do not produce some metabolites under non-natural conditions, and specific environmental requirements of the organisms should be taken into account (Scherlach and Hertweck 2009).

Fig. 9. Expression of TS genes of *Tgam* in different liquid substrates

Total RNA was extracted from 4-day-old mycelium of *Tgam* grown on minimal medium without sucrose (FRIESM) (basal condition, $2^{-\Delta\Delta Ct} = 1$), or minimal medium with 0.9% sucrose (FRIES), 0.5 mM H_2O_2 (FRIESM H_2O_2), low N concentration (FRIESM N), or 200 mM NaCl (FRIESM 200salt). Colour bars represent relative expression values of TS genes on each condition. The β-tubulin gene was used as control for data normalization. Values are means of three independent biological replicates with the corresponding standard deviation. Fold change in sample relative to control is expressed as $2^{-\Delta\Delta Ct}$. Statistically significant values are indicated with asterisks ($P \geq 0.05$ no significative; $0.05 > P \geq 0.01$ = *; $0.01 > P \geq 0.001$ = **; $P < 0.001$ = ***).



Experiment performed has a single time-point, so we cannot exclude that the activation of this gene takes place at earlier/later growth stages, or even that the environmental

signals that trigger its expression are different from those tested or they are more related to biotic interactions. As observed in *tri5*, *ts5* and *ts7* transcripts were not detected in any of the tested conditions.

Availability and type of C sources affects SMs production and expression levels of the involved genes in a strain-dependent manner (Calvo et al., 2002; Jiao et al., 2008). Addition of 0.9% of sucrose to the culture medium had contrasting effects on the expression of TS genes in *Tgam*. While the transcript levels of *ts6* and *ts11* did not change significantly, a 0.1-fold down-regulation was found in *ts3* ($P = 0.000$). On the other hand, 0.9% sucrose up-regulated 2.8-fold *ts1* ($P = 0.000$) and 2.6-fold *ts9* ($P = 0.000$), but the highest effect was observed in *ts4*, which was 18.7-fold up-regulated ($P = 0.000$).

Association of oxidative stress with SMs biosynthesis in filamentous fungi has been extensively demonstrated, and it has been suggested that it is induced to prevent ROS damage in fungi (Hong et al., 2013). As observed in cultures amended with 0.9% sucrose, addition of 0.5 mM H_2O_2 to the substrate induced opposite changes on TS expression in *Tgam*, indicating different regulation of TS genes in response to oxidative stress. A 0.3-fold down-regulation was found in *ts3* ($P = 0.000$), while *ts6* was 0.6-fold down-regulated ($P = 0.022$). In contrast, 0.5 mM of H_2O_2 promoted a 2.7-fold up-regulation of *ts9* ($P = 0.000$). Since *ts9* was predicted to encode an indole diTS, it suggests that production of indole-terpenes in *Tgam* might occur in response to oxidative stress, as it has been also observed in *Aspergillus* spp. (Fountain et al., 2016). No effects were observed in the expression levels of *ts1* and *ts11*.

Nitrogen availability has a considerable impact on secondary metabolism in fungi (Hautbergue et al., 2018). In *Fusarium fujikuroi*, it affected the expression of 35 out of 40 BGCs, and 2 diTS and 1 sesquiTS, from a total of 10 TS-encoding genes (Wiemann et al., 2013). In our experiment, low N concentration tends to negatively regulate the expression of TS genes in *Tgam*, suggesting terpenoid biosynthesis does not confer particular advantages to the fungus to overcome this stress. N deficit down-regulated 0.4-fold *ts3* and *ts11* ($P = 0.001$ and 0.013, respectively), and, although not statistically significant, a downward tendency was found in *ts1*, *ts6* and *ts9* expression.

Some salt-stress tolerant *Trichoderma* isolates could be used as BCAs against phytopathogens, plant-growth promoters and inducers of salt-tolerance in crop plants

grown on saline environments, as recently demonstrated by Sánchez-Montesinos et al. (2019) and Daliakopoulos et al. (2019). Saline stress can induce both quantitative and qualitative changes in SMs production, as it has been shown for VOCs produced by *T. harzianum*, with inhibitory effects against *B. cinerea* (Bualem et al., 2015). In order to induce a stress response, the growth of *Tgam* was evaluated on PDA with different salt concentrations (50, 100, 150 and 200 mM NaCl). No significant differences were detected in the diameter of the colony between 50, 100 and 150 mM NaCl and the control (data not shown), but the growth of the fungus was slightly reduced at 200 mM NaCl. Thus, this concentration was selected to test the expression of TS genes. Addition of 200 mM NaCl to the medium did not change the expression levels of *ts1*, but dramatically down-regulated the other's TS expression comparing with the effects observed in N starvation and oxidative stress. The highest repression was observed in *ts3* (0.02-fold change, P = 0.000), followed by *ts11* (0.18-fold change, P = 0.002), *ts6* (0.26-fold change, P = 0.001) and *ts9* (0.33-fold change, P = 0.012). Thus, and similar to what observed in N starving conditions, this suggests that terpenoid biosynthesis does not have a particular role under saline stress conditions.

4.2 *Tgam* – wheat – *Fgra* interaction

Terpenoid production plays a crucial role in *F. graminearum* to successfully colonize and defend its niches, enabling infection on wheat spikes and suppressing the release of chitinases by mycoparasites (Venkatesh and Keller, 2019; Audenaert et al., 2013). In *T. arundinaceum*, *tri* gene expression is affected when grown in dual cultures with *B. cinerea*, while polyketides and HA produced by the first induce changes in some *B. cinerea* genes linked to its virulence (Malmierca et al., 2015). This is an example illustrating how a molecular crosstalk between SMs determines the outcome of the interactions between antagonistic organisms. In this context, and since *Tgam* is able to suppress *Fgra* on wheat spikes and to reduce DON production by the pathogen (Matarese et al., 2012; Sarrocco et al., 2013, 2019), a differential response of the TS genes in *Tgam* could occur when the beneficious fungus is in an FHB scenario.

Three-player interactions involving *Trichoderma*, plant and fungal pathogen have received less attention comparing to the dual systems due to a higher difficulty in studying such a complex scenario, which usually require -omics approaches (Vinale et al., 2008).

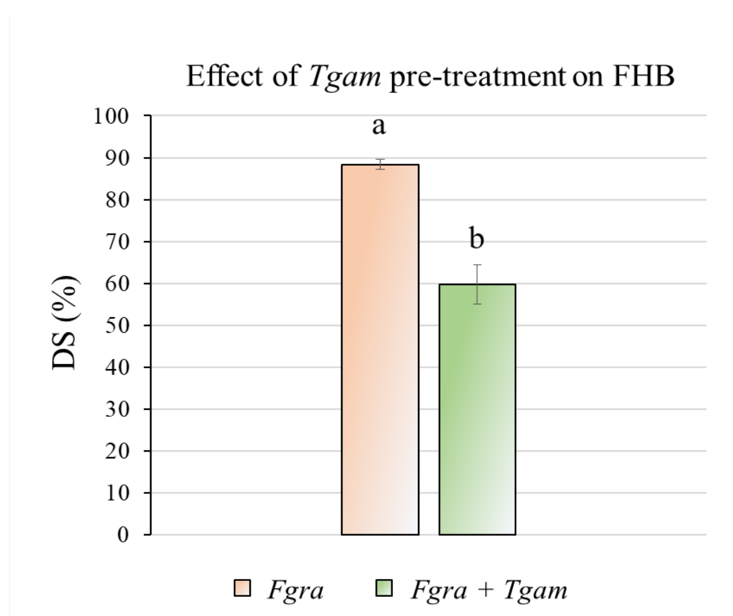
For that reason, and in order to establish a starting point towards the study of the involvement of terpenoids in the biocontrol activity of *Tgam* against FHB, we screened changes in TSs expression when *Tgam* was inoculated on wheat spikes along with *Fgra* comparing when *Tgam* was alone.

Wheat plants of *Triticum aestivum* cv. *Apogee* were used to reproduce FHB disease under controlled conditions. This cultivar is very useful for lab-scale experiments due its short propagation cycle comparing with other wheat varieties, enabling to optimize trial timings. FHB disease severity (DS) was evaluated in plants inoculated with *Tgam* + *Fgra* in comparison with those inoculated with *Fgra* alone, and spikelets were considered as diseased when glumes were slightly darkened and pinkish, characteristics of the early stages of FHB, or showed premature bleaching.

Analysis of variance revealed a significant reduction of 25.4% of the DS of FHB (P = 0.011) when plants were treated with *Tgam* 48 h before *Fgra* inoculation (Fig. 10). Plants inoculated with *Fgra* alone showed 88.3 ± 1.2% of diseased spikelets, while *Tgam* + *Fgra* plants showed 57.9 ± 4.7% of diseased spikelets.

Fig. 10. Effect of spike inoculation with *Tgam* in the Disease Severity of FHB on wheat.

Tgam was inoculated on wheat spikes at anthesis 48 h before inoculation of *Fgra*. Disease Severity (DS) was calculated 6 days after inoculation of the pathogen on ten spikes as percentage of diseased spikelets. Bars represent the average and DS values out of three biological replicates with the corresponding standard deviation. Different letters represent statistically different values.



In addition, qualitative differences were observed between plants inoculated with *Fgra* alone and those inoculated with both *Tgam* and the pathogen (Fig. 11). Six days after inoculation of the pathogen, diseased spikelets from plants inoculated with *Fgra* alone showed to a greater extent the characteristic premature bleaching of FHB.
Instead, when spikes were pre-treated with *Tgam*, most of the diseased spikelets showed brownish and pink spots at the base of the glumes, while only few of them were completely bleached.

Fig. 11. Effect of spike inoculation with *Tgam* in FHB symptoms on wheat.

Tgam was sprayed as spore suspension (10^7 spores mL^{-1}) on wheat spikes at anthesis 48 h before inoculation of *Fgra* (10^5 spores mL^{-1}). Reduction of FHB symptoms was evaluated 6 days after inoculation of the pathogen. The image shows qualitative differences between control spikes (left) and spikes inoculated with the pathogen (centre), and between these and spikes pre-treated with *Tgam* (right).

These results indicate that *Tgam* not only reduces the number of infected spikelets, but it is also able to delay the development of disease symptoms in infected spikelets. These results are in agreement with experiments performed in field, in which a reduction from 23.1% to 7.4% of the Disease Severity, and from 65.7% to 30.3% of the Disease Index (% of infected spikes), was observed when *Tgam* was used as spike inoculant at anthesis (Sarrocco et al., 2020).

The methodology employed to simulate FHB under laboratory conditions enabled us to reproduce results obtained in the field, thus demonstrating its utility for small-scale experiments. Reproducing FHB under controlled conditions, and the biocontrol effect exerted by *Tgam* against *Fgra* on wheat spikes, will be useful for assessing fungal growth, gene expression and many other physiological parameters, without introducing the external variations usually occurring in nature, such as changes in temperature, humidity or the interaction with natural (micro)organisms.

4.2.1. TSs expression in *Tgam* on FHB scenario

Aimed to shed light on whether a differential production of terpenes in *Tgam* could take place when the fungus interacts with *Fgra* on wheat spikes, relative expression of TS genes was evaluated in co-inoculated spikes (*Tgam* + *Fgra*), using spikes treated only with *Tgam* alone as *Tgam* control.

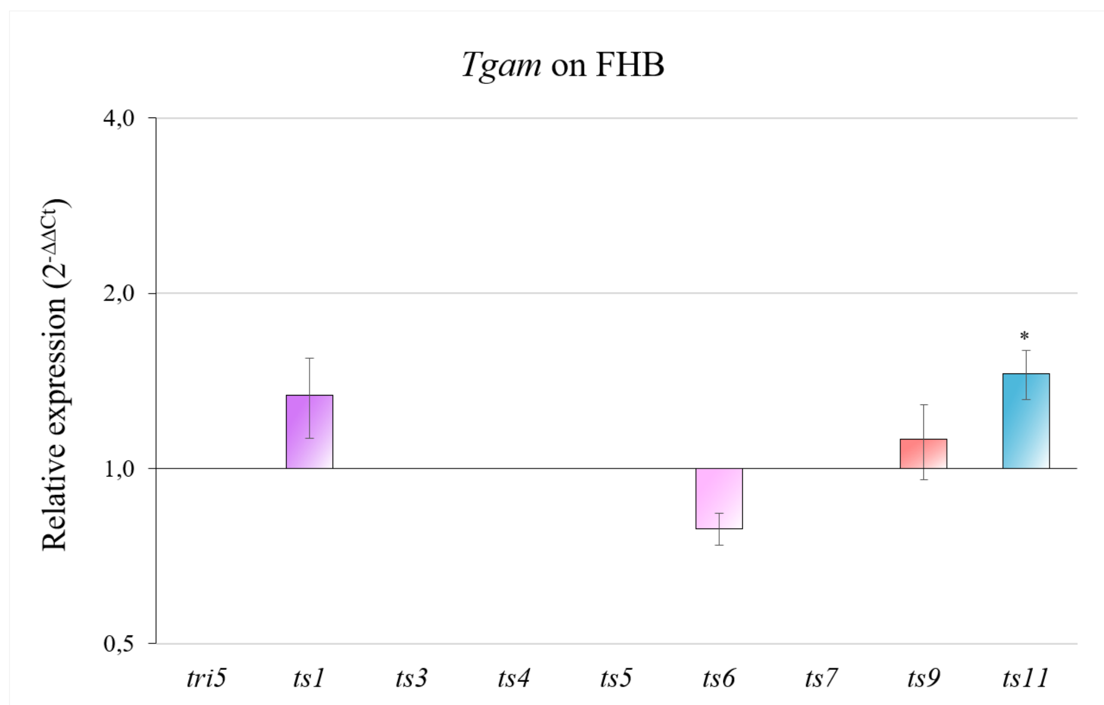
Analysis of expression revealed 4 TS genes – *ts1*, *ts6*, *ts9* and *ts11* – active when *Tgam* was on spikes with or without *Fgra* (Fig. 12). No transcripts of *tri5*, *ts3*, *ts4*, *ts5* and *ts7* were detected. No specific transcripts were found in either conditions. Expression of *ts1*, *ts6* and *ts9* did not change significantly between the two theses, suggesting these genes are not particularly involved in the triple interaction under the conditions tested.

Instead, *ts11* was slightly up-regulated (1.45-fold, $P = 0.011$) when *Tgam* was on spikes with *Fgra*, suggesting the presence of *Fgra* directly induced changes in its expression or could mediate physiological changes in spikes that promoted changes on *ts11* expression. Additional analyses are required to determine whether this gene could be involved in that reduction of the FHB symptoms by *Tgam* observed in wheat plants six days after

inoculation of the pathogen. Interestingly, neither the presence of the plant nor the pathogen triggered the activation of *tri5* in the tested conditions.

Fig. 12. Expression of TS genes of *Tgam* in the presence of *Fgra* on wheat spikes.

Total RNA was extracted from wheat spikes colonized by *Tgam* alone (basal condition, $2^{-\Delta\Delta Ct} = 1$) or by *Tgam* + *Fgra*, six days after inoculation of the pathogen. The *β*-tubulin gene was used as control for data normalization. Colour bars represent relative expression values of each TS gene. Values are means of three independent biological replicates with the corresponding standard deviation. Fold change in sample relative to control is expressed as $2^{-\Delta\Delta Ct}$. Statistically significant values are indicated with asterisks (P ≥ 0.05 no significative; 0.05 > P ≥ 0.01 = *; 0.01> P ≥ 0.001 = **; P < 0.001 = ***).



Overall results show that *Tgam* did not induce prominent changes in terpene biosynthesis when interacted with *F. graminearum* on wheat spikes in the tested conditions. Gene expression patterns are highly dynamic, and more extensive time-course experiments are needed to provide more information on whether a modulation of terpene biosynthesis in *T. gamsii T6085* could play a role when the fungus interacts with *F. graminearum* on wheat spikes.

4.3. *Tgam* – wheat roots interaction

Root colonization by *Trichoderma* is an intimate relationship involving a tightly regulated exchange of molecular signals including SMs (Hidangmayum and Dwivedi, 2018). When *Trichoderma* colonizes the roots, it releases a wide variety of SMs that promote substantial changes in plant biochemistry, which in turn, cause a reprogramming of the fungal physiology (Contreras-Cornejo et al., 2018).

As many other *Trichoderma* spp., *Tgam* is able to colonize the rhizosphere and establish a beneficial interaction with wheat roots, behaving as an endophyte and inducing the plant defence responses (Sarrocco et al., 2020). In order to assess whether a differential regulation on terpene biosynthesis occurs in these conditions, changes in TS gene expression were assessed in *Tgam* when interacting with wheat roots. For this purpose, *Tgam* was incubated for 3 days on PDA in presence of 1-day-old wheat seedlings, while *Tgam* grown alone on PDA under the same conditions was used as control.

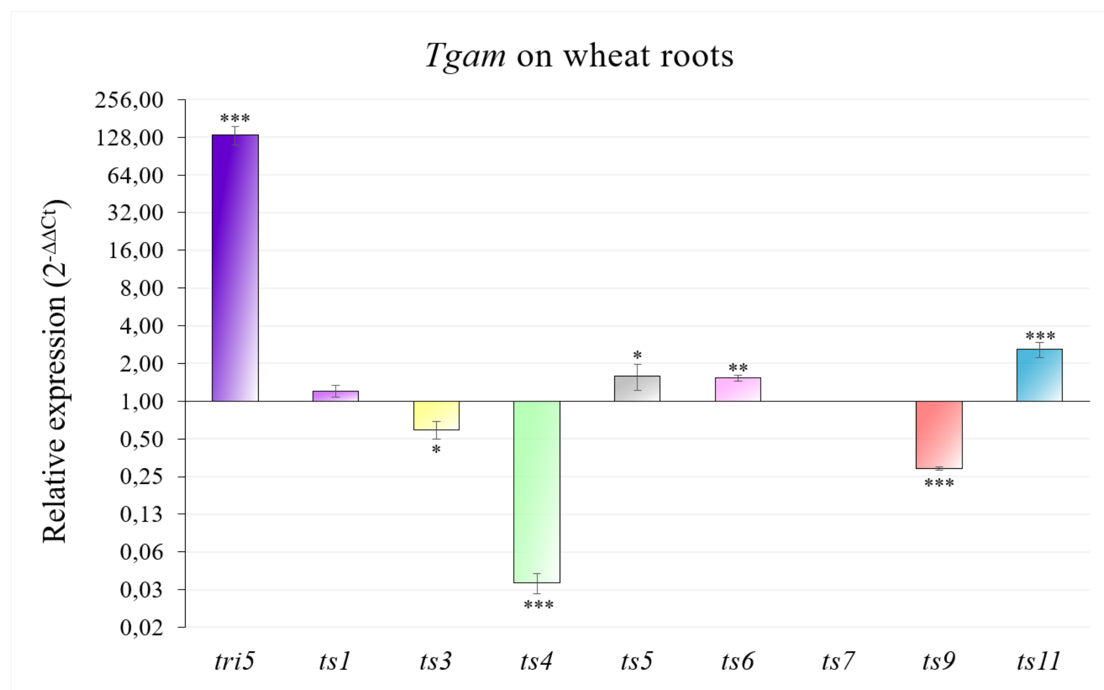
Root colonization affected TS expression in *Tgam*, which differentially regulated most of its TS genes, with the exception of *ts7*, whose transcripts were not detected in any condition, and *ts1*, which did not change its expression (Fig. 13). This clearly indicates a reprogramming in terpene biosynthesis in *Tgam* when colonizing the wheat roots.

In particular, a modulation in sesquiterpene biosynthesis occurred in *Tgam* during root colonization. Whereas *ts5* was slightly up-regulated (1.58-fold; (P = 0.045), the contact with the roots strongly repressed the expression of *ts4* (0.03-fold; P= 0.000) and in a lesser extent, that of *ts3* (0.59-fold; P= 0.021). Nevertheless, the most remarkably difference was found in the relative expression of *tri5*, which was 133.6-fold up-regulated (P = 0.000) during root colonization. This strong overexpression suggests that signals from the roots are the responsible of triggering the expression of this gene in *Tgam*. Although activation of *tri5* usually leads to the production of phytotoxic compounds, such as trichodermin in *T. brevicompactum* (Tijerino et al., 2011a), HA produced by *T. arundinaceum* lacks on phytotoxic activity, and has proven to have a crucial role in plant protection against *B. cinerea* (Malmierca et al., 2012). This example illustrates how different ecological demands led to an adjustment in a metabolic pathway governed by the same genes in species belonging to the same fungal genus (Mukherjee et al., 2013). In this context, and since *Tgam* establishes a beneficial interaction with the plant roots,

we can imagine the involvement of *tri5* in the biosynthesis of a sesquiterpenic compound that could play a role in promoting this relationship. Further analyses involving both the monitoring of *tri5* expression before contact with roots, at contact, and when the fungus penetrates the root tissues, as well as assessing the behaviour of a *tri5* knock-out mutant in root colonization are required to infer a possible role in beneficial interaction with roots. On the other hand, the differential response of the plant to the WT fungus and the *tri5*-deletion mutant will shed light into the role of *tri5* in *T. gamsii* T6085 and its possible involvement in the establishment of a beneficial fungus-plant interaction.

Fig. 13. Expression of TS genes of *Tgam* in interaction with wheat roots.

Total RNA was extracted from mycelium of *Tgam* grown on PDA for 3 days (basal condition, $2^{-\Delta\Delta Ct} = 1$), and from wheat roots colonized by *Tgam* for 3 days. The β-tubulin gene was used as control for data normalization. Colour bars represent relative expression values of each TS gene. Values are means of three independent biological replicates with the corresponding standard deviation. Fold change in sample relative to control is expressed as $2^{-\Delta\Delta Ct}$. Statistically significant values are indicated with asterisks ($P \geq 0.05$ no significative; $0.05 > P \geq 0.01$ = *; $0.01 > P \geq 0.001$ = **; $P < 0.001$ = ***).



The role of fungal sesquiterpenes in plant root colonization is not well documented. Nevertheless, it has been shown that sesquiterpenes produced by ectomycorrhizal fungi re-programme root architecture by enhancing lateral root and root hair development,

which is thought to provide nutritional benefits to root-associated fungi and to enhance the probabilities of successful root colonization (Ditengou et al., 2015).

In addition, an up-regulation of 1.52-fold and 2.58-fold was found on *ts6* (P = 0.003) and *ts11* (P = 0.000), respectively, when *Tgam* was interacting with wheat roots. Since *ts6* was predicted to encode a squalene synthase, results suggest that triterpene biosynthesis is modulated in *Tgam* in during root colonization.

Interestingly, *ts9* was found strongly down-regulated (0.28-fold change; P = 0.000) in *Tgam* colonizing wheat roots in comparison to *Tgam* alone in PDA, suggesting that root colonization induce a repression on indole-diterpene biosynthesis in the fungus.

Results reveal that root colonization induces an overall modulation of TS gene expression in the fungus, suggesting that reprogramming of terpene biosynthesis has likely an impact in the outcome of this beneficial interaction, and led to hypothesize that some terpenoid may have a role on supporting it.

4.4 Metabolic profiles of *Tgam* in different substrates

As mentioned throughout this work, the number of isolated metabolites compared with the genetic potential to produce them, found by mining fungal genomes, suggests that many of the SMs genes and BGCs are inactive under laboratory conditions. Therefore, cultivation-based techniques have been extensively used aimed to stimulate the activation of these “silent” pathways. The principles behind these techniques have been joined under the concept “one strain many compounds” (OSMAC) by Zeeck and co-workers (Bode et al., 2002), which explains how a single strain can produce different compounds when grown under different environmental conditions. In this context, we examined quantitative and qualitative differences on metabolite production by *Tgam* grown in different liquid substrates and in their ½ dilutions by LC-MS analysis.

Principal Component Analysis (PCA) was used to determine the relationship between the SMs pattern composition and the liquid substrates used. As shown in Fig. 14, the first two components accounted for 73.63% of the total variance. Metabolites found in non-diluted substrates (FRIES, ME and PDB) clustered separately, and despite dilution by ½ of FRIES and ME did not affect significantly metabolite composition compared to the full-strength media, qualitative differences were observed when diluting PDB compared with the non-diluted substrate, as shown in Fig. 15.

Fig. 14. PCA of metabolite profiles obtained from *Tgam* grown in different liquid substrates for 21 days.

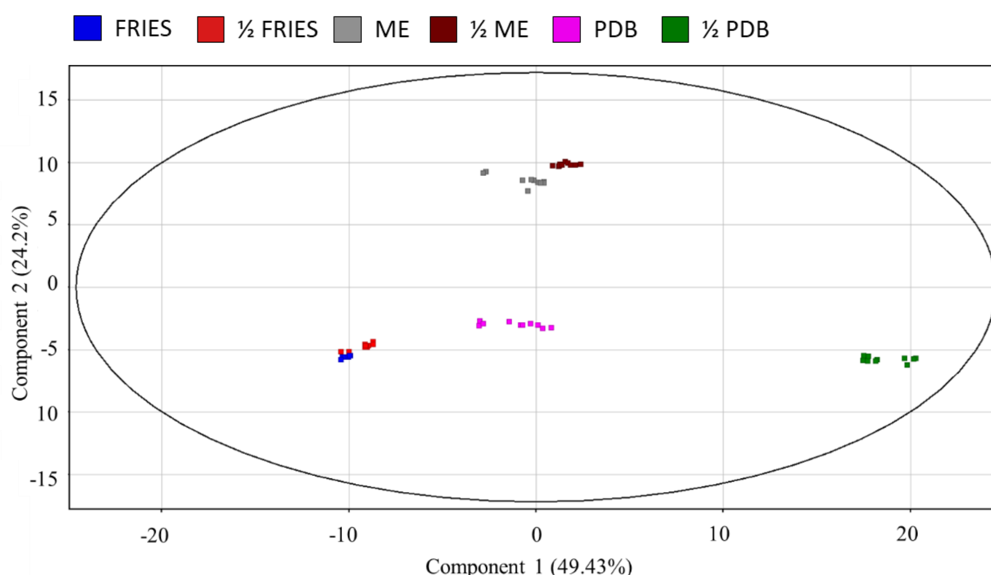
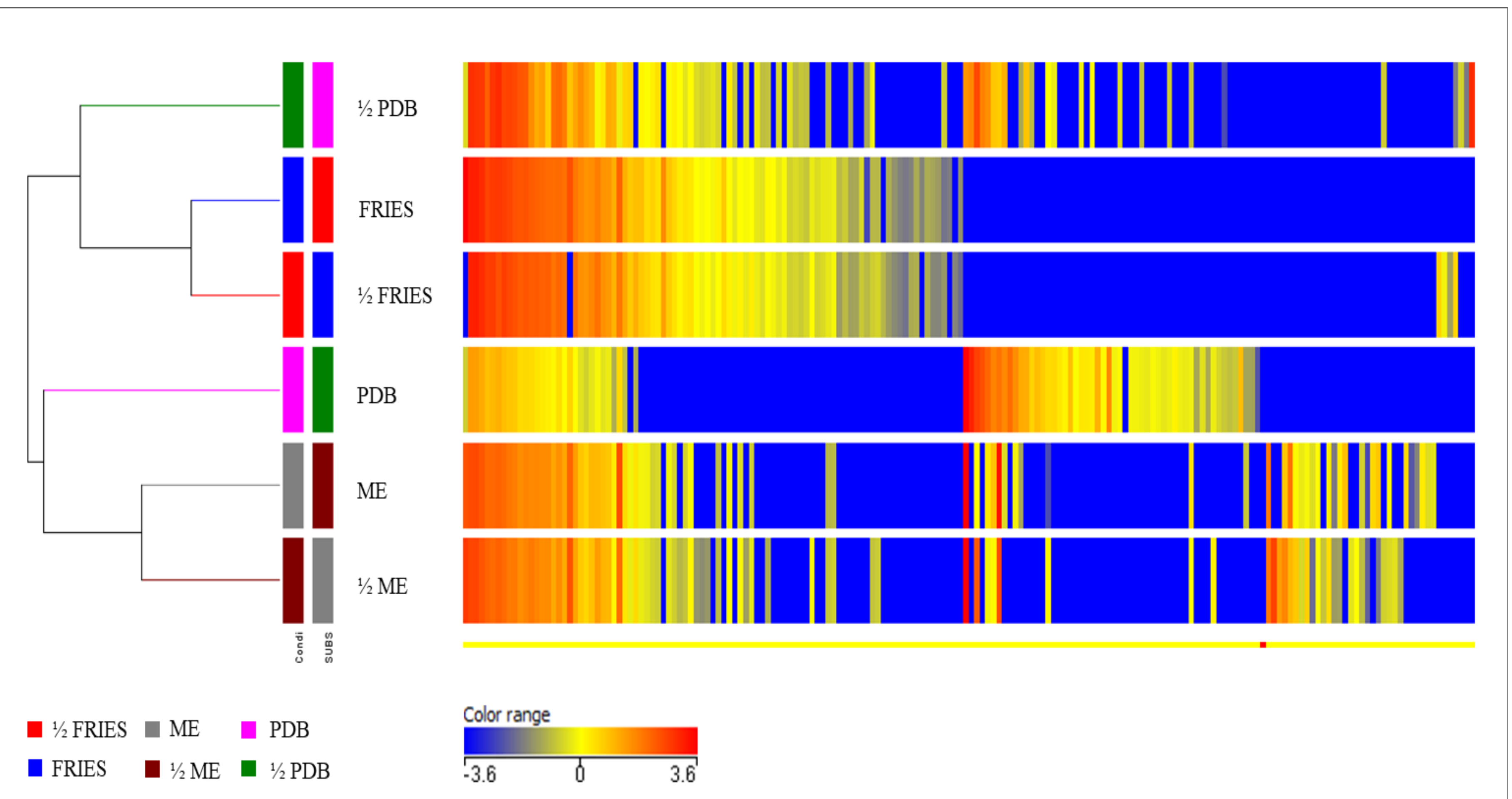


Fig. 15. Comparison between metabolites found in liquid cultures of *Tgam* in different substrates.

The heatmap shows qualitative and quantitative differences between metabolites differentially produced in filtrates from 21-day-old static cultures of *Tgam* analysed by LC-MS. Each bar represents a single metabolite and the colour range is referred to their abundance, where -3.6 (blue) means the absence of the compound and +3.6 means the maximum quantity found comparing to the other substrates.

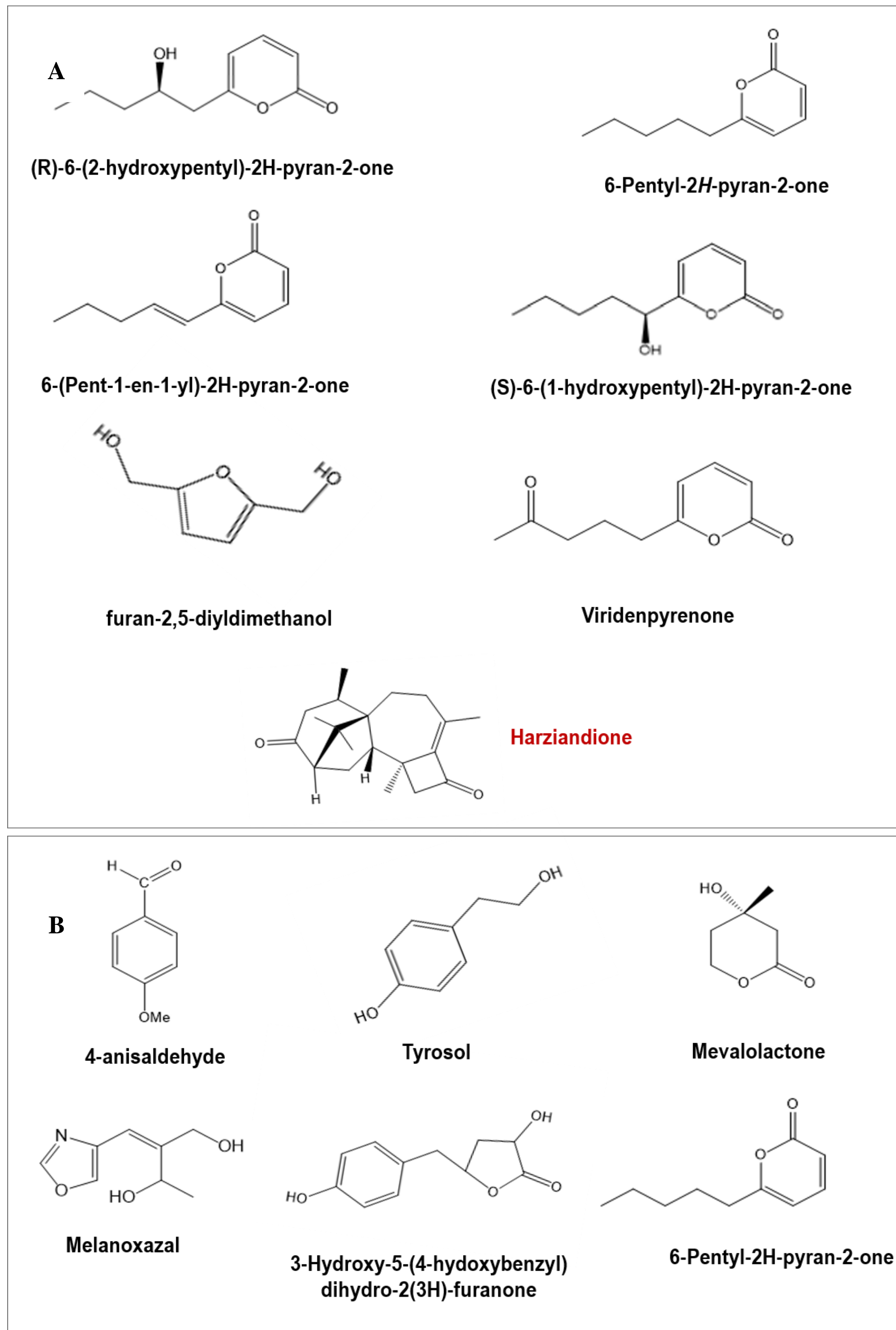


Nevertheless, the most remarkably differences were found between PDB half strength and the other substrates, both at quantitative and qualitative level. Although all the substrates yielded metabolites which are not present in the other media, PDB showed the bigger number of unique metabolites, followed by ME. For that reason, PDB and ME were selected to perform an analysis to preliminary identify SMs differentially produced, with particular attention to terpenes. Most of the reports regarding the production of SMs are focused on the use of liquid cultures; however, filamentous fungi seem to be more adapted to solid substrates (Shakeri and Foster, 2007; Viniegra-González, 2014). On this basis, metabolic profile was determined in solid ME (MEA) substrate, along with PDB.

^{1}H-NMR and ^{13}C-NMR analysis of extracts obtained from 12-day-old PDB filtrates and 15-day-old MEA cultures yielded 7 and 6 major compounds, respectively (Fig. 16). The most abundant metabolite found in both cultures was 6-pentyl-2H-pyrone (6-PP) – already detected in hug amount in the first analysis performed by LC-MS– 16.3 mg in PDB and 9.9 mg in MEA, which was not surprising due the high intense coconut aroma issued by *Tgam* comparing with other *Trichoderma* isolates (Collins et al., 1972). In addition, compounds derived from chemical modifications of 6-PP were the major constituents of the extracts obtained from MEA cultures: 6 (Pent-1-en-1-yl)-2H-pyran-2-one (1.21mg); (S) 6 (1-hydroxypentyl)-2H-pyran-2-one (2.7 mg); (R) 6 (2-hydroxypentyl)-2H-pyran-2-one (2.35 mg); viridenpyrenone (2.14 mg), this last was isolated for the first time in *T. viride* and shows antifungal properties (Cooney and Lauren, 1999; Evidente et al., 2003). A broad range of pyrones have been isolated in *Trichoderma* spp., acting not only as biological weapons against other organisms, but also as molecules able to elicit the plant defense responses (Claydon et al., 1987; Parker et al., 1997; Keszler et al., 2000; Patil et al., 2016; Manganiello et al., 2018). The strong relationship found between the biosynthesis of these compounds and the biocontrol activity of *Trichoderma* (Scarselletti et al., 1994; Worasatit et al., 1994) suggest that the ability of *Tgam* to produce significative amounts of pyrones probably support the biocontrol activity observed against *Fusarium* spp. involved in FHB and the induction of the plant defense responses.

Fig. 16. Metabolites identified in the major fractions of solid and liquid cultures of *Tgam*.

A) Metabolites obtained from 15-day-old solid cultures on MEA. B) Metabolites from 12-day-old liquid cultures in PDB. The organic extracts from both cultures were separated by column chromatography using ethyl acetate/hexane as solvent. Final purification was carried out by HPLC and the isolated metabolites were characterized by extensive spectrometric and spectroscopic analysis by HRMS, ^{1}H-NMR and ^{13}C-NMR.



Other compounds, such as furan-2,5-diyldimethanol (1.2 mg) and harziandione (2 mg) were identified in MEA cultures. As mentioned, harziandione is a diterpene without known antifungal activity, unlike its analog compounds (Sivasithamparam and Ghisalberti, 1998). Not many diterpenes have been reported in *Trichoderma* (Adelin et al., 2014), and their production is likely restricted to certain strains. The presence of harziandione in solid cultures of *Tgam* brings out the possibility that this fungus is also able to produce other diterpenes, as suggested for *T. harzianum* by Ghisalberti (2000), who hypothesized an elaborate biosynthetic pathway when observing the complexity of this compound.

Regardless 6-PP, tyrosol was the most abundant compound found in PDB cultures (9.38 mg) (Schneider et al., 1996). Tyrosol [2-(4-hydroxyphenyl) ethanol] is a phenolic antioxidant that seems to act as a quorum-sensing molecule able to inhibit competitors, with a role in decreasing the length of the lag phase of growth in fungi (Albuquerque and Casadevall 2012; Chang et al., 2019). Tyrosol has been detected in PDB cultures of *T. parareesei*, playing a role as allosteric modulator in the shikimate pathway (Pérez et al., 2015). Interestingly, overexpression of *tri5* in *T. brevicompactum* led to an increased production of trichodermin, tyrosol and its related metabolite hydroxytyrosol in PDB cultures, while a repression of *tri5* was observed in cultures supplemented with tyrosol (Tijerino et al., 2011a). These results likely link the starting point of the sesquiterpene pathway with this major fungal regulator.

Other compounds found in the major fractions of PDB cultures were melanoxazal (1.03 mg), a melanin biosynthesis inhibitor (He et al., 2015), 4-anisaldehyde (1.54 mg), hydroxy-5 (4-hydroxybenzyl) dihydro -2 (3H)-furanone (1.05 mg). In addition, accumulation of modified intermediaries of the mevalonate pathway in the culture broth, such as mevalolactone (1.21 mg) (Sanghvi and Parikh, 1976), suggests a down-regulation in the biosynthesis of isoprenoids. Furthermore, analysis of filtrates from PDB revealed the absence of trichothecene production in *Tgam* in all the fractions, despite the culture conditions were very similar to those described for trichothecene accumulation in *T. brevicompactum* (Tijerino et al., 2011a), which is in agreement with the bioinformatic data reported in section 3.4, in which the inability of *Tgam* to produce these compounds has been discussed. This last information is of importance in view of using this isolate as bioactive ingredient of commercial products in the biocontrol of FHB on wheat.

CONCLUSIONS & PERSPECTIVES

In the first part of this work, we reported a comparative genomic study focused on the genes and BGCs involved in SMs biosynthesis of 21 isolates belonging to 17 *Trichoderma* species, which represents the most extensive work carried out on this topic and provides an overview of the SMs diversity and potential within the genus.

The phylogenetic and ecologic diversity of the species selected for the analyses enabled to evaluate the impact of these two factors on the genes responsible for SMs and on the BGCs content in *Trichoderma* genus. The ability of *Trichoderma* spp. to produce SMs is strongly dependent on their evolutive history, and species establishing complex interactions with their environment have likely expanded their SMs inventories.

Around half of the SMs genes potentially cooperate in different metabolic pathways in *Trichoderma*. This brings out the complexity of the SMs biosynthetic machinery of these species and the great ability to shape their SMs repertory.

Although *Trichoderma* genomes are rich in SMs synthases, PKSs determine the variability in the SMs inventories among the clades, while production of species/isolate-specific metabolic skills seems to rely in the biosynthesis of bioactive peptides. Given the scarce availability of information about the diversity of RiPPs in *Trichoderma*, future research should be focused on the isolation and characterization of these metabolites and their related biosynthetic genes.

The presence of DMATSs in some genomes indicates that *Trichoderma* spp. have at least the potential to synthesize alkaloids or their derivates under certain conditions, which must be taken into account in case of using these isolates for industrial purposes or as BCAs. This opens an interesting pathway for future research aimed to determine the environmental signals regulating these genes, the products they synthesize, and their impact in the ecology of *Trichoderma*. Thus, screening the factors triggering their activation, combined with metabolic profiles of these fungi and functional gene analysis should be further addressed.

Although polyketides and NRPs are the compounds which likely contribute the most to SMs production in *Trichoderma*, the impressive number of TS-encoding genes we found in the genomes analysed demonstrates that terpenoid biosynthesis has a great impact in the diversity and complexity of SMs in *Trichoderma*.

The limited information available about the diversity within the TS-gene family in *Trichoderma* aimed us to provide a more detailed vision of the TS inventory within this genus. For this purpose, we developed an *in silico* approach that enabled the identification of 15 groups of prenyl transferases, terpene cyclases and chimeric proteins, thus generating the first overview of the terpenoid inventory and the diversity of these enzymes within the genus, and paving the way for future functional analyses on TS genes.

Trichoderma spp. share similar terpenoid potential, but the diversity found within their TSs inventories reflect the evolution of specific terpenoid biosynthetic abilities to adapt better to different physiological and environmental demands. In this context, it would be interesting addressing the study of those TSs that are specific of certain species, such as the HAD-like proteins, or the proteins belonging to uncharacterized groups which are mainly distributed among the *Harzianum* and *Viride* species. Similarly, and since the potential for indole diterpene biosynthesis was found widely distributed across the species, further studies must be focused on providing information about these TSs and the roles of these metabolites in the ecology of *Trichoderma*.

Trichoderma spp. have a huge potential for sesquiterpenoid biosynthesis, and a variety of sesquiTSs contribute in generating clade-specific TSs inventories. Diterpenoid biosynthesis is scarce and not very widespread within the genus, but some species have at least the potential to produce sesterterpenes.

The presence of trichodiene synthase-encoding genes (*tri5*) in non-trichothecene producer species lacking on the *TRI* genes required for the biosynthesis of these compounds, leaves open some questions about the role of *tri5* in beneficious *Trichoderma* spp. Given that in *T. gamsii*, *tri5* is included in a BGC completely different to the clusters found in the genomes of trichothecene-producer fungi, we hypothesize that *tri5* might be involved, in this species, in the biosynthesis of sesquiterpene/s other than trichothecenes, and that the presence of a new *tri5*-containing cluster in *T. gamsii* could imply that *tri5* participates in different metabolic pathways in *Trichoderma*. Why not working in concert with tyrosol as a co-regulator of quorum sensing and primary metabolism, as described in other species of *Trichoderma*? This finding underlines the importance of assessing the genomic context of a given gene across the species, in order to shed light on its possible metabolic role.

The striking genomic potential for terpenoid production of *Trichoderma* spp. found in this work suggests that functional differentiation of gene family members is the driver for the high TS gene numbers of these species. Here, we provide a picture showing that different terpene synthase genes are differentially regulated, a strong indication of different biological functions.

The imposed stress conditions – saline and oxidative stresses, N starvation– negatively regulated TS genes, indicating terpenoid production does not participate in overcoming these stresses. However, production of indole-diterpenes could be enhanced in response to oxidative stress.

On spikes, the presence of the pathogen did not induce prominent changes in TS expression in *Tgam*, although *ts11*, encoding a protein with unknown function, was found slightly upregulated. Evaluation of gene expression at different stages of the interaction will provide more information about the implication of terpenoids when *Tgam* interacts with *Fgra* on wheat spikes.

Root colonization induced a significative reprogramming in terpene biosynthesis in *Tgam*. Repression in indole diterpene biosynthesis seems to occur during root colonization. In addition, the contrasting effects observed in the expression of sesquiTS, as well as the up-regulation of the squalene synthase, suggest that root colonization induce a modulation on sesquiterpene and triterpene biosynthesis in the fungus through FPP as central node.

Expression studies on *tri5* suggest a different regulation of *tri5* in *Tgam* compared to *T. brevicompactum*. The lack of *tri5* expression in the substrates tested suggests that induction of *tri5* in *Tgam* occurs under a more restricted range of conditions than in *T. brevicompactum*. In fact, the strong up-regulation of *tri5* found when *Tgam* colonizes the roots suggests that signals from the plant elicit its expression. This could indicate a possible role in the beneficial interaction the fungus establishes with the wheat roots, a question that must be further addressed. Further research will be directed to obtain *tri5*-disruption mutants of *Tgam* and to assess their behaviour during root colonization, their ability to endophytically colonize the roots, as well as determining the impact on plant defence responses activation, in order to infer a possible role of *tri5* in supporting this beneficial partnership. The lack of *tri5* expression observed in response to wheat spikes was very interesting, as it could suggest that signals from the roots are the responsible of

activating this gene in *Tgam*. Gene expression is usually modulated over time, and further time-course experiments aimed to assess *tri5* expression at earlier stages of the *Tgam*-spike interaction, as well as in minimal media with plant cell-wall components, must be evaluated before hypothesizing a possible specific response of *tri5* to the plant roots.

Recent transcriptomic data obtained in our laboratory resulting from the interaction between *Tgam* and *Fgra* at pre-contact (5 mm distance) revealed that the presence of the pathogen down-regulates the expression of *tri5* in *Tgam*, compared when *Tgam* is in self-interaction (Zapparata et al., 2020, manuscript in preparation). It is necessary to study *tri5* expression in other stages of the interaction with *Fgra*, such as at contact or after the contact of both fungi, both in culture media and on wheat spikes, as well as the behaviour of a *tri5*-deletion mutant in this interaction, in order to determine the relevance of this gene in the relation with the pathogen. Isolation of the metabolite derived from the activation of *tri5* is required to determine the relevance of this gene in the biology of *Tgam*, as well as assessing whether the rest of the genes enclosed in the putative cluster are co-regulated, which would mean the production of a trichodiene-derived compound. Furthermore, similar studies on *tri5* of *T. asperellum* and *T. guizhouense* will help to shed light on determining the role of this gene in beneficial *Trichoderma* spp., or if it participates in different metabolic pathways. In order to deepen into the role of *tri5* in *T. gamsii*, a plasmid for *tri5* silencing in *Tgam* has been constructed and fungal transformation is ongoing.

Metabolic extracts of *Tgam* are abundant in pyrones, which probably contribute to support its biocontrol activity against FHB agents and the induction of the defence responses observed in wheat plants. The presence of harziandione in MEA extracts suggests the possibility that *Tgam* is able to produce other diterpenes. Although culture conditions were similar to those used to induce the accumulation of trichothecenes in *T. brevicompactum* and *T. arundinaceum* described in other works, the absence of these compounds in the fractions extracted from *Tgam* indicates this fungus is unable to produce them, which is in agreement with our genomic data.

In conclusion, we adopted an integrated approach of computational and molecular biology that provides: i) the most complete view of the SMs potential of *Trichoderma*, ii) the diversity of the TS-gene family within the genus, iii) a picture of the regulation of TS genes in different aspects of the ecology of *Tgam*, iv) a step forward to deciphering the regulation of *tri5* in *Tgam* and its relevance in the relation with the plant, and v) open

interesting questions about the biological significance of *tri5* in beneficious *Trichoderma* spp.

Supplementary

Nucleotide sequences of *Tgam* genes

TGAM01_v205548: *β-tubulin*

```
   1  aaaataggtt cacatccaga ccggtcagtg cgtaagtcaa caacctccaa aagtccgcca
  61  tcgaagcggt gtccaggagc tcaccagatt ccacagggta accaaatcgg tgccgccttc
 121  tggcaaacca tttccggcga gcacggtctc gacagcaatg gtatctacaa cggctcttcc
 181  gagctccagc tggagcgcat gaacgtctac ttcaacgagg tatgtaatga taagtgaaaa
 241  agaggacacc tggaggactt tcgcgctgac cctgatggtg caggcctcca acaacaagta
 301  tgttcctcgc gctgtcctcg tcgatctcga gcccggcacc atggacgccg ttcgtgccgg
 361  tcctttcggt cagctcttcc gtcccgacaa cttcattttc ggccagtctt cagccggaaa
 421  caactgggcc aagggccact acaccgaggg tgctgagctc gtcgaccaag tcctcgacgt
 481  tgtccgccgc gaggccgaag gctgcgactg cctccagggc ttccagatca cccactccct
 541  cggtggtggt accggatctg gtatgggaac tctcctgctt tccaagatcc gcgaggagtt
 601  tcccgaccga atgatggcca ctttctctgt tatgccatcc cccaaggtgt ccgacaccgt
 661  cgttgagccc tacaacgcca ctctctccgt ccaccagctg gtcgagaact ctgacgagac
 721  cttttgcatt gataacgagg ctctttacga catctgcatg cgcaccctca agctgaacaa
 781  ccctgcctac ggtgacctga actacctcgt ctctgccgtc atgtcaggca tcaccacctg
 841  cttgcgattc cccggtcagc tcaactctga tctccgcaag ctggccgtga acatggttcc
 901  tttccctcgt ctccacttct tcatggtcgg cttcgctccc ctgaccagcc ccggtgctca
 961  ctctttccgt gccgtcaccg tgcccgaact cacccagcag atgttcgacc ccaagaacat

                                                  TUBF ►
                                       G CTACCTGACC TGCTGCTCTA T
1021  gatggctgct tctgacttcc gcaacggtcg ctacctgacc tgctgctcta tcttgtaagt
1081  aacgatgatg ccttggcgtg gcaagatgtg gtgctaacag acatatgtag ccgtggcaag
1141  gtcgccatga aggaggttga ggaccagatg cgaaacgtgc agaacaagaa ctccacctac

                        ◄ TUBR
                  TAGGGGTT GTTGTAGGTC TGA
1201  ttcgttgagt ggatccccaa caacatccag actgccctct gcgccatccc ccctcgtggc
1261  ctgaagatgt catccacctt cattggtaac tccacctcca tccaggagct gttcaagcgt
1321  gtcggcgagc agtttagcgc catgttccgt cgcaaggctt tcttgcattg gtacactggc
1381  gagggtatgg acgagatgga gttcactgag gccgagtcca acatgaacga cttggtgtct
1441  gagtaccagc aataccagga ggctggtatt gacgaggagg aggagtacga ggatgaggcc
1501  cccatggagg ccgaggagta a
```

<u>TGAM01_v203716: *ts1*</u>

```
  1   atggcctctc cccccttttgg ccaggccgcc ctcattaccg cccttaaagg ccaaaccctc

                                                              TS1F
                                                         T  CAACGCCCAC
 61   cgcatcccca gcctccaagc cctcttcacc tcatggccct ccccacgcct caacgcccac

         TS1F ▶
      TACCCAGA
121   tacccagagc tcgtcgcctt cgccagcgcc accatcctcg aaatcgccgc ggccgcgcct

181   cacctgcgca ttgagcgtcg gcttcgcgac gacattgccc tcctgacgtg cctgctgttc

                                                               ◀ TS1R
                                                                 GACG
241   ccaacggcgc cgcggagacg catcgaggcg ctggtgctgt acatggtgtg gctcgtctgc

            TS1R
      ACCCTGCTGT  GCCA
301   tgggacgaca cggtggacac gaatgagggc gacttggctg ctgatttcgc ccgcgcggag

361   gagtggcgca acaggacgct ggaagtgata aaagtagctc ttcagctccc tgaggaagcc

421   cagcagcgtg gagaggtgga tgctatcaat gcggtgctcg tcagctttgg ccagcgctac

481   tatggcacac atgagagggc tcctgtcgag cagcgccaga ggttgtatga cgaaatcagc

541   atcttcgttc gtgcgtgcgc cacggagcag aggctacgtc ttgacggcgc cattcccagc

601   tttgacgagt acatggtcct ccgagagggc actgtggcag gcggcacgct ctgcgcgttg

661   gttccctacg ccatgcccca gtttgtgccg ccggagctgc tcgacatgcc gcagtttgca

721   gttttccgga agcaggccaa tgtgctgttc ggcctgctca acgacttgat ctccttgaaa

781   aaggaactcg ctggggactg cgtcatcaat gccgtctgta cgctgctgcg gccggagacg

841   cccctggatg aagtcatggc gcagatttac cagaagctgc aggacgcggt gcggcttttc

901   gacaaggcgg ccagggagct gttgggtcaa ttcacggaca atgaccttta ctacgacctc

961   tcagaagagt tgattgatgg gcatagaagt gtagtcaccg gcacgctcga attcatgtga
```

TGAM01_v205612: *ts3*

```
  1  atggccattc ccgaggtatg cttcaatatc caatgactca agtagccccg tccactaaca

 61  attatagaca tccctctcca ccatccgtga agttctttca catttcgtct ccgaggttac

121  cggaggctac aaaatcgatg aaaaggctct tagcttaaca cccattgaca agctgcgtgc

181  tgcagttgtc tcagagttgc agcagtccag cctcaaggac cacctggact acctaaattc

                                                       TS3F ►
                                              GAT TTTACATCAT CCCACCCC
241  aaaagaccag ttcaccatgg cgtgctatgt agcagctgat tttacatcat cccacccccct

301  tgagtaccag gtcgtggttg cccaacttac cattttcttc ttccacgccg aggatatcct

                     ◄ TS3R
                GACTTCAGG AGGCGGTTGA C
361  cgaagagaag cctgaagtcc tccgccaact gcagctcaac gtagcctctg gcaagccatg

421  cggtgaccca gtccttgact ggtacgcccg tgagcttgtg ccccatctgt ggaagcactt

481  tgaccccctt gttgccaaca tgattaccat tgcctgctat gactttatca acggcattgg

541  aatcgagtcc ctgaccaagg acgttgaaat ccaaccccag gcagtggctt tccctgattg

601  gctgcgcttc aaaacggggc tttccccaat gtatgcactg ttggctctag cacgaccctc

661  agatccgaaa ttgtctactg gtggactgga caagtacgtc caggtcatcc ctgatgttat

721  cgtcttcacc aacattgtca acgatgtcat ctccttctac aaggagctcc tggccgagga

781  gaagggcaac tacattgaca tgcgcgccca gagagagaac atcaccgtcc tggaggcact

841  gagcaccctt gctgacgagg gtatccgagt tcgtgagcgt gttctcaagg ttcttgagga

901  tgagcccgag taccgagcca actttgatac atatgccaag ggtatcaccc acttccacac

961  ctcatctccg cgttaccgca tgatgggatt gtttgagaag cagtaa
```

TGAM01_v207465: *ts4*

```
                                                                                    TS4F
                                                                                       A
  61  caagtaacca gaagtcatct gccatcattc gtgatacccт gacaactttt gttgatagga
```

TGAM01_v202085: ***ts5***

```
                                       TS5F ►
                        TGCTTAG  TGTTACCGTC  CTTCTG
   1  atggatcctg aactgcttag tgttaccgtc cttctgccgg acatgttcca aacatttctg

  61  aagcaagaag ctctggtgaa tccatattat gagaaagtca agagggagtc agaagagtgg

 121  ctgagcgagt aagttgtccc atcattgctt atcgatctac ttctgcgcca acgttgactc

                                                ◄ TS5R
                                    CGGGT  TCTACTTCTT  CTTCTAAT
 181  gctctgctag cttttgttca ttcgggccca agatgaagaa gaagattaac aagtgtgact

 241  tttcctactt ttgcgcaatt gcagcgccgt ttgctccccg atcaaatttt cgtacgattt

 301  gtgattgggg caattgggtg agagagccat tatctactca cgttggtcta gaacgtatca

 361  ctaatgaatc tattggacaa tgaacaggtg ttcccgtatg atgacagtaa gtccatatat

 421  ctgtgtataa cgtaacgcca tatgctgagc gaatccagtg tttgacaatg gatgtctccg

 481  agacaagcct gaagaatctc aacgagtcat ggagagtttg atgatgccca tgatggatga

 541  ttcctcttgc tcgagcaaca aagagaggct gcgtattgtg caagctcacg acacagtctt

 601  tcaacgaatt gccgcggtat agtactttta attattttga tagatggcta ttttgagcta

 661  actgaaagct tgacaggaag cgcctacagg tacgatctca taagagcaca ctaaaacaac

 721  tactaacaag cctcatgtaa aaggcgttca aaagagattt gcactggcca tgcaaggcta

 781  ctgccaagga gcgttgatcc agatcgacaa ccactttgca agcaagacgc cctctctgga

 841  agaaatcgtt ctcatcaggc aaaggtcagc aggatgcaag cctctgtacc atcttgtcga

 901  gtatgcgcac ggccttttaa tcccagacga ggtatttgac aatcccatca tccaagaact
 961  ggagtgcctt ggcatggata tggtggccat gtaagaagcc ttgggaaata tctagaacgc

1021  agaagctgat gtttatagat caaacgacat cttatcgtac cgcaaggaac aggtaaggcc

1081  cttttaatca cccccatacc tttgtatagc caacgtactt gctcttaaca cattatatct

1141  aggctgaagg cgtgccgcac aatatggtca cagtctgttg ctcaaatgga atgtcgcagc

1201  aaaaagcctt caatacagta ggcaaactcc ttgagcagcg gtacaagcat tgggacgaag

1261  ctgaagcaag cgtgccgagc tggggtgaag agactgatgc gcaagtccga agatacattg

1321  agggcatcaa atgcgtagtc aaggcaaatc ttaactggag gtga
```

TGAM01_v202927: *ts6*

```
   1 atgggcgcgc tctactattt actgcatccg gcccaattgc ggtccatgat tcaatggtaa

  61 gtgtcatcgt tgagaagaaa ttttgagagg ggtctcagac aagattactt gaacatgctg

 121 ctaactttaa cctctgcagg aagctttggc atgaccctgt tcacagacgc gacccttcga

                                                                  TS6R
                                                             GCAGTTTCT
 181 acgagtcggc gacattatca gaatgctttc gctatttgaa catgacaagc cgcagtttct

            TS6R ►
     CGGCTGTCAT TC
 241 cggctgtcat tcaagaactc aaccacgagc ttttggtccc aatcaccctt ttctacctgg

 301 tactacgtgg cctcgacaca attgaggatg atatgaccat tcccctcgac gtcaaggtcc

                                                            ◄ TS6F
                                                         G GTGCTGTCGT
 361 ctatgctacg aaacttccat gtgacaatgg agcaagatgg ctggcagtac cacgacagca

       TS6F
     TTCTTTTCC
 421 aagaaaagga tgcagagctt ctagagcact ttgatgtcgt gattaccgaa ctcaagaagc

 481 tcaagaagcc ttactacgag atcatcaagg acatgaccgt taagatgggt aacggcatgg

 541 ctgattacgc tcaaaacacc gagatgattg aaaatggtgt acagaccatc gaagaatatg

 601 agctgtactg ccactacgtt gcggggctgg tcggcgaagg cctgacgcgg ctatttgtgg

 661 cttctgagct tgccaacccc aagctggctg agagacccgc cttgaccgag tccatgggtc

 721 agttcctgca gaaaacgaac atcatccgtg atatccatga ggattggggc gaccgccggc

 781 ggtggtaccc aaaggagatt tggagcaagc atgttgacaa gtgggaagac ctgtttgatc

 841 ccaagtatga accgcaggct atcaactgcg tatcagaaat ggtacttgat gcgctgaagc

 901 acgttgaaga ctgtttgttt tacatggccg gcatgaggga tcaaagtgct ttcaactttg

 961 ttgccattcc gcagggcatg gccattgcca ccctggaact atgcttcagg aacccggccg

1021 tgctgaaaag ccatatcaag atcaccaagg gagatgcttg ccaaatcatg ttggagagca

1081 cacagaacct tcaggtgctt tgcgatgttt tccgaagata cgccaggcgc attcaaaaga

1141 agaatgaccc tcgggatccc aacttcttgg caattagcgt acagtgtgcc aaggtacgaa

1201 gaagttattc tcttggagtt gaaccttaac ctttgctgac aaaatcaccc ctctagattg

1261 aacaattcat cgagaccttg ttcccaaagc aagatgcgaa gaagctcacc aaggaggccc

1321 aggagaagca gagacaagaa cccggcatga ccaccggcga aacctttatg ttgattgctg

1381 tcgttctcgg cgttttgctg accatgacgg gcgttatggt aagacatact cacacttatt

1441 ccgcgcccca gatttgactt ccacattgtt caatctaaca ttgtacagct tggcattgct

1501 tggttcttcg gtgcgagatt tgacggcaca ttttctgaca ctgccaaact atggtcgggt

1561 gctggcggtg cgcctccagt attagaccgt gacgaattgt aa
```

TGAM01_v202761: *ts7*

```
   1  atggcaagcg tcttccacaa tctactctct cgaccccagt ttgacatctt gagttttgcg
  61  gatgtcaggc ctcttgcaaa acatgagaaa gcggagataa tcaagtgtct agatgctgca
 121  ctgtcatctt tcctctcaga aatcaacttc tcccactgtt tttccccaag tgattccaag
 181  cttcgtgacg atctgtcgtc ctggagcaaa gaaaatctaa agaaggtctg catttgcgac
 241  gacaaagtct tgaatggaat acttgacgag gctgcagcta ttgtcgagta ttactatccg
 301  catgctcacc atgacactcg tctgcaaatg gctataggtc tggcagcagg gctcactgcg
 361  gatgacggcg tcactcccgc ggcttctagg aaggcttttg caagtttcca ctatcaatta
 421  tggtgcggag tgtctgattc tagaagggat gaatggtcaa ctatgtatct caaagtcatt
 481  agaaatttcg ctcggcattt tggagccaat gatcctcgga tgggaactat tggcgccaat
 541  ggctgggcca gctttgcaga ggcatcattt atggaagaca gcctggcgaa ggccctacca
 601  ccgcatttct cgcaaatcca gccgggtatg gatgatcatg gttgctgccc aaatggattc
 661  gcatacttct tcagaagtct tacagggctc tctggccctc tcatgcttgg tttgttcaaa
 721  cctgccattg attttgaagt cccactagaa tactggatta taagcgtggt gcctcttacg
 781  acgttcatta atctgatcaa tgacctgctg tcattcccca aggagatact ggctggtgag
                                                                 TS7F
                                                   GGTCA TCTCTCCGCA
 841  gcccacaact acatgtctct ccagacacaa gccaagcgcc agagcggtca tctctccgca
        TS7F ►
      TTTCCC
 901  tttccccaga aaggtaattc tgatacgcga tggaactttc gagacacatt atgcgagact
 961  atggaccttt tgtgtgacgc cattcgctct ttggataaag cctttatcca gttccacagg
1021  tccgttgaac ttttgattat ccgaatgtag ggtgtcatac actaatgcgt attttcttag
                                            ◄ TS7R
                            TTGTC GCTACTTACC TCATCACTC
1081  cgtctgtttg aattacaaca atagcaacag cgatgaatgg agtagtgagc tggcaagcca
1141  ggtgtggata gcattcagaa atggcattat ttcttggcat atcaaccgcc caagatatgg
1201  gcttactact ctgagaagca ccttcaccaa agacatatca atcactgagc agtaa
```

TGAM01_v2JGI9898: *ts9*

```
   1 atgccttccg acatcaactt ctcttccaac gccctccctt cgacaacctc tagcagcggt
  61 ctgccctctc cagccctaca ccaggacttt gcatttccca acactcttgg ctcgtccctg
 121 tcgcctctca aacccttcgc tcccggcggc ctcggacccc taatcgaaag cgaggctgag
 181 cgtctaggca agcattccgg atcagagaag ctctcaggac atactggaaa gaggtctttg
 241 cactctgagt cgccgccgca aaggagcagc aagaggataa atcgcggcgt gctcagcatg
 301 cagcaccaga acaatccggg gccgcccccc gacccctcgc ggttcgccaa cgaagacttc
 361 gacttcagtc gccgaggaac atggaacgac gacaaggaga ggatcgtaag gggtccgttt
 421 gactatgtcg tcagccaccc gggcaaggat ttccgtgccc agctcatctc ctccttcaac
 481 gccttcctcg atgtgcccgc cgcgagcctc gccatcatca ccaaggttgt gggcatgctt
 541 cacgagtcct ccctcctcgt cgacgacgta caagattcgt ccgagctccg ccgcggcttc
                                                TS9F ►
                                  AAACCATAA ACTCAGCCAA CTACG
 601 cctgttgccc acaacatctt cggcgtggcc caaaccataa actcagccaa ctacgtatac
 661 tttgctgcct tgcaagagct cgtcaagctc ggcaaccaga atgtcatccc gctgtttgcc
 721 gaggagctcc tcaatctaca ccgcggccaa ggcatggatt tattctggag agacactctc
                                       ◄ TS9R
                              CTC TACCACCCCT TGTTCTGAC
 781 acctgcccca cggaagacga ctacctcgag atggtgggga acaagactgg tggcctgttc
 841 cgcctcggcg tcaagctgat gcaggcggaa tcaagcacca acgtcgacct ggtgcctctc
 901 atcaacctcg ttggcctcat cttccagatc cgcgatgatt acatgaatct ttcttcaagc
 961 gaatatagcc acaacaaggg catgtgtgaa gacctgacgg aaggcaaatt ctccttcccc
1021 gtcattcaca gcatccgcgc cgatcccgtt gatcttcagt tgatcagcat actgaaacaa
1081 aagactacgg atgagcaggt taaacgctat gctgtctcct atatggagtc caagggcagt
1141 ttcgactata cccgtaaagt ggttgagact ctcatcgaga gggcgcgcaa gctagtcgag
1201 ggactagacg ccgggtctgg aaaggccgct ggcatccaca agattctgga taagatggtc
1261 atatcgtcat ga
```

TGAM01_v208552: *ts11*

```
   1  atgcatcgtc ccggcattct aggcaatggc ctgaaaaggc tcgccagcgc gcctcggagc

                                                              TS11R
                                                 TCGGCG ATACTGTTTG
  61  cgtcgcagct tcgcaacggc gacggacgct gacgtcgaat cagctcggcg atactgtttg

       TS11R ►
      AAGCA
 121  aagcagcttc agtatggcgt caatccgttc cccagccctt tttttctttg acatgacaag

 181  gcaactttac cgccttgttg ctttctgcta cagcttctaa cgacttctgc cttatcacag

 241  gaccagcgac tacgatgccc acctcatcca ccgcttcatc ccgccgcccg tccaggacac

                                          ◄ TS11F
                                     CAGGCG GACGGGCTCA AC
 301  ttatgccgcc ctccgcgcat tgaacctcga gctcgtccgc ctgcccgagt tggtgtccaa

 361  tcccaccatt ggcgtcatgc gcatgaagtt ctggcaagag tccattgaca agacgttcgc

 421  cgggcagcct ccccgtgagc caatctgcat cctgctccac cgcggactgc agcagctcga

 481  gctacgggcc ggcagctcgt cgacggcaaa gtccatcaaa ttctgggtct cgcgcctgat

 541  ccgcacgcgc gagaggcaca tggacaatcg gcccttcgcc accctggcca gcctggagga

 601  ctacgccgag cacacgtact ccacgatcat gtatgcgact ttggcatcca tgccgctgcg

 661  gtcaatgcac gtggatcacc tggccagcca tatcggcaag gcgtgcggca tcgtcgctgt

 721  gcttcggggc attcccatcc tcgccgcccc cgcgcagccc gtcaggagcc cttctggcct

 781  cgataccccg agccgcaccc aagccatcct gctgccgctg gacatcatgg ccgaggaggg

 841  tgtcaaggag gaggaggtct tccggcaagg ccccaacgcc cccggactac aggatgccat

 901  cttcaaggtc gccacccgcg ccaatgacca tctgatcacg gcgcgagaga tgctcaagag

 961  gttaaaagcg gggcaggatc caggccacga gtttgagcac cagggcgagg gcgagcacca

1021  gtactttgag gaggagaaca acgacacggc gcgagacata cggcgcggtt tcggggcctt

1081  gttagaagct gtgcccgcag cacaatggtt gttaagcctg gaaaaggcca actttgaccc

1141  gtttgccgtg aagagcagcg ggtggaggct gccgtggggt gtatggcaag ctctgtctaa

1201  ggagaggata tag
```

TGAM01_v209595: *tri5*

```
   1  atggttgagc tcaatgatgt tcctaacgaa gagcagtttc ctcgagctac ttatctacag

  61  gccatggtcc atctgttaga cactgtgagc tacaaggatg agaattttgc agatgaagaa

 121  cgtgtttcat atctgaaaaa ctgctatgca acagctgctg agcatttcgc tcagcctcat

 181  gttcagcaag ctctcaaagt gcctcctaag cgtctggatg ctgctctcaa gactattgtc

 241  ggcatgtgcg tttactcttg gtgccgtgtg agtcaggacg ttatggcaga tctcagcatc

 301  cattacacat atactctact tcttgatgat agcagagaag agcctgccga taccatggtg

 361  acctggtatg aagatcttct gaatgccaaa ccacaagctc atagttggtg gagattggtc

                              TRI5sF ►
                        T TCTGCGTCAT TATGGAGGC
 421  aacgatttca ttcccaatgt tctgcgtcat tatggaggct actgccagat gaacatagtg

 481  agaagcacaa tagattgtaa gcttacatac tttacatctt taattgacca ttagaagcta

 541  acattaccaa atatagtttt ccagggttgt tggattgaac agcacaactt caagggcttt

                       ◄ TRI5sR
                  CGCTGATAGG ACCGAAGGAA
 601  cgcggctcga gcgactatcc tggcttcctt cgccgtataa acgggcttgg tcactgtgtt

 661  ggcgcttcta tctggccgat cgaattactt gacgaagagg aacattttct cgaaatcact

 721  acagccattg cccagatgga aaattggatg gtctggacga acgatctctt ctctttttac

 781  aaggagtact ttacagagcg cgatcagacc tctctagtca acaactatgt ggcgtgcgac

 841  ggcatcacta tggagcaggc tctcgataaa ctctgcaaag atacaatccg cagttctgaa

 901  gagatcatcc aagtgttcca tgacaaagat cccaagatgt acgaaattct cagccgcttt

 961  atccaaggct acatcacttg gcacttgtgc gatgaccgat accgcctcgt cgaggtgtac

1021  gaatccgcag gagacgactc tattgcccaa aggtttaaaa aatatgttga atctgctcgc

1081  cgtgtcggct taatagatcc tgctaagtat tgcaggccgt cagttgccga gctatgccac

1141  cgtaaaatgg caatgcaatc taccagcagg agctgggact ttggcctttt tagacttgtg

1201  agcagtatta cgtctgttga gtggtga
```

Supplementary Table 1A. SM clusters in *Trichoderma*

Distribution of gene clusters putatively involved in SM biosynthesis in *Trichoderma* spp. and *Beauveria bassiana* is shown in different purple shades, depending on the amount of each family type.

Strain	*B. bassiana* ARSEF2860	*T. virens* Gv29-8	*T. pleuroticola* Tr1	*T. pleuroti* TPhu1	*T. harzianum* CBS 226.95	*T. harzianum* TR274	*T. guizhouense* NJAU 4742	*T. atrobrunneum* ITEM 908	*T. afroharzianum* T6776	*T. atroviride* IMI 206040	*T. gamsii* A5MH
t1PKS	9	14	17	19	20	20	18	18	18	12	12
NRPS	15	16	12	13	9	13	12	10	11	10	8
TS	8	10	10	10	9	9	10	7	9	8	8
RiPP	0	0	0	0	1	0	1	0	0	0	0
DMATS	0	0	0	0	0	0	0	0	0	0	0
NRPS-t1PKS	5	6	7	4	7	6	7	3	5	3	2
t1pks/nrps/betalactone	0	0	1	0	1	0	1	1	1	0	0
t1pks/nrps-like	0	0	0	0	0	0	0	0	1	0	0
t1pks/terpene	1	0	1	0	0	0	0	0	1	0	0
nprs/indole	0	1	0	0	0	0	0	1	0	0	0
t1pks/nrps/nrps-like	0	0	0	0	0	0	0	0	0	1	1
t1pks/nrps/terpene	0	0	1	1	0	0	0	0	0	0	0
other	0	0	0	0	0	0	0	0	0	0	0
nrps/betalactone	0	0	0	1	0	0	0	0	0	0	0
t1pks/fungal-RiPP	0	0	1	0	0	0	0	0	0	0	0
nprs-like/terpene	0	0	0	0	0	0	0	0	0	0	0
t3pks/t1pks	1	0	0	0	0	0	0	0	0	0	0
nprs/terpene	0	1	0	0	0	0	0	0	0	0	0

Supplementary Table 1B. SM clusters in *Trichoderma*

Distribution of gene clusters putatively involved in SM biosynthesis in *Trichoderma* spp. and *Beauveria bassiana* is shown in different purple shades, depending on the amount of each family type.

Strain	*T. gamsii T6085*	*T. hamatum GD12*	*T. asperellum TR356 v1.0*	*T. asperellum CBS 433.97*	*T. arundinaceum IBT 40837*	*T. brevicompactum IBT 40841*	*T. citrinoviride TUCIM 6016*	*T. longibrachiatum ATCC 18648*	*T. parareesei CBS 125925*	*T. reesei RUT C-30*	*T. reesei QM6a*
t1PKS	12	14	11	11	13	22	8	9	9	8	9
NRPS	9	14	10	12	9	15	8	6	6	7	6
TS	7	7	11	11	8	8	7	6	7	8	8
RiPP	0	0	0	0	0	0	0	0	0	0	0
DMATS	0	0	0	0	0	1	0	0	0	0	0
NRPS-t1PKS	3	4	5	5	9	8	4	3	4	3	4
t1pks/nrps/betalactone	0	0	0	0	0	0	0	0	0	0	0
t1pks/nrps-like	1	0	0	0	1	1	0	0	0	0	0
t1pks/terpene	0	0	0	0	1	1	0	0	0	0	0
nprs/indole	0	0	0	0	1	1	0	0	0	0	0
t1pks/nrps/nrps-like	0	0	1	1	0	0	0	0	0	0	0
t1pks/nrps/terpene	0	0	0	0	0	0	0	0	0	0	0
other	0	0	0	0	0	0	1	0	1	0	0
nrps/betalactone	0	0	0	0	0	0	0	0	0	0	0
t1pks/fungal-RiPP	0	0	0	0	0	0	0	0	0	0	0
nprs-like/terpene	0	0	0	0	0	1	0	0	0	0	0
t3pks/t1pks	0	0	0	0	0	1	0	0	0	0	0
nprs/terpene	0	0	0	0	0	0	0	0	0	0	0

Supplementary Table 2A. Characterization of TS proteins from *Trichoderma*.

TS proteins found in *Trichoderma* spp. are highlighted in colours according with their conserved domains described in the text and Fig. 7. Number of TS belonging to each group found per specie is shown in the second column, while number of total TSs per species is shown in the second row. Specific portions of the terpenoid inventory are boxed in red.

Strain	TS/group	*B. bassiana* ARSEF2860	*T. virens* Gv29-8	*T. pleuroticola* Tr1	*T. pleuroti* TPhu1	*T. harzianum* CBS 226.95	*T. harzianum* TR274	*T. guizhouense* NJAU 4742	*T. afroharzianum* T6776	*T. atrobrunneum* ITEM 908	*T. atroviride* IMI 206040	*T. gamsii* A5MH
TS-family size	387	16	20	23	23	18	18	19	20	16	18	15
HAD-like	7	0	0	0	0	0	0	0	0	0	1	1
Uncharacterized 1	8	1	0	0	0	0	0	0	0	0	1	0
TRI5	7	1	0	0	0	0	0	1	0	0	0	1
Uncharacterized group 2	14	0	1	0	0	0	0	0	0	0	2	2
Longiborneol synthases	15	1	1	1	1	1	1	1	1	1	0	0
Presilphiperfolan-8β-ol synthases	16	0	1	2	2	1	1	1	2	0	0	0
Pentalenene synthases	22	0	1	1	2	1	1	1	1	1	1	1
Uncharacterized group 3	7	0	0	1	1	1	1	1	1	1	0	0
Uncharacterized group 4	58	0	5	6	4	3	3	3	3	3	3	1
Squalene synthases	22	1	1	1	2	1	1	1	1	1	1	1
GGTases 1	21	1	1	1	1	1	1	1	1	1	1	1
GGTases 2	21	1	1	1	1	1	1	1	1	1	1	1
FTases	21	1	1	1	1	1	1	1	1	1	1	1
Uncharacterized group 5	21	2	1	1	1	1	1	1	1	1	1	1
Oxidosqualene cyclases	21	1	1	1	1	1	1	1	1	1	1	1
Diterpene synthases	8	0	0	0	0	0	0	0	0	0	0	0
GGPP synthases	22	1	1	1	1	1	1	1	1	1	1	1
FPP synthases	31	1	1	2	2	2	2	2	2	1	1	1
Indole diTS	23	1	2	1	1	1	1	1	1	1	2	1
Chimeric-like	22	3	1	2	2	1	1	1	2	1	0	0

Supplementary Table 2B. Characterization of TS proteins from *Trichoderma*.

TS proteins found in *Trichoderma* spp. are highlighted in colours according with their conserved domains described in the text and Fig. 7. Number of TS belonging to each group found per specie is shown in the second column, while number of total TSs per species is shown in the second row. Specific portions of the terpenoid inventory are boxed in red.

Strain	TS/group	*T. gamsii* T6085	*T. hamatum* GD12	*T. asperellum* TR356 v1.0	*T. asperellum* CBS 433.97	*T. arundinaceum* IBT 40837	*T. brevicompactum* IBT 40841	*T. citrinoviride* TUCIM 6016	*T. longibrachiatum* ATCC 18648	*T. parareesei* CBS 125925	*T. reesei* RUT C-30	*T. reesei* QM6a
TS-family size	**387**	16	15	21	20	20	21	18	15	16	17	18
HAD-like	7	1	0	2	2	0	0	0	0	0	0	0
Uncharacterized 1	8	1	1	1	1	0	0	0	1	0	1	1
TRI5	7	1	0	1	1	1	1	0	0	0	0	0
Uncharacterized group 2	14	2	2	2	2	1	0	0	0	0	0	0
Longiborneol synthases	15	0	0	0	0	1	1	1	1	1	1	1
Presilphiperfolan-8β-ol synthases	16	0	0	0	0	0	1	1	1	1	1	1
Pentalenene synthases	22	1	1	1	1	1	1	1	1	1	1	1
Uncharacterized group 3	7	0	0	0	0	0	0	0	0	0	0	0
Uncharacterized group 4	58	1	2	2	2	3	4	3	1	2	2	2
Squalene synthases	22	1	1	1	1	1	1	1	1	1	1	1
GGTases 1	21	1	1	1	1	1	1	1	1	1	1	1
GGTases 2	21	1	1	1	1	1	1	1	1	1	1	1
FTases	21	1	1	1	1	1	1	1	1	1	1	1
Uncharacterized group 5	21	1	1	1	1	1	1	1	1	1	1	1
Oxidosqualene cyclases	21	1	1	1	1	1	1	1	1	1	1	1
Diterpene synthases	8	0	0	1	1	1	1	1	0	1	1	1
GGPP synthases	22	1	1	1	1	1	1	2	1	1	1	1
FPP synthases	31	1	1	1	1	3	3	1	1	1	1	1
Indole diTS	23	1	1	1	1	1	1	1	1	1	1	1
Chimeric-like	22	0	0	2	1	1	1	1	1	1	1	2

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