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Estudo químico e biológico de linhagens de *Trichoderma* da Amazônia

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Tese apresentada ao Programa de Pós-Graduação em Química da Universidade Federal do Amazonas, como parte do requisito para obtenção do título de Doutor (a) em Química, com ênfase na linha de pesquisa Produtos Naturais e Biomoléculas.

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Estudo químico e biológico de linhagens de *Trichoderma* da Amazônia.

KAMILA RANGEL PRIMO FERNANDES

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DEDICATÓRIA

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EPÍGRAFE

"*Não importa o que aconteça, continue a nadar*. "

(Walters Graham; Procurando Nemo, 2003)

RESUMO

Trichoderma é um gênero que desperta a atenção de pesquisadores de diversas áreas, devido à sua diversidade de espécies e importância industrial, médica e biotecnológica. Fungos deste gênero são encontrados em plantas, água e solos de regiões temperadas e tropicais, entre outros ambientes. Entre as substâncias produzidas por espécies de *Trichoderma* têm sido descritas enzimas, peptídeos, policetídeos, especialmente da classe das koninginininas, alcaloides e terpenos. Apesar de toda essa versatilidade, existem poucos estudos a respeito das linhagens de *Trichoderma* isoladas dos diferentes hábitats no Brasil, sobretudo da Amazônia, onde são poucos os grupos de pesquisa que investigam o gênero. Este trabalho de tese teve como objetivo contribuir com a investigação química e biológica de linhagens de *Trichoderma* e foi motivado pelos seguintes questionamentos: 1- Seria possível encontrar similaridade química e biológica entre espécies de *Trichoderma* de origem amazônica? 2- Qual é o nível de correlação entre linhagens da mesma espécie com relação a sua produção metabólica? 3- Seria possível encontrar novos peptaibois em microrganismos amazônicos? 4- Esses peptaibois possuiriam atividade antimicrobiana significante? A fim de responder tais questionamentos foram realizados os seguintes estudos: agrupamento através de análises morfológicas, moleculares (rDNA) e de perfis químicos; avaliação de atividade antimicrobiana dos extratos extra e intracelulares de espécies de *Trichoderma*; caracterização de metabólitos secundários e repetição dos bioensaios para as frações pertencentes a uma espécie em destaque. As trinta e sete linhagens de *Trichoderma* spp. investigadas foram agrupadas em nove espécies: *T. asperellum* (2), *T. harzianum* (9), *T. lentiforme* (12), *T. spirale* (1), *T. koningiopsis* (2), *T. atroviride* (3), *T. asperelloides* (2), *T. afroharzianum* (4) e *T. reesei* (2). Dentre eles, *T. lentiforme* se destacou por ter mais de 30% de todas as cepas estudadas e identificadas. Esta espécie pertence ao complexo Harzianum, cujas linhagens têm alta frequência de isolamento em todo o mundo. No estudo quimiométrico dos perfis químicos por espectrometria de massas das trinta e sete linhagens de *Trichoderma* foi observada uma grande diversidade de agrupamento metabólico, havendo três grupos com maior coerência nesse agrupamento em relação à sua proximidade filogenética: o primeiro grupo formado pela junção de linhagens de *T. asperellum* e *T. asperelloides*, o segundo com *T. atroviride* e o terceiro formado por *T. reesei*. Os extratos intracelular e extracelular de dezessete linhagens foram testados quanto à atividade antimicrobiana. Sendo que sete extratos (cinco linhagens) apresentaram atividade contra *Candida albicans* e *Staphylococcus aureus*. Os extratos T.145 CM e T.221 MY apresentaram atividade bactericida com CIM de 500 $\mu g.mL^{-1}$ para *Enterococcus faecalis*. Destas linhagens, a T.145 (*T. asperelloides*) foi selecionada para a produção de extratos e isolamento de metabólitos secundários por meio de técnicas cromatográficas, seguida de caracterização por espectrometria de massas, bem como testes antimicrobianos e citotóxicos de determinadas frações. Estudos químicos revelaram quinze sequências de peptaibois pertencentes à subfamília das asperelinas, nove inéditas. Por fim, o estudo da cinética metabólica de duas linhagens de *Trichoderma* permitiu comparar os resultados de ambas, promover compreensão de seus comportamentos metabólicos, avaliar indiretamente suas fases de crescimento e as similaridades das fases fisiológicas e a produção de substâncias majoritárias para cada espécie. Neste trabalho avançamos no conhecimento químico e biológico de espécies do gênero *Trichoderma* de origem Amazônica, utilizando diferentes abordagens e análises com trinta e sete linhagens, selecionadas de uma coleção de trabalho.

Palavras-chave: Amazônia, *Trichoderma*, Diversidade biológica, Perfil químico, Quimiotaxonomia, Peptaibois.

ABSTRACT

Trichoderma is a genus of interest to researchers from different areas, due to its species diversity and industrial, medical and biotechnological importance. Fungi of this genus are found in plants, water and soils in temperate and tropical regions, among other environments. Some the substances produced by *Trichoderma* species have been described as enzymes, peptides, polyketides, especially from the class of koninginins, alkaloids and terpenes. Despite all this versatility, there are few studies on *Trichoderma* strains isolated from different habitats in Brazil, especially in the Amazon, where there are few research groups that investigate genus. This thesis work aimed to contribute to the chemical and biological investigation of *Trichoderma* strains and was motivated by the following questions: 1- Would it be possible to find chemical and biological similarities between species of *Trichoderma* of amazonian origin? 2- What is the level of correlation between strains of the same species concerning their metabolic production? 3- Would it be possible to find new peptaibols in Amazonian microorganisms? 4- Would these peptaibols have significant antimicrobial activity? To answer these questions, the following studies were carried out: grouping through morphological, molecular analyzes (rDNA) and chemical profiles; evaluation of the antimicrobial activity of extra and intracellular extracts of *Trichoderma* species; characterization of secondary metabolites and repetition of bioassays for fractions belonging to a highlighted species. The thirty-seven strains of *Trichoderma* spp. investigated were grouped into nine species: *T. asperellum* (2), *T. harzianum* (9), *T. lentiforme* (12), *T. spirale* (1), *T. koningiopsis* (2), T. *atroviride* (3), *T. asperelloides* (2), *T. afroharzianum* (4) and *T. reesei* (2). Among them, *T. lentiforme* stood out for having more than 30% of all strains studied and identified. This species belongs to the Harzianum complex, whose strains have a high frequency of isolation worldwide. In the chemometric study of the chemical profiles by mass spectrometry of the thirty-seven *Trichoderma* strains, a great diversity of metabolic grouping was observed, with three groups with greater coherence in this grouping about their phylogenetic proximity: the first group formed by the combination of *T. asperellum* and *T. asperelloides* strains, the second with *T. atroviride* and the third formed by *T. reesei*. The intracellular and extracellular extracts of seventeen strains were tested for antimicrobial activity. Seven extracts (five strains) showed antimicrobial activity against *Candida albicans* and *Staphylococcus aureus*. The extracts T.145 CM and T.221 MY showed bactericidal activity with MIC of 500 $\mu g.mL^{-1}$ for *Enterococcus faecalis*. From these strains, the T.145 (*T. asperelloides*) was selected for the production of extracts and isolation of secondary metabolites, using chromatographic techniques, followed by characterization using mass spectrometry, as well as antimicrobial and cytotoxic tests of certain fractions. Chemical studies have revealed fifteen sequences of peptaibols belonging to the asperelin subfamily, nine new. Finally, the study of the metabolic kinetics of two strains of *Trichoderma* allowed us to compare the results of both, promote understanding of their metabolic behaviors, indirectly evaluate their growth phases and the similarities of the physiological phases and the production of major substances for each species. In this work, we advance the chemical and biological knowledge of species of the genus *Thichoderma* of Amazonian origin, using different approaches and analyzes with thirty-seven strains, selected from a collection of work.

Keywords: Amazon, *Trichoderma*, Biological diversity, Chemical profile, Chemotaxonomy, Peptaibols.

SUMÁRIO

1 INTRODUÇÃO

Apesar do alto número de artigos publicados sobre *Trichoderma*, existem poucos estudos de linhagens do gênero isoladas da região amazônica, e até mesmo no Brasil, por existirem poucos grupos de pesquisa para este gênero, menores ainda na região norte, com trabalhos não publicados, produtos não registrados legalmente e baixo incentivo financeiro em pesquisas científicas.

Gerar conhecimento científico sobre o gênero *Trichoderma* foi o principal motivo de estudo deste trabalho, pois o gênero desperta curiosidades e estímulos para diversas subáreas de pesquisas. Assim, o presente trabalho visou estudar a química e biologicamente espécies de *Trichoderma* oriundos de diferentes hábitats da região amazônica preservados na coleção do grupo de pesquisa LABMICRA - Laboratório de Bioensaios e Microrganismos da Amazônia, situado na Universidade Federal do Amazonas - UFAM.

Para a melhor compreensão das linhagens selecionadas, iniciou-se com o estudo da variabilidade morfológica, identificação molecular através de seu material gênico (genoma) e suas relações filogenéticas. Seguiu-se a investigação de metabólitos expressos, através do estudo de perfil químico, as correlações e comparações entre as linhagens (quimiotaxonomia) e a busca por espécies com metabólitos bioativos (metabolômica). Por fim, uma espécie foi investigada quimicamente, com elucidação estrutural de moléculas inéditas, contribuindo para aumentar o conhecimento da diversificação dos metabólitos das linhagens de *Trichoderma*.

Este trabalho de tese foi organizado em capítulos no formato de manuscritos científicos na língua inglesa e de acordo com o formato das revistas previamente selecionadas, com seus respectivos materiais suplementares, a fim de evidenciar de forma completa os trabalhos submetidos e a serem publicados.

2 OBJETIVOS

2.1 Geral:

Investigar químicamente e biologicamente linhagens de *Trichoderma*.

2.2 Específicos:

- Identificar por análises morfológicas e moleculares linhagens de *Trichoderma* da coleção de trabalho do grupo de pesquisa;

- Estabelecer por espectrometria de massas os perfis químicos de extratos das linhagens de *Trichoderma*;

- Aplicar a técnica hierárquica de agrupamentos aos dados de perfis químicos das linhagens para avaliar seu potencial para a identificação taxonômica dos microrganismos estudados, em comparação com os resultados com classificações morfológicas e moleculares;

- Investigar a cinética da produção de extratos e respectivos metabólitos de duas linhagens de *Trichoderma*;

- Ensaiar extratos das linhagens de *Trichoderma* quanto à atividade antimicrobiana e outras atividades biológicas;

- Caracterizar metabólitos de uma ou duas linhagens de *Trichoderma*, cujos extratos apresentarem valores significativos para atividade antimicrobiana;

- Submeter aos mesmos ensaios supracitados, frações e metabólitos isolados de uma espécie promissora de *Trichoderma*.

3 REVISÃO BIBLIOGRÁFICA

3.1 A importância dos fungos

Até 1969, os fungos eram classificados como vegetais, sendo reclassificados para reino Fungi através das diferenças entre as características morfológicas, citológicas, bioquímicas e metabólicas. Estes são aclorofilados, uni (leveduriformes) ou pluricelulares (filamentosos), eucarióticos, em sua maioria saprófitas, com parede celular constituída principalmente por quitina e β-glucanos, ultilizando glicogênio e lipídeos como reserva energética (ALEXOPOULUS et al., 1996; LOBATO et al., 2009).

São organismos de extrema importância, não só por suas funções nos biossistemas, mas também por toda influência que têm sobre os seres humanos e atividades relacionadas a eles, provenientes de seus metabolismos, produtos naturais e suas diversas funcionalidades (CUI et al., 2016).

Os fungos são importantes na indústria alimentícia, com produtos fermentados e substâncias utilizadas na produção de alimentos; na área farmacêutica e na saúde, com metabólitos bioativos de alto valor econômico, inovações em medicamentos, processos de biotransformação e atividade enzimática (BORGES et al., 2009; ZHAO et al., 2010; RAMOS et al., 2011; PEREIRA et al., 2017); na área ambiental, relacionados com o ciclo do carbono, com processos de biorremediação, biodegradação e tratamento de efluentes; na ecologia como decompositores e mantenedores do equilíbrio dos ecossistemas; e na área agrícola, como protetores contra patógenos e estimuladores de crescimento (RHODES et al., 2014; BENEVIDES e MARINHO, 2015).

Hawksworth (1991, 2001, 2004c) estimou que o reino Fungi continha aproximadamente 1,5 milhão de espécies, habitando todos os ecossistemas da terra, porém Arnold et al. (2000) e Muller et al. (2007) o estimaram em 8,25 milhões de espécies. De acordo com Abreu et al. (2015), o número de espécies já descritas é de aproximadamente 100 mil, ou seja, considerando a menor estimativa acima, menos de 7% dos fungos já foram descritos e estudados.

Levando em consideração esses dados estatísticos, o reino Fungi ainda é pouco explorado, catalogado e aprofundado em pesquisas científicas, e se extinto geraria o desaparecimento de muitas espécies de animais, plantas e ecossistemas, isso por que são responsáveis por muitos ciclos biológicos. Isto torna essencial estudos voltados para esses

organismos, principalmente em regiões tropicais, onde é mais evidente a extinção de espécies devido ao desmatamento (DA SILVA et al., 2016).

Os trabalhos voltados ao gênero *Trichoderma* geralmente são sobre aspectos morfológicos, taxonômicos, moleculares e de metabólitos secundários, com pesquisadores pioneiros como John Bisset, Garry. J. Samuels, Walter Gams, Walter Jakltish, Al Mahmoud Rifai e entre muitos outros autores; porém, são poucos os trabalhos publicados com linhagens isoladas de origem amazônica.

3.2 O gênero *Trichoderma*

O gênero *Trichoderma* foi descrito pela primeira vez por Persoon (PERSOON, 1794) há mais de 200 anos de acordo com Samuels (1996), a primeira classificação foi proposta por Wittaker (1969), entretanto essa classificação sofreu várias alterações ao longo do tempo, incluindo uma classificação dentro do reino Fungi proposta por Alexopoulus (1996) e fundamentada nas relações filogenéticas que o classificou no Filo Ascomycota. A classificação mais atual encontra-se no banco de dados Genbank (NCBI) que utiliza o index *fungorum* (www.indexfungorum.org) como referência à classificação do gênero, descrito na tabela 1.

Tabela 1. Classificação do gênero *Trichoderma*.

Ordem taxonômica	
Domínio	Eucaryota
Grupo	Fungi/ Metazoa
Reino	Fungi
Filo	Ascomycota
Subfilo	Pezizomycotina
Classe	Sordariomycetes
Subclasse	Hypocreomycetidae
Ordem	Hypocreales
Família	Hypocreaceae
Gênero	*Trichoderma*

Fonte. *Genbank* (NCBI); www.indexfungorum.org; acesso em 12.11.19

O código internacional de Nomeclatura de Algas, Fungos e Plantas (ICN) passou a adotar uma única nomenclatura para as duas fases reprodutivas. No caso de *Trichoderma* a fase sexuada é conhecida como *Hypocrea* (PRADO et al., 2011; ROOSMAM et al., 2013; JAKLITSH et al., 2015). *Trichoderma* foi classificado como gênero através da lei de prioridade e por consenso dos pesquisadores representados pela Subcomissão Internacional de *Trichoderma* e *Hypocrea* (ISTH) (BISSETT et al., 2015).

Com mais de 300 espécies descritas, provenientes de diferentes matrizes como madeira morta, casca e rizosfera, as espécies de *Trichoderma* também são encontradas em ambientes aquáticos (mares ou rios), em plantas (epifíticos ou endofíticos), gramíneas e herbáceas; demostrando assim, a capacidade de sobreviver em diferentes hábitats (SAMULES et al., 2006; HARMAN et al., 2012; ATANOVA, 2014).

Podem apresentar comportamento nutricional saprófita ou biotrófico, com diversidade genética, morfológica e metabólica, e por isso estes vêm sendo descritos quanto aos detalhes moleculares conservados e aqueles que variam entre as espécies. São utilizados para o biocontrole de patógenos vegetais e estimuladores de crescimento. A tabela 2 apresenta alguns outros mecanismos de ação do gênero (MUKHERJEE et al., 2013a; MUKHERJEE et al., 2013b; CONTRERAS - CORNEJO et al., 2013; MARTINEZ - MEDINA et al., 2014; MENDOZA - MENDOZA et al., 2017). Além disso, possuem também espécies (*T. longibrachiatum, T. koningiopsis)* descritas na área médica como patógenos humanos, entre outras relatadas (SANDOVAL et al., 2014).

Tabela 2. Mecanismos de ação de *Trichoderma.*

Mecanismos de ação	Referências
Biocontrole: parasitismo, hiperparasitismo, e micoparasitismo	Hany et al. (2018); Harmam (2000); Jung-Bae et al. (2016); Melo (1998); Stadinik e Betiol (2000); Pascale et al. (2017); Szabó et al. (2012).
Atividade antimicrobiana e competição	Betiniz et al. (2004); Broun et al. (2018); Narendran et al. (2016); Zhang et al. (2018).
Ação enzimática	Cherkupally et al. (2017); Gajera et al. (2012); Marrufo et al. (2016); Souza et al., (2018).
Indução de crescimento e resistência	Harmam et al. (2004a, b); Filho et al. (2008); Lucon (2009); Pascale et al. (2017); Vasquez - Romeiro (2007).
Biodegradação	Adnan et al. (2015); Lipsa et al. (2016); Nykiel - Ssymaska et al. (2018); Szcsepaniak et al. (2015).

Fonte. Baseado em Machado et al. (2012) com atualizações.

3.3 Biologia do gênero: características morfológicas e sistemáticas

Uma das primeiras características macroscópicas de *Trichoderma* foram relatadas e discutidas por Rifai (1969), onde se reconheceu mais de uma espécie de *Trichoderma*, chamando-as de “espécies agregadas”, o que enfatizou o trabalho de Harz (1981) com a primeira delimitação do gênero através das características morfológicas comuns (SAMUELS et al., 1994).

Isolados de *Trichoderma* podem ser reconhecidos pelas características morfológicas, tais como: rápido crescimento em cultura e rede micelial transparente, septada e bastante ramificada. Muitas linhagens não possuem ciclo sexual conhecido, porém o ciclo assexual é caracterizado pela formação de células conidiogénas (brancas, verdes ou amarelas), contidas ou não em estruturas especializadas ou por fragmentação do talo micelial com abudância na produção de conídios, soltos ou campactados em tufos, conforme ilustrado na figura 1 (RIFAI, 1969; BISSET, 1991, 2003; KRUGER E BACCHI, 1995; HARMAN et al., 2004a, b).

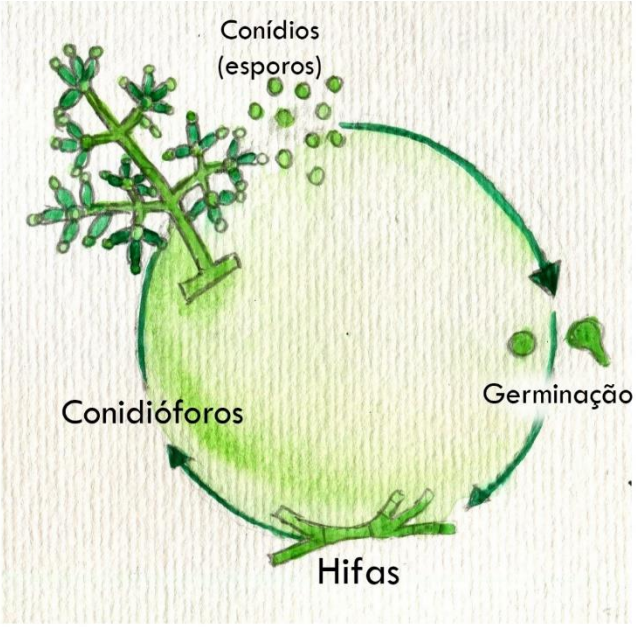

Figura 1. Ciclo de vida assexual de *Trichoderma*.
Fonte. Transcrito de Machado et al. (2012).

A estrutura e o tamanho dos conidióforos são utilizados para diferenciação entre as espécies, com conídios subglobulosos, ovóides e elipsóides ou ainda elípticos cilíndricos, que geralmente são formados em série e acumulado no ápice das filíades com menos de 15 µm de diâmetro, podendo ser lisos ou rugosos (BISSET, 1991; SAMUELS et al., 1999; PERES e MELO 1995). Em condições laboratoriais, linhagens de *Trichoderma* apresentam crescimento rápido em torno de 25°C a 28°C e temperaturas entre 10°C a 17°C diminuem o crescimento micelial de forma significativa (BONFIM et al., 2010).

Até o segundo dia de crescimento apresentam superfície lisa e quase transparente sobre o meio de cultivo, posteriormente tornam-se flocosas ou compactadas com tufos. Podem apresentar halos concêntricos de conídios ou células conidiógenas distribuídas na superfície do meio de cultura, com coloração marrom ou amarelo no dorso do ágar devido a produção e difusão de seus metabólitos; porém a cor da colônia é de acordo com a coloração dos conídios (CORABI - ADEEL et al., 2003; FIPKE et al., 2015).

3.4 Aspectos moleculares e filogenéticos de *Trichoderma*

A identificação de espécies de *Trichoderma* é um trabalho difícil devido as similaridades morfológicas, com poucas diferenciações que variam com minuciosidade, levando a sobreposição das mesmas. Este fato explica por que tão poucas espécies foram descritas ao longo de mais cem anos de estudos do gênero, bem como as identificações errôneas pelos taxonomistas relatadas em trabalhos durante muitos anos (KULLNING et al., 2001; SAMUELS, 2006).

Segundo Druzhinina et al. (2010) a quantidade de espécies de *Trichoderma* morfologicamente reconhecidas (espécies morfológicas) eram bem menores que a de espécies reconhecidas através de genealogia em concordância com o loci de DNA, isto é, as filogenéticas. Esses fungos apresentam ainda sinapomorfia, características morfológicas primitivas de espécies ancestrais que acabam dificultando a identificação. Por tudo isso, para definir espécies de *Trichoderma* com precisão, devem ser somados aspectos morfológicos aos moleculares (SUMMERBELL, 2003; KRÉDICS et al., 2014).

O uso da metagenômica tem favorecido a problemática da identificação entre as espécies e também as várias ferramentas moleculares disponíveis vêm auxiliando no estudo da biodiversidade do gênero. As primeiras tentativas de *primers* utilizados foram projetados para o fragmento ITS1 do *cluster* do gene rRNA (HAGN, 2007). Porém, foi possível verificar que o ITS1 sozinho não era suficiente para algumas espécies, devido o compartilhamento do mesmo alelo, ou seja, insuficiente para distinguir espécies do mesmo clado (CHAVERRI, 2004; DRUZHININA e KUBICEK 2005).

De acordo com VOS et al. (1997); KULLNING et al. (2000) e GOES et al. (2002) algumas técnicas chamadas de “impressão digital” vêm sendo utilizadas em estudos de diversidade do gênero, a fim de realizar o mapeamento genético, análise parental, fornecer características quantitativas, identificar espécies, definir populações e desenvolver marcadores do DNA, tais como *Amplified Fragment Length Polymorphismo* (AFPL), *Restriction Fragment Length Polymorphism* (RFLP) e *Random Amplified Polymorphic* (RAPD), todas baseadas na *Polymerase Chain Reaction* (PCR).

O mais comum é o uso das regiões ITS1, ITS2 e 5,8s do rDNA, com vários estudos taxonômicos e filogenéticos realizados, porém a alta conservação dessa região (casos de análises comparativas), não discriminam espécies muito próximas devido ao baixo

polimorfismo da região (CHAVERRI et al., 2011, 2015). Por isso houve o crescente desenvolvimento de genes como codificadores de proteínas como exemplo, o fator de alongamento (*traslation elongation*), calmodulina, actina e a subunidade II da RNA polimerase, que têm sido utilizados em trabalhos filogenéticos (KULLNING - GRADINGER et al., 2002; SAMUELS, 2004; DRUZHININA et al., 2008).

Vale ressaltar ainda que os avanços em bioinformática tais como, o uso de algoritmos específicos, procedimentos e coeficientes estatísticos, fatores e critérios de alinhamentos de sequências, inferências, uso de programas online como *Trich*OKEY (DRUZHINA e KUBICEK, 2005), *Tricho*BLAST (KOPCHINKIY et al., 2005), *Tricho*CHIT (NAGY et al., 2007) têm feito um diferencial nas análises a nível de espécie e na busca de novas espécies de *Trichoderma* (KRÉDICS et al., 2014).

3.5 Diversidade: ocorrência e distribuição

Estudos voltados para biodiversidade de *Trichoderma* levam em consideração metodologias de coleta das amostras, meios de cultivo seletivo e padrão, forma de isolamento, manutenção e purificação das culturas, identificação morfológica e molecular (CORABI - ADELL et al., 2003; HARMAN et al., 2004c; KRÉDICS et al., 2014).

Trabalhos de diversidade fúngica baseados em metodologias com meio de cultura, enfrentam a problemática de não mostrar a real diversidade dos microrganismos, pois algumas espécies são facilmente isoladas em determinados meios e outras não, com espécies onipresentes (ELAD et al., 1981; PAPAVIZAS e LUMSDEN, 1982; ASKEW e LAING, 1993; WILLIAMS et al., 2003; HOYOS - CARVAJAL et al., 2009; KRÉDICS et al., 2014). A técnica de isolamento mais adequada destes microrganismos é a diluição seriada em meios de cultura seletivos ou semiseletivos, auxiliando na caracterização, isolamento e purificação (DAVET, 1979; CORABI - ADELL et al., 2003).

A quantidade de trabalhos já publicados sobre a diversidade de *Trichoderma* destaca o gênero, pois estes relatam o descobrimento de novas espécies, suas funções, propagação nos mais diferentes ambientes e ocorrência pelo mundo (KRÉDICS et al., 2014). O primeiro trabalho a relatar a biodiversidade de *Trichoderma* ocorreu em 1973 por Danielson e Davey, em uma variedade de solos florestais nos estados do sudeste dos Estados Unidos (EUA) e Washington, identificando linhagens como, *T. harzianum*, *T.*

polysporum, *T. pseudokoningii, T. hamatum, T. koningii* e *T. viride*, sendo as últimas três descritas como as mais abrangentes.

Alguns autores são destaques nos estudos de biodiversidade do gênero, como: Widden-Abitibol (1980) com espécies de *Trichoderma* isoladas no Canadá, Vajna (1983) com espécies isoladas na Hungria, Bisset (1991a, b, c, 1992) com diversidade do gênero na América do Norte e alguns de regiões da Europa, Vasanthakumari e Shivanna (2011) com espécies isoladas na Índia.

Outros autores como Kubicek et al. (2003) descrevem espécies de *Trichoderma* no sudoeste da Ásia, Zahang et al. (2005) relatam espécies deste gênero na China, Tsurumi et al. (2010) com epécies isoladas na Asia, Japão, Mongólia e Vietnã, Abda-Eslan et al. (2010) revelam espécies de *Trichoderma* em isolados na Arábia Saudita, Wuczkowski et al. (2003) com diversidade do gênero na Europa, Hoyos-Carvajal et al. (2009) citam espécies de *Trichoderma* isoladas na Colômbia e regiões adjacentes, Sadfi-Zouaoui et al. (2009) com espécies isoladas em quatro zonas bioclimáticas da Tunísia.

Muitas publicações a respeito da diversidade de *Trichoderma* são de origem do solo, porém há um crescimento de estudos voltados para biodiversidade de isolados de plantas, como endofíticos ou epifíticos, em animais e humanos, como parasitas e patógenos isolados de madeira em decomposição, como exemplo, o trabalho de Jaklitsch e Voglmayr (2015) com 652 espécies de *Trichoderma* isoladas de seis países do sul da Europa.

Em trabalhos mais atuais, como o de Braithwaite et al. (2017), a diversidade e ocorrência de *Trichoderma* na Nova Zelândia é descrita com 320 cepas sequenciadas e quatorze espécies ainda não descritas. O trabalho de Du Plessi et al. (2018) relata a diversidade de *Trichoderma,* com isolados do solo, pertencentes a África do Sul, revelando cinco novas espécies: *T. beinartii, T. caeruleimontis, T. chetii, T. restritum e T. undulatum.*

No trabalho de Sandoval-Denis (2014) as 73 linhagens de *Trichoderma* pertenciam a amostras clínicas de humanos e de animais, sendo muitos isolados do trato respiratório humano. Gazis e Chaverri (2010) estudaram a biodiverdidade do gênero como endófitos de *Hevea brasilienses* (seringueira).

No Brasil estudos de diversidade de *Trichoderma* geralmente estão associados a espécies de plantas importantes para o comércio agrícola, como por exemplo o trabalho de Inglis et al. (2020), relatando cinquenta e quatro linhagens de *Trichoderma* isoladas de

amostras de solo, coletadas de culturas de cebola e de alho e ainda descrevendo duas novas espécies: *T. azevedoi* e *T. peberdyi*.

Poucos trabalhos relatam a diversidade do gênero na região amazônica. Hanada et al. (2003) apontam espécies de *Trichoderma* isoladas de madeira amazônica, Lima et al. (2017) descrevem a diversidade de fungos amazônicos isolados do Rio Solimões, incluindo espécies do gênero *Trichoderma*, Souza et al. (2018) relatam nove linhagens de *Trichoderma* de origem amazônica com alta capacidade celulolítica. Com isso, podemos considerar que há muito a ser estudado sobre a diversidade de *Trichoderma* no Brasil e região amazônica.

3.6 Química do gênero: produção de metabólitos secundários

Existem evidências diretas e indiretas de que ações e benefícios descritos para o gênero *Trichoderma* (biocontrole, biorremediação, antibiose, indução de crescimento entre outros) são decorrentes de seus metabólitos primários e secundários (ZEILINGER et al., 2016). A biossíntese desses metabólitos geralmente envolve vias bioquímicas (rotas biossintéticas) únicas e incomuns, podendo variar através de mecanismos de reação, rearranjos e interferências moleculares para produzir uma diversidade de substâncias. Isso ocorre a partir de moléculas precursoras fundamentais, de origem do mebabolismo primário, como, acetil-CoA, mevalonato, metileritritol fosfato, chiquimato e aminoácidos que compõem os principais esqueletos de moléculas sintetizadas (DEMAIN e FANG, 2000; KELLER et al., 2005; DEWICK, 2009).

Muitas moléculas produzidas por *Trichoderma* são originadas a partir de genes biossintéticos que envolvem enzimas centrais, tais como sintetases peptídicas não ribossomais (NRPS), policetídeos sintase (PKSs), terpeno sintases e ciclases ou enzimas específicas como ainda citocromo P450s, oxidorredutases, metil transferase, entre outras. Alguns casos envolvem genes transportadores e fatores de transcrição (MUKHERJEE et al., 2012a; MURKHERJEE et al., 2013a; ZEILINGER et al., 2016; BANSAL e MURKHERJEE, 2016a, BANSAL e MURKHERJEE, 2016b). Assim são produzidas inúmeras substâncias voláteis e não-voláteis com diferentes funções, incluindo a classe dos peptaibois, de origem não ribossômica, sideróforos, gliotoxina, gliovirina, policetídeos,

terpenos, pironas, compostos similares a dicetopiperazinas e metabólitos de isocianos (MEYER e REUSSER, 1967; BREWER et al., 1987; HERMOSA et al., 2014).

A produção destas substâncias depende de fatores bióticos e abióticos, acarretando em um metabolismo individual distinto e a produção de moléculas diferentes, conforme a diversidade do gênero (KRÉDICS et al., 2014). Assim, o trabalho de Keswani et al. (2013), apresenta um levantamento de metabólitos secundários específicos secretados por dezenove espécies de *Trichoderma* com suas potencialidades de aplicações (Tabela 3). De forma semelhante, Krédics et al. (2014) em seu estudo, apresenta uma tabela com mais de seis espécies de *Trichoderma* de origem marinha, as mais diferentes moléculas isoladas e relatadas em trabalhos publicados entre 2003 a 2012. As estruturas químicas de alguns desses compostos são relatadas na fig. 2.

Tabela 3. Metabólitos secundários de *Trichoderma* e suas aplicações.

Espécie	Substâncias	Aplicações	Referências
T. polysporum ***T. harzianum*** ***T. viride***	**Antraquinonas**: Emodina	Estimulante catártico, inibidor tumoral.	Ali et al. (2004); Huang et al. (2006); Donnelly e Sheridan (1986); Lin et al. (2012); Slater et al. (1967).
T. viride ***T. atroviride*** ***T. hamatum***	**Alcalóides**: Gliotoxina, gliovirina, camptotecina	Antimalárico, imuno supressor.	Pahl et al. (1996); Tanaka et al. (1998); Brian et al. (1945); Pu et al. (2013).
T. harzianum ***T. koningii*** ***T. koningiopsis*** ***T. reesei***	**Policetídeos:** Harzianolidas, T.39Butenolida, Koningininas, Trichodermacetonas, Trichodermatidas, Koningiopisinas, Ácido harzianico	Antifúngico, Indutor de crescimento de plantas, antiofídico.	Cutler et al. (1991a); Ghisalberti e Rowland (1993); Parker et al. (1995a, b); Souza et al. (2008); Vinale et al. (2006); Kobaiashi et al. (1993); Souza et al. (2008); Sun et al. (2008);
T. harzianum ***T. koningii***	**Pironas**: 6 - Pentil - 2H- Piran - 2- ona 6-Pentil 1α-pirona	Antifúngico (fitopatôgeno), crescimento em plantas, aroma de coco.	Claydon et al. (1987); Parker et al. (1997); Vinale et al. (2008).
T. viride	**Amino alquil citrato**: Viridiofuginas A-C	Potencial anticâncer, bactericida.	Harris et al. (1993); Mandala et al. (1997).
T. hamatum ***T. koningii***	**Esteróides**: Viridiol, Viridina	Antienvelhecimento, antifúngico de amplo espectro, composto antineoplásico, antiaterosclerose.	Heraux et al. (2005b); Sakuno et al. (2000); Brian e Mc Gowan (1945); Dodge et al. (1995); Hussain et al. (1975); Tamura et al. (1975).
T. virens	**Sesquiterpenos:** Trichocarotinas A-H, Trichocadininas	Inibição contra plâncton marinho.	Zhen Shi et al. (2018).
T. asperellum ***T. harzianum*** ***T. viride*** ***T. longibrachiatum*** ***T. saturnisporum*** ***T. atriviride*** ***T. polysporum*** ***T. reesei***	**Peptaibois:** Trichotoxinas, Harzianinas, Tricharzianinas, Trichovirinas, Hypomurocinas, Trichokindinas, Trichorzinas, Trichostomaticinas, Trichostrigocinas, Tricholonginas, Trichovorinas, Asperlinas, Trichokoninas, Atroviridinas, Neoatroviridinas, Trichodecenina, Suzucacilinas, Alamecetinas, Paracelsina, Saturniosporinas, Longibrachinas, Trichoginas, Trichosporinas, Cyclosporinas, Pseudikoninas.	Antimicrobianos, atividade citotóxica, antifúngico de amplo espectro, indutor de defesa vegetal.	Landreau et al. (2002); Luo et al. (2010); Ruiz et al. (2007); Ren et al., (2013); Xiao-Yan et al. (2007); Wada et al. (2004); Daniel et al. (2007).

Fonte. Keswani et al. (2013) com atualizações.

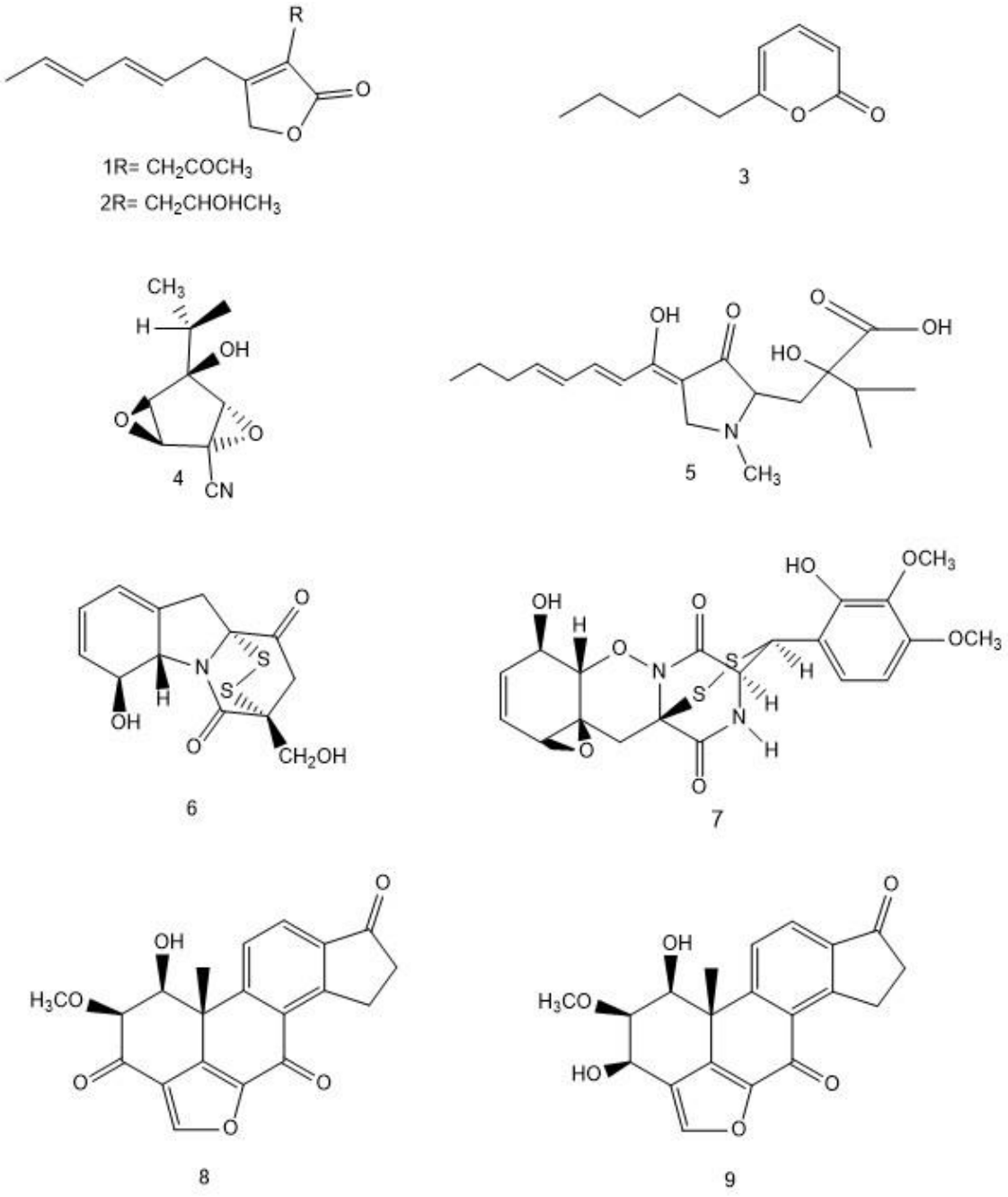

Figura 2. Estruturas químicas de metabólitos secundários de *Trichoderma* spp. 1- T.39 Butenolida; 2- harzianolida; 3- 6 Pentil-1α-pirona; 4- Trichoviridina; 5- Ácido harzianico; 6- Gliotoxina; 7- Gliovirina; 8- Viridina; 9- Viridiol; 10- Trichorzianina.

3.7 Peptaibois: moléculas marcadoras

Os peptaibois são moléculas peptídicas lineares ou cíclicas formadas por 4 a 21 aminoácidos, com peso molecular entre 500 a 2100 daltons; sendo sintetizados por diversos gêneros de fungos, tais como, *Trichoderma*, *Emericellopsis*, *Stibella*, *Gliocadium* e *Acremonium* (ZEILINGER et al., 2007). Possuem características peculiares, como a presença marcante de aminoácidos não-proteinogênicos, tais como α-aminoisobutirato (Aib, A), Isovalina (Iva, B), Etilnorvalina (EtNor, C) e Hidroxiprolina (Hyp, D) (GORRES & RAINES, 2010; DANIEL et al., 2007). (Fig. 3).

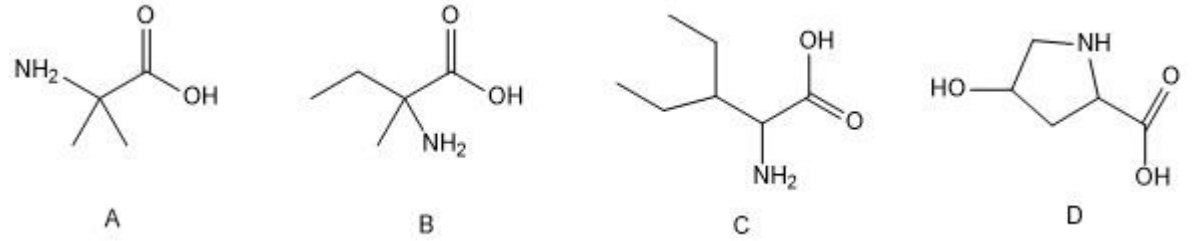

Figura 3. Amonoácidos não-proteinogênicos Aib (A), Iva (B), EtNor (C) e Hyp (D) encontrados em peptaibois.
Fonte: DANIEL et al. (2007); BRITO, (2014).

A nomenclatura "peptaibois" está diretamente ligada as principais características químicas de suas moléculas, formada por **pep**tídeo, **Aib** e amino álco**ol**. Além disso, essas substâncias possuem na extremidade N-terminal um grupo acil (peptaibióticos) ou acetil (peptaibois). No C-terminal álcool geralmente ocorrem fenilalaninol (Phe-OH, E), leucinol (Leu-OH, F), prolinol (Pro-OH, G) e valinol (Val-OH, H) (DANIEL et al., 2007; STOPPACHER et al. 2013). (Fig 4).

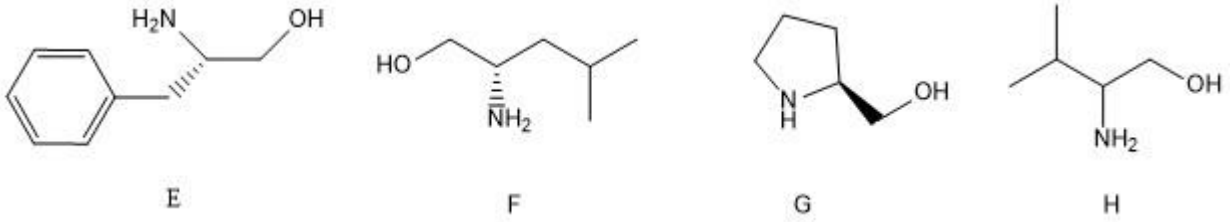

Figura 4. Estrutura de amino álcoois encontrados na extremidade C-terminal de peptaibois: Phe-OH, E; Leu-OH, F; Pro-OH, G; e Val-OH, H.
Fonte: DANIEL et al. (2007); BRITO, (2014).

Estão incluídos dentro da família dos peptaibois as subfamílias de asperelinas, trichotoxinas, harzianinas, trichobrachinas e outras subfamílias (Ren et al., 2013; Ruiz et al., 2007). Entre os peptaibióticos estão os lipopeptaibois (TONIOLO et al., 2001) e lipoaminopeptídeos (DEGENKOLB et al., 2003; BRITO et al., 2014). Muitas pesquisas vêm ganhando força com esta família de moléculas não só pelas funções e aplicações que desempenham, mas pelas estruturas inéditas que continuam a ser encontradas (KESWANI et al., 2013; KRÉDICS et al., 2014; CHUTRAKUL at al., 2008; DEGENKOL et al., 2008; LECLERC et al., 2001; MADDAU et al., 2009; PANIZEL et al., 2013; REN et al., 2013).

O gênero *Trichoderma* é um dos principais produtores de peptaibois, sendo relatadas e isoladas de várias espécies, tais como, *Trichoderma asperellum*, *T. reesei, T. longibachiatrum*, *T. brevicompactrum*, *T. citrinoviridae*, *T. atroviride*, *T. virens*, *T. harzianum* e *T. viride*. A primeira molécula de peptaibois isolada e relatada foi oriunda de *T. viride* nomeada de alameticina F-30 (MEYER e REUSSER, 1967; BREWER et al., 1987; RUIZ et al., 2007). Alguns autores afirmam que os padrões de produção e subfamílias de peptaibois tendem a ser específicos por espécie (DEGENKOLB et al., 2006a, b, 2008a; KUBICEK et al., 2008a).

Os peptaibois não são resultado de transcrição e tradução de um gene, e por isso se encaixam em um grupo de moléculas denominadas peptídeos não ribossomais, ou seja, são produzidos por síntese não ribossômica, que envolvem complexos multi enzimáticos chamados de **S**intetases de **P**eptídeos **N**ão-**R**ibossomais – **NRPSs.**

As NRPSs possuem vários tipos de agentes (complexos) enzimáticos com múltiplos módulos que ligam, ativam e condensam cada aminoácido específico a fim de formar um produto peptídico final (MARAHIEL et al., 1997; SCHWARZER et al., 2003). O número de resíduos de aminoácidos, a organização e a ordem no complexo enzimático reflete no tamanho, complexidade e sequência do peptídeo formado (KLEINKAUF e VON DOHREN 1990; RUIZ et al., 2007). Em resumo, estes complexos multi enzimáticos são formados por um conjunto de módulos, onde cada um possui domínios catalíticos responsáveis pelas etapas de síntese até o produto final (SCHWARZER et al., 2003)

De acordo com Stachelhaus et al. (1999) as especificidades destes domínios podem variar, de modo que alguns sítios de ligação parecem ser altamente específicos enquanto outros não. Como exemplo é o sítio para Aib, que parece aceitar Iva, Val e Ala e desta forma podem gerar uma mistura de peptídeos durante a biossíntese, as sequências

homólogas. No entanto, mesmo com essas variações, três domínios são comuns, domínio A, PCP e C. (MURKHERJEE et al., 2011; STOPPACHER et al., 2013; BRITO et al., 2014).

A biossíntese de peptaibois se inicia com a entrada do aminoácido no domínio A (Adenilação), ativado por adenilação (Fig 5a). No domínio PCP ("Peptidyl Carrier Protein") o aminoácido ativado é ligado a um cofator (HS-4'PP) da proteína PCP, que funciona como um transportador entre os centros catalíticos (Fig 5b). E por fim, no domínio C (Condensação) ocorre a ligação peptídica (Fig 5c) (MAHARIEL & MOOTZ, 1997; MOOTZ et al.; 2002; SCHWARZER et al., 2003).

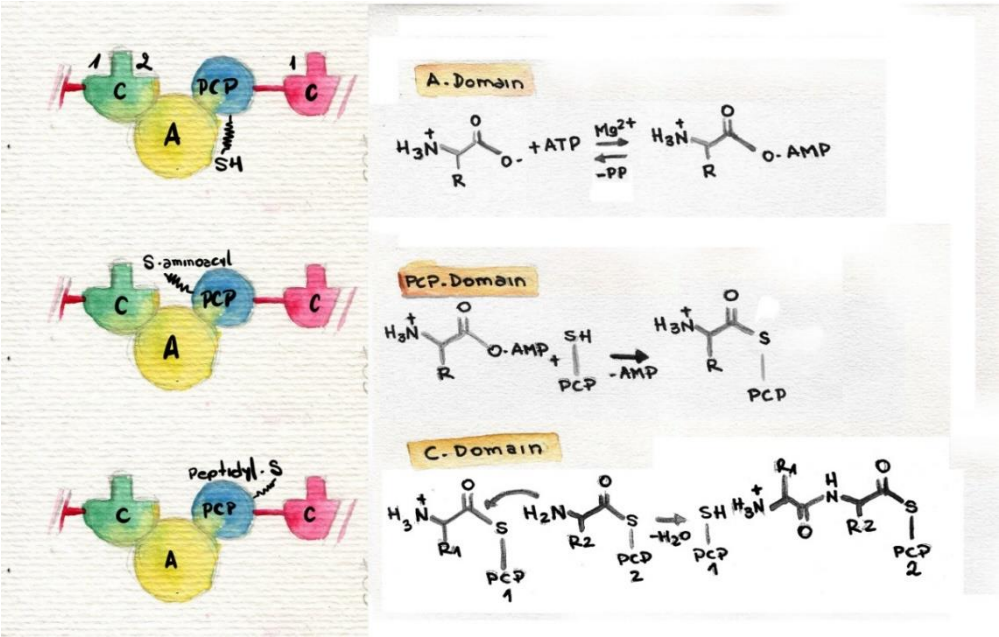

Figura 5 – Biossíntese de peptaibois em uma NRPS
Fonte: Transcrito de SCHWARZER et al. (2003).

Em torno de 317 estruturas de peptaibois já foram depositadas no banco de dados *Peptaibol Database* (http://www.cryst.bbk.ac.uk/peptaibol), agrupados em nove subfamílias diferentes, sendo 190 descritas e produzidas por espécies de *Trichoderma* e *Hypocrea*, (NEUHOF et al., 2007; DEGENKOLB et al., 2008a). Porém, atualmente novos compostos vêm sendo relatados em várias espécies de *Trichoderma* (MUKHERJEE et al., 2011; DEGENKOLB et al., 2012). A tabela 4 mostra algumas moléculas pertencentes as subfamílias de peptaibois.

Tabela 4. Subfamílias de peptaibois.

Peptaibois		Sequência	Quantidade de resíduos
N°	Molécula representante		
Subfamília 1	Alamenticina_F30	UPUAUAQUVUGLUPVUUEQF	17 a 20
Subfamília 2	Trichotoxina A40	UGULUEUUUAUUPLUJQV	14 a 16
Subfamília 3	Emerimicina_II_A	WIQUITULUOQUOUPF	16 a 17
Subfamília 4	Harzianina_HC_I	UNLUPSVUPULUPL	11 a 14
Subfamília 5	Trichoginina_A_IV	UGLUGGLUGIL	7 a 11
Subfamília 6	Ampullosporina C	WAUULUQUAUQLUQL	15
Subfamília 7	LP237_F5	UPYUQQUZQAL	11
Subfamília 8	Clonostachina	UOLJOLJOUJUOJI	14
Subfamília 9	Peptaibolina	LULUF	5 a 7

Fonte: Chugh et al. (2001). http://www.cryst.bbk.ac.uk/peptaibol; acesso: 11 de novembro de 2019.

Tais moléculas já foram descritas com variadas atividades biológicas como: antimicoplasmática, neuroléptica em camundongos, antibacteriana, antifúngicos e antiviral. O que depende das características estruturais das moléculas e disposição dos aminoácidos. Alguns estudos sugerem que sua atividade biológica está relacionada com sua natureza anfipática, permitindo que esses compostos formem canais iônicos nas membranas lipídicas (LUCACIU et al., 1997; BÉVEN et al., 1998).

Os peptaibois geralmente apresentam estrutura helicoidal e anfipática fazendo com que se auto-montem, isso ocorre justamente pela presença do resíduo Aib, que são indutores conformacionais capazes de formar estrutura helicoidal, devido a dupla substituição no carbono alfa por dois grupos alquilos (CASTRO, 2011). Essas estruturas permitem interações com bicamadas naturais ou artificiais, através da formação de poros ou canais iônicos, o que resulta na capacidade de romper as membranas biológicas, causando choque osmótico e vazamento de material intracelular (EL HAJJI et al., 1989; AUVIN GUETTE et al., 1993; RITZAU et al., 1997; REBUFATT et al., 2000; YUN et al., 2000; LECLERC et al., 2001; LANDREAU et al., 2002; BERG et al., 2003; ZELEZETSKY et al., 2005a, b; CHUGH et al., 2001).

São exemplos de interação desses peptídeos com membranas celulares: o trabalho de Mikkola et al. (2012), em que dois peptaibois de *T. longibrachiatum,* com 11 e 20 resíduos de aminoácidos, denominados de trilonginas, capazes de formar canais de Na^+ e K^+, apresentaram voltagem em células espermáticas de javali e quando juntos, atuaram em

sinergia formando canais iônicos; e o trabalho de Rahaman & Lazaridis (2014), em qual foi feita a simulação da dinâmica molecular de canais iônicos formados por alamenticinas, um peptaibol de 20 resíduos de aminoácidos produzido por *T. viride* (Fig 6).

A figura 7 mostra o mecanismo de ação para a entrada de peptídeos em membranas biológicas de acordo com o modelo *Barrel-Stave*, em que o peptídeo anfipático encontra-se na forma helicoidal, posiciona-se paralelamente a superfície da membrana e posteriormente penetra na camada lipídica para induzir a formação de poros que são orientados perpendicularmente ao plano da bicamada (BENCHINGER, 1999; YEAMAN & YOUNT, 2003). Este mecanismo foi proposto inicialmente por Baumann e Muller (BAUMANN & MUELLER, 1974) e confirmado no trabalho de He e colaboradores, He et al. (1996), em experiências com o peptídeo antibiótico alamenticina. Essa versatilidade na interação com as células ilustra a importância farmacológica dos peptaibois e torna necessários estudos sobre a sua biossíntese, manipulação e síntese, permitindo a criação de novas drogas (MOOTZ et al., 2002).

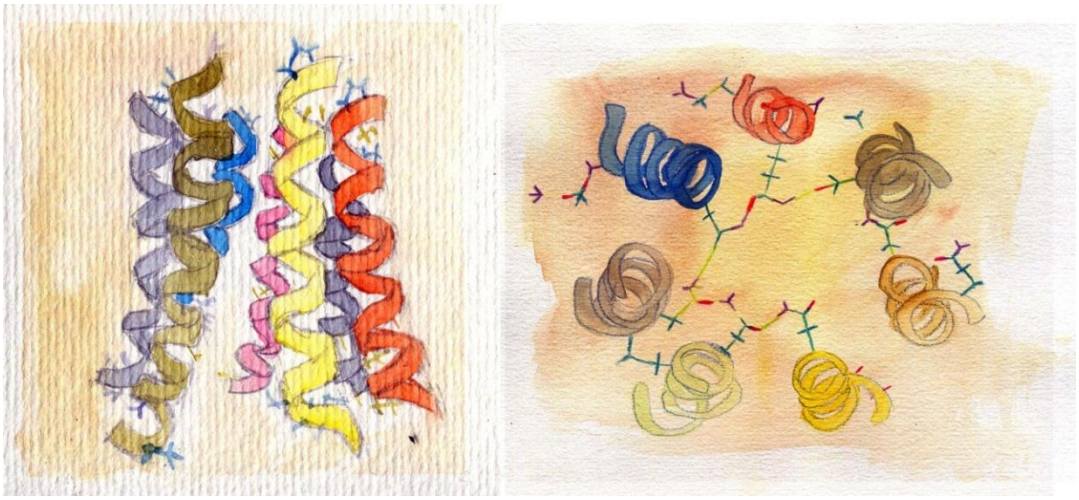

Figura 6. Formação de canais iônicos (imagem lateral e superior), heptâmero de alamentecinas.
Fonte: Transcrito de RAHAMAN & LAZARIDIS, (2014); BRITO, (2014).

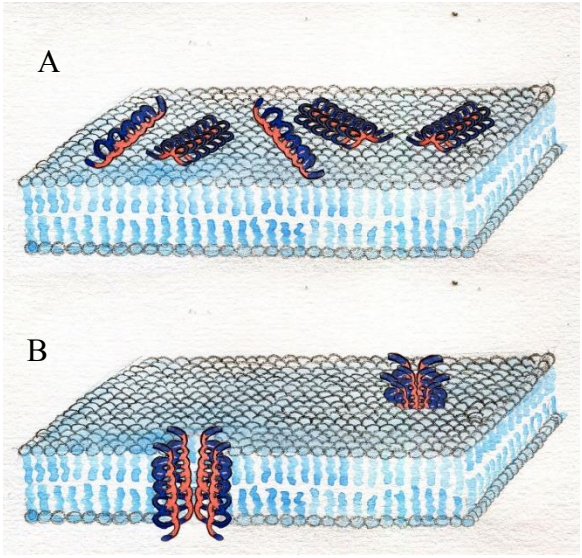

Figura 7. Mecanismo de entrada de peptídeos em membranas biológicas (bicamada lipídica), proposto por *Barrel-Stave*. (A): O azul escuro das hélices representa a face hidrofóbica e o vermelho, hidrofílica. (B): O modelo propõe que as faces hidrofóbicas das hélices voltam-se para fora de forma a estarem aptas a interagirem com as cadeias apolares dos fosfolipídeos.
Fonte: Transcrito de BROGDEN, (2005) e CASTRO, (2011).

Para a formação de poros, uma pequena quantidade de peptídeos pode ser suficiente, pois organizam-se entre si na membrana a ponto de formar um poro capaz de iniciar a ruptura das funções celulares e segundo Hancok e Chapple, não é favorável energeticamente a inserção de um único peptídeo anfipático em uma membrana, visto que o recrutamento de vários monômeros é essencial para a formação de um canal transmembranar estável energeticamente (HANCOK & CHAPPLE, 1999).

3.7.1 Identificação e elucidação estrutural de peptaibois

Geralmente a produção de peptaibois ocorre a partir do crescimento de *Trichoderma* spp. utilizando meios de cultura para obtê-los como metabólitos secundários extracelulares. A extração ocorre através do uso de solventes orgânicos: metanol, butanol e/ou acetato de etila. O isolamento e purificação ocorrem através de diferentes métodos cromatográficos com solventes de média e alta polaridade: metanol, acetonitrila e água. Já a identificação e análise estrutural tem sido realizada principalmente por espectrometria de massas, muitas vezes complementada por análises por RMN (BRITO, 2014; DEGENKOLB et al., 2003; DANIEL et al., 2007).

A espectrometria de massas (MS) permite determinar massas moleculares de peptídeos, proteínas, carboidratos, lipídeos dentre tantas outras substâncias, através da relação entre a massa e a carga (*m/z*) de espécies ionizadas que, via de regra, são separadas em analisadores de diversos modelos, detectadas em um detector específico, e convertidas por um processador de dados em um espectro de massas do tipo *full scan* (BRITO, 2014; FERREIRA et al., 2009). Os métodos de ionização mais utilizados para identificar peptaibois são *electrospray* (ESI) e ionização por MALDI, úteis para estudos químicos de biomoléculas de altos pesos moleculares e compostos lábeis ou não voláteis.

A ionização por fonte *electrospray*, utilizada para o desenvolvimento desta tese, torna a repulsão eletrostática entre as espécies carregadas muito alta, o que promove sucessivas explosões coulômbicas. A transferência dos íons para fase gasosa ocorre quando a tensão superficial da gota não pode mais suportar tantas cargas (limite de Rayleigh) (Fig.8).

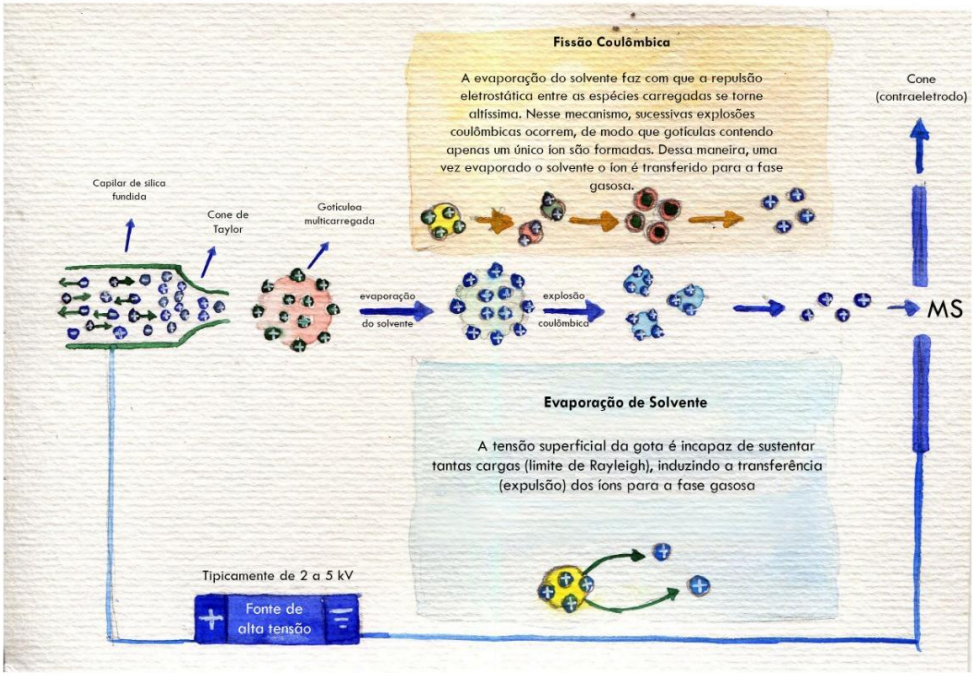

Figura 8. Representação esquemática da ionização por *electrospra*y (ESI)
Fonte: Transcrito de CANTÚ et al., 2015

Após a detecção dos íons correspondentes às respectivas moléculas, íons precursores, a elucidação estrutural dos peptaibois requer a fragmentação dos mesmos, para obter a sequência dos aminoácidos. Essa fragmentação ocorre quando os peptídeos

são direcionados e acelerados para uma região do espectrômetro totalmente preenchida por um gás inerte (hélio, argônio ou nitrogênio), gerando colisão com ele e fragmentando os íons peptídicos em unidades menores de um ou mais aminoácidos.

O conjunto dos íons fragmentos gerados formam os pares de íons complementares e opostos entre si: a/x, b/y, c/z (Fig 9). Os pares de íons b e y, oriundos da clivagem das ligações peptídicas, são os mais comuns, pois necessitam de menos energia para a quebra (TABB et al., 2003; DONGRE et al., 1996; CANTÚ et al., 2008).

O diferencial da identificação de peptaibois está na identificação dos resíduos de aminoácidos não-proteinogênicos, pois como são incomuns, não são inseridos nos *softwares* de busca mais utilizados. Consequentemente, a identificação e elucidação estrutural desses compostos geralmente são realizadas de forma artesanal no espectro. A tabela 5 mostra as massas monoisotópicas dos resíduos de aminoácidos não-proteinogênicos mais encontrados em peptaibois.

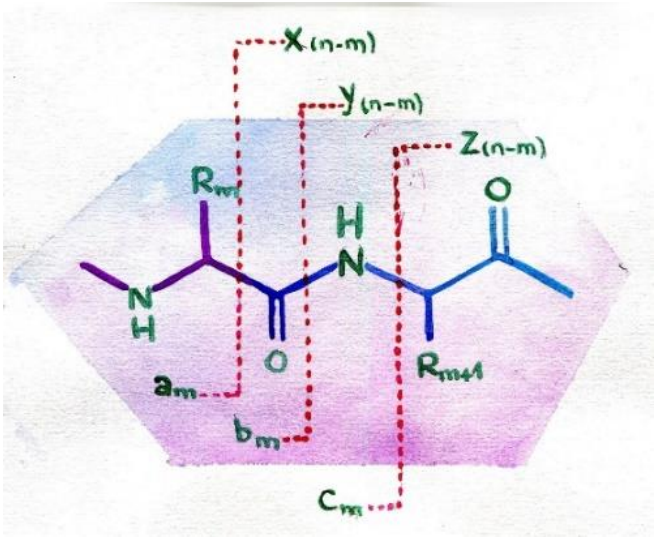

Figura 9. Fragmentos formados devido a transferência de energia para o peptídeo; n: número total de resíduos no peptídeo; m: número de resíduos correspondentes aos íons a, b ou c.
Fonte: Transcrito de CANTÚ et al. (2008).

Tabela 5. Dados de aminoácidos não-proteinogênicos encontrados em peptaibois

Aminoácidos Não-proteinogênicos	Código	Fórmula Molecular (aminoácido)	Fórmula Molecular (resíduo)	Massa Monoisotópica (aminoácido)	Massa Monoisotópica. (resíduo)
α-Aminoisobutirato	U ou Aib	$C_4H_9NO_2$	C_4H_7NO	103,063332	85,052764
N- Etil-L-Norvalina	Z ou EtNor	$C_7H_{15}NO_2$	$C_7H_{13}NO$	145,110275	127,099671
Isovalina	J ou Iva	$C_5H_{11}NO_2$	$C_5H_{11}NO$	117,078979	99,068414
Hidroxiprolina	O ou Hyp	$C_5H_9NO_3$	$C_5H_7NO_2$	131,058244	113,047679
Grupo C-terminal	**Código**	**Fórmula Molecular**		**Massa monoisotópica**	
Prolinol	P-OH	$C_5H_{11}NO$		101,084064	
Valinol	V-OH	$C_5H_{13}NO$		103,099714	
Leucinol	L-OH	$C_6H_{15}NO$		117,115364	
Fenilalaninol	F-OH	$C_9H_{13}NO$		150,091889	
Prolina	P ou Pro	$C_5H_9NO_2$		115,063329	
Hidroxiprolina	O ou Hyp	$C_5H_9NO_3$		131,058244	
Hidroxiprolinol	Hypol	$C_5H_{11}NO_2$		117,078979	
Grupo N-terminal	**Código**			**Massa monoisotópica**	
Acetil	Ac			43,01839	

3.8 Asperelinas: pequenas sequências de peptaibois

As asperelinas são uma subfamilia de peptaibois, com nove a dez resíduos de aminoácidos, N-terminal acetilado e C-terminal amino-alcool que geralmente é o prolinol. O Aib é o aminoácido não-proteinogênicos com maior frequência. As primeiras asperelinas foram descobertas em 2009, relatadas por Ren e colaboradores. Eles elucidaram seis sequências através de técnicas espectroscópicas (RMN e ESI-MS/MS) com as asperelinas A a F, a partir da fermentação (simulando condições marinhas) de um fungo marinho, *Trichoderma asperellum,* isolado de sedimentos da ilha Antártica dos Pinguins, Islândia. Eles puderam verificar os deslocamentos químicos e correlações dos resíduos, padrões de fragmentação e estereoquímica levógira (REN et al., 2009).

Em 2013, Ren e colaboradores relataram 35 sequências de asperelinas (Tabela 6) proveniente do mesmo fungo que trabalharam em 2009, denominadas de asperelinas A a Z_{13}, acrescentando 29 sequências inéditas, duas diferenciadas, com C-terminal hidroxiprolinol e prolina (Z_{12} e Z_{11}) não descritos anteriormente. Este trabalho abordou a identificação dos aminoácidos por método de Marfey e os diferentes padrões de fragmentação, com detalhamento nos espectros de massas (Ren et al. 2013). A título de exemplo, na figura 10 apresenta-se a identificação da asperelina G, por fragmentação do tipo y (modo negativo) e tipo a (modo positivo do íon do tipo $[M+Na]^+$).

Chen e colaboradores em 2013 relataram as asperelinas A, C, D, E, F, G e H, moléculas oriundas de outra linhagem de *T. asperellum,* isolada de sedimentos marinhos coletados da ilha Fujian, na China. Essas asperelinas foram determinadas por métodos espectroscópicos, difração de raio-x e derivação química. Foi o primeiro estudo que relatou asperelinas com C-terminal acelitado com estrutura cristalina (CHEN et al., 2013). Destaque-se que as asperelinas G e H de Chen et al. (2013) são diferentes das asperelinas G e H de Ren et al. (2013), possivelmente devido a publicações simultâneas.

Brito e colaboradores em 2014 foram os últimos autores a relatar asperelinas, as descritas anteriormente asperelinas A e E, elucidadas por MALDI-TOF e oriundas também de *T. asperellum*, porém de uma linhagem isolada de solo brasileiro. Até o ano de 2020, não foram encontrados relatos de asperelinas isoladas de outras espécies de *Trichoderma*.

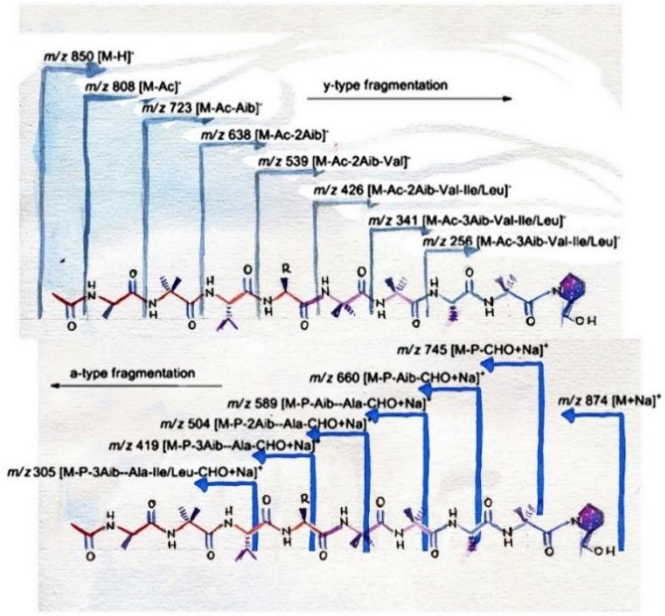

Figura 10. Fragmentação do tipo y (modo negativo) e a (modo positivo), para identificação da asperelina G.
Fonte: Transcrito de REN et al. (2013).

Normalmente, as asperelinas encotram-se em misturas complexas, gerando uma árdua tarefa para o isolamento, às vezes impraticáveis segundo Ren et al. (2013). Quando o isolamento ocorre o rendimento é ineficiente para realizar ensaios biológicos, sendo necessário alta produção no processo de fermentação para uma obtenção quantitativa, por esse motivo, alguns trabalhos sugerem modelação, simulação estrutural e testes *in sílico* de tais moléculas, como por exemplo o trabalho de Castro et al. (2011)

Entre os poucos estudos de bioatividade das asperelinas, destaca-se o trabalho de Chen et al. (2013), com avaliação de atividade citotóxica a células de leucemia P388 e Hl-60 e células de melanoma H375, das asperelinas A, C, D, F, G e H, através do método colorimétrico MTT, o qual é fundamentado na avaliação do metabolismo celular para determinar a atividade. Todas apresentaram valores de IC50 maiores que 50 μM, ou seja, apresentaram alta toxicidade frente a essas células.

Tabela 6. Asperelinas relatadas até o presente trabalho.

Asperelina	$[M+H]^+$	MW	$[M-H]^-$	Sequência	Referências
A	937	936	935	Ac-Aib-Aib-Val-Aib-Ile-Aib-Aib-Ala-Aib-Prolinol	Ren et al. (2009); Ren et al. (2013); Chen et al. (2013); Brito et al. (2014).
B	923	922	921	Ac-Ala-Aib-Val-Aib-Lxx-Aib-Aib-Ala-Aib-Prolinol	Ren et al. (2009); Ren et al. (2013).
C	923	922	921	Ac-Aib-Aib-Val-Aib-Ile-Aib-Ala-Ala-Aib-Prolinol	Ren et al. (2009); Ren et al. (2013); Chen et el. (2013).
D	923	922	921	Ac-Aib-Aib-Val-Aib-Val-Aib-Aib-Ala-Aib-Prolinol	Ren et al. (2009); Ren et al. (2013); Chen et al. (2013).
E	953	952	951	Ac-Aib-Aib-Val-Aib-Ile-Aib-Aib-Ser-Aib-Prolinol	Ren et al. (2009); Ren et al. (2013); Chen et al. (2013). Brito et al. (2014).
F	951	950	949	Ac-Aib-Val-Val-Aib-Ile-Aib-Aib-Ala-Aib-Prolinol	Ren et al. (2009); Ren et al. (2013); Chen et al. (2013).
G	852	851	850	Ac-Aib-Aib-Val-Ile-Aib-Aib-Ala-Aib-Prolinol	Ren et al. (2013).
H	852	851	850	Ac-Aib-Val-Aib-Ile-Aib-Aib-Ala-Aib-Prolinol	Ren et al. (2013).
G	995	994	993	Ac-Aib-Aib-Val-Aib-Ile-Aib-Aib-Ser-Aib-Prolinol+Ac	Chen et al. (2013).
H	979	978	977	Ac-Aib-Aib-Val-Aib-Ile-Aib-Aib-Ala-Aib-Prolinol+Ac	Chen et al. (2013).
I	909	908	907	Ac-Ala-Ala-Val-Aib-Lxx-Aib-Aib-Ala-Aib-Prolinol	Ren et al. (2013).
J	909	908	907	Ac-Ala-Aib-Val-Aib-Lxx-Aib-Ala-Ala-Aib-Prolinol	Ren et al. (2013).
K	909	908	907	Ac-Aib-Ala-Val-Aib-Lxx-Aib-Ala-Ala-Aib-Prolinol	Ren et al. (2013).
L	909	908	907	Ac-Aib-Aib-Ala-Aib-Lxx-Aib-Aib-Ala-Aib-Prolinol	Ren et al. (2013).
M	909	908	907	Ac-Ala-Aib-Val-Aib-Val-Aib-Aib-Ala-Aib-Prolinol	Ren et al. (2013).
N	909	908	907	Ac-Aib-Ala-Val-Aib-Val-Aib-Aib-Ala-Aib-Prolinol	Ren et al. (2013).
O	909	908	907	Ac-Aib-Aib-Val-Aib-Val-Aib-Ala-Ala-Aib-Prolinol	Ren et al. (2013).
P	909	908	907	Ac-Aib-Aib-Val-Ala-Val-Aib-Aib-Ala-Aib-Prolinol	Ren et al. (2013).
Q	923	922	921	Ac-Aib-Aib-Val-Aib-Lxx-Ala-Aib-Ala-Aib-Prolinol	Ren et al. (2013).
R	923	922	921	Ac-Ala-Val-Val-Aib-Lxx-Ala-Aib-Ala-Aib-Prolinol	Ren et al. (2013).
S	923	922	921	Ac-Aib-Aib-Val-Aib-Lxx-Aib-Aib-Ala-Ala-Prolinol	Ren et al. (2013).
T	923	922	921	Ac-Aib-Aib-Val-Aib-Lxx-Aib-Aib-Ala-Ala-Prolinol	Ren et al. (2013).
U	923	922	921	Ac-Aib-Ala-Val-Aib-Lxx-Aib-Aib-Ala-Aib-Prolinol	Ren et al. (2013).
Z	925	924	923	Ac-Aib-Aib-Val-Aib-Val-Ala-Aib-Ser-Aib-Prolinol	Ren et al. (2013).
Z_1	925	924	923	Ac-Aib-Aib-Val-Aib-Val-Aib-Ala-Ser-Aib-Prolinol	Ren et al. (2013).

Z_2	937	936	935	Ac-Aib-Val-Val-Aib-Lxx-Aib-Aib-Ala-Ala-Prolinol	Ren et al. (2013).
Z_3	937	936	935	Ac-Ala-Val-Val-Aib-Lxx-Aib-Aib-Ala-Aib-Prolinol	Ren et al. (2013).
Z_4	937	936	935	Ac-Aib-Aib-Val-Aib-Leu-Aib-Aib-Ala-Aib-Prolinol	Ren et al. (2013).
Z_5	939	938	937	Ac-Aib-Aib-Val-Aib-Lxx-Aib-Ser-Ala-Aib-Prolinol	Ren et al. (2013).
Z_6	939	938	937	Ac-Aib-Aib-Val-Aib-Lxx-Ala-Aib-Ser-Aib-Prolinol	Ren et al. (2013).
Z_7	939	938	937	Ac-Aib-Aib-Val-Aib-Lxx-Aib-Ala-Ser-Aib-Prolinol	Ren et al. (2013).
Z_8	939	938	937	Ac-Aib-Ala-Val-Aib-Lxx-Aib-Aib-Ser-Aib-Prolinol	Ren et al. (2013).
Z_9	939	938	937	Ac-Aib-Aib-Val-Aib-Val-Aib-Aib-Ser-Aib-Prolinol	Ren et al. (2013).
Z_{10}	939	938	937	Ac-Aib-Aib-Val-Aib-Lxx-Ser-Aib-Ala-Aib-Prolinol	Ren et al. (2013).
Z_{11}	951	950	949	Ac-Aib-Aib-Val-Aib-Lxx-Aib-Aib-Ala-Aib-Prolina	Ren et al. (2013).
Z_{12}	953	952	951	Ac-Aib-Aib-Val-Aib-Lxx-Aib-Aib-Ala-Aib-Hypol	Ren et al. (2013).
Z_{13}	967	966	965	Ac-Aib-Val-Val-Aib-Lxx-Aib-Aib-Ser-Aib-Prolinol	Ren et al. (2013).

3.9 Koningininas: moléculas marcadoras

As koningininas são policetídeos isolados principalmente de *Trichoderma koningii, T. koningiopsis, T. neokoningii, T. aureoviride e T. ovalisporum*. Foram relatadas até recentemente as koningininas de A a V (CUTLER et al. 1999; DANG et al., 2010; ZHOU et al., 2014; LANG et al., 2015; HU et al., 2016; LIU et al., 2016; SHI et al., 2020; BIASETTO et al., 2020).

Elas são sintetizadas por enzimas especializadas e complexas da classe dos policetídeos sintases (PKSs), que catalisam a construção de moléculas a partir dos precursores propionil-CoA e metilmalonil-CoA, através de reações de condensação e descarboxilações sucessivas (KELLER et al., 2005; ZEILINGER et al., 2015; ZEILINGER et al., 2016). A importância farmacológica das konigininas é destacada por suas propriedades antimicrobianas, anticancerígenas, imunossupressoras e antiofídicas (PARKER et al., 1995; SOUZA et al., 2008; DEWIC, 2009; KHOSLA, 2009; HERMOSA et al., 2014). Na tabela 7, e figura 11 encontram-se dados e estruturas de algumas koningininas já relatadas.

Tabela 7. Dados de koninginininas de A a V.

Koninginina	Massa exata	$[M+H]^+$	$[M+Na]^+$	Fórmula molecular	Referências
A	284,198760	285,206585	307,188530	$C_{16}H_{28}O_4$	Almassi et al. (1991); Cole et al. (2003); Cutler et al. (1989); Dang et al. (2010).
B	282,183110	283,190934	305.172880	$C_{16}H_{26}O_4$	Cole et al. (2003); Cutler et al. (1991); Liu e Wang (2001).
C	284,198760	285,206585	307,188530	$C_{16}H_{28}O_4$	Cole et al. (2003); Parker et al. (1995b).
D	298,178025	299,185850	321,167795	$C_{16}H_{26}O_5$	Cole et al. (2003); Dunlop et al. (1989); Liu e Wang (2001).
E	282,183110	283,190934	305,172880	$C_{16}H_{26}O_4$	Cole et al. (2003); Parker et al. (1995a); Ghisalbert et al. (1993).
F	298,178025	299,185855	321,167795	$C_{16}H_{26}O_5$	Liu e Wang (2001).
G	302,209325	303,217150	325,199095	$C_{16}H_{30}O_5$	Cutler et al. (1999).
H	298,178025	299,185850	321,167795	$C_{16}H_{26}O_5$	Tarawneha et al. (2013).
I	296,162375	297,170200	319,152145	$C_{16}H_{24}O_5$	Zhou et al. (2014).
J	296,163775	297,170200	319,152145	$C_{16}H_{24}O_5$	Zhou et al. (2014).
K	312,157990	313,165115	335,147060	$C_{16}H_{24}O_6$	Zhou et al. (2014).
L	280,167460	281,175285	303,157230	$C_{16}H_{24}O_4$	Lang et al. (2015).
M	280,167460	281,175285	303,157230	$C_{16}H_{24}O_4$	Lang et al. (2015).
N	280,167460	281,175285	303,157230	$C_{16}H_{24}O_4$	Liu et al. (2016).
O	280,16460	281,175285	303,157230	$C_{16}H_{24}O_4$	Liu et al. (2016).

P	298,178025	299,185859	321,167795	$C_{16}H_{26}O_5$	Liu et al. (2016).
Q	312,193675	313,201500	335,183445	$C_{17}H_{28}O_5$	Liu et al. (2016).
R	340,188590	341,196415	363,178360	$C_{18}H_{28}O_6$	Hu et al. (2016).
S	296,162375	297,170200	319, 152145	$C_{16}H_{24}O_5$	Hu et al. (2016).
T	328,188590	329,196415	351,178360	$C_{17}H_{28}O_6$	Biasetto et al. (2020).
U	210.125595	211,133420	243, 123190	$C_{12}H_{18}O_3$	Biasetto et al. (2020).
V	370,235540	371,243365	393, 225310	$C_{20}H_{34}O_6$	Shi et al. (2020)

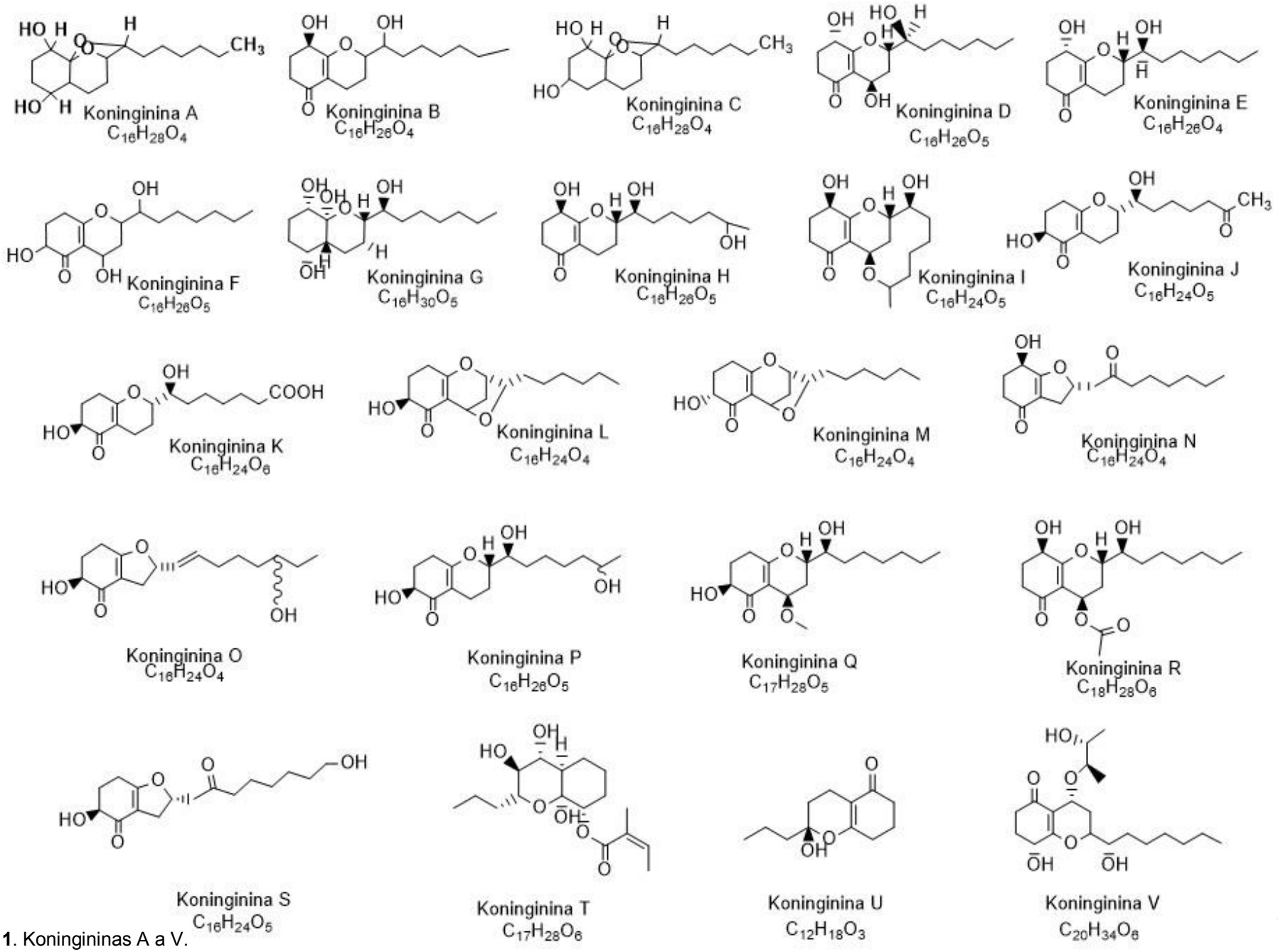

Figura 11. Koningininas A a V.

3.10 Quimiotaxonomia

Para identificação de espécies de *Trichoderma* são necessários somatórios de resultados morfológicos e moleculares, mas existem obstáculos que dificultam esse trabalho taxonômico, como limitações morfológicas, pequeno número de especialistas, e identificações incorretas através das sequências de DNA, que embora seja uma técnica precisa e poderosa não está isenta de falha humana. Estima-se que aproximadamente 40% das sequências de banco de dados de espécies de *Trichoderma* foram identificadas erroneamente ou permanecem não identificadas quanto à espécie (KULLNING et al., 2001; DRUZHININA et al., 2006; DAEJUNG et al., 2010).

Como método complementar a quimiometria é uma técnica que permite a aplicação de ferramentas matemáticas e estatísticas à química, através do tratamento, interpretação e previsão de dados químicos (KOWALSKI, 1975; MALINOWSKI, 1991). Associada a quimiometria, a quimiotaxonomia permite a utilização dos dados gerados a partir dos metabólitos para diferenciar espécies de fungos, da mesma forma que tem sido utilizada para estudos de classificação taxonômica de espécies de plantas. Diversas pesquisas quimiotaxômicas têm sido desenvolvidas, mas particularmente para fungos é muito pequena a proporção de espécies quimicamente estudadas (FRISVAD, 2008).

A quimiotaxonomia está ligada com a crescente evolução de equipamentos e programas computacionais e como consequência tem sido utilizada em inúmeras abordangens, como desenvolvimento de métodos analíticos de desreplicação e estudos de fragmentação para detecção e elucidação de novas moléculas (MONGRAND et al., 2001; SKALTA et al., 2001; DEGENKOLB et al., 2008).

Poucos são os estudos sobre quimiotaxonomia de *Trichoderma,* alguns desenvolvidos e relatados por Degenkolb et al. (2008); Thrane et al. (2001); Respinis et al. (2010); porém apenas o estudo de Daejung et al. (2010) identificou as seguintes substâncias: sorbicilinol, bisorbicilinol, trichodermanona C, ácido harzianico, ácido demeteharzianico, ácido homoharzianico, koningininas B, D e E e determinou o porquê dos diferentes agrupamentos entre as espécies de *Trichoderma*.

Os perfis de metabólitos de uma ou mais amostras é frequentemente usado para detectar compostos desconhecidos em misturas complexas, porque torna possível a observação simultânea de metabólitos de diversos tipos. Para isso, são utilizadas técnicas

como cromatografia líquida (LC), espectrometria de massas (MS) e ressonância magnética nuclear (RMN). A mais utilizada e aplicada para detecção específica de metabólitos desconhecidos em complexos químicos e biológicos é a espectrometria de massas, pela sua robustez e sensibilidade (POPE et al., 2007; KIM et al., 2009).

De acordo com os trabalhos de Svendsen & Frisvad (1994); Smedsgaard & Frisvad (1996; 1997); Assis et al. (2011); De Respinis et al. (2010) e Agustini (2014), a análise da composição de extratos de fungos por injeção direta em um espectrômetro de massas no modo *electrospray* (ESI-MS) ou MALDI, permite uma razoável classificação quimiotaxônomica de centenas de linhagens de fungos das mais variadas origens, bem como facilita a identificação de novas espécies e a escolha de linhagens para o isolamento de diversas substâncias inéditas ou de interesse.

Esses estudos de perfis químicos são normalmente associados a quimiotaxonomia, ou seja, utilizam abordagens metabolômicas que incluem análises estatísticas, como a análise de componentes principais (PCA) e agrupamento por métodos hierárquicos (HCA) (RESPINIS et al., 2010; DAEJUNG et al., 2010).

Segundo Ferreira (2016), a PCA é um método para projetar os dados multivariados em espaço de dimensão menor, reduzindo a dimensão original sem afetar os resultados; o que facilita a visualização e interpretação, já que na quimiotaxonomia o montante de variáveis pode atingir com facilidade a casa dos milhares, e a HCA foi desenvolvida para estudar a semelhança entre organismos de diferentes espécies, gênero, família entre outros, sendo um método de reconhecimento de padrões naturais e comportamento entre as amostras com base no seu perfil químico multivariado.

CAPÍTULO 1

Diversidade e quimiotaxonomia de *Trichoderma* spp. da Amazônia

O presente capítulo está relacionado ao primeiro, segundo e terceiro objetivos desta tese e foi elaborado como manuscrito a ser submetido ao periódico Fungal Diversity, um jornal internacional de micologia (https://www.springer.com/journal/13225). Na sequência, apresentamos um resumo em língua portuguesa, o manuscrito em língua inglesa e os materiais suplementares, os quais viabilizam uma análise mais completa do que se pretende publicar.

Resumo

Objetivo: apresentar a diversidade de uma amostra de linhagens de *Trichoderma* obtidas de diferentes hábitats tropicais, em sua maioria no Amazonas, avaliadas através de análises morfológicas, moleculares e quimiotaxonômicas.

Metodologia: as linhagens foram obtidas da coleção de trabalho do Laboratorio de Bioensaios e Microrganismos da Amazônia da Universidade Federal do Amazonas (LABMICRA/UFAM) e avaliadas por meio de análises macro e micromorfológicas, utilizando nesta segunda as microscopias óptica e eletrônica de varredura. As análises filogenéticas foram realizadas utilizando sequências de regiões do espaçador transcrito interno (ITS) do rDNA e porções do gene do fator de alongamento da tradução 1-α (*TEF1*-α). Dados de perfis químicos obtidos por espectrometria de massas foram utilizados para obter agrupamentos entre as linhagens por HCA, bem como para prever o potencial das linhagens para a produção de peptaibois e outros metabólitos.

Resultados e discussão: as 37 linhagens foram classificadas em nove espécies de *Trichoderma*: *T. asperellum* (2), *T. harzianum* (9), *T. lentiforme* (12), *T. spirale* (1), *T. koningiopsis* (2), *T. atroviride* (3), *T. asperelloides* (2), *T afroharzianum* (4), *T. reesei* (2). Uma linhagem de *Clonostachys rosea* foi utilizada como linhagem de referência (out group). *T. lentiforme* e *T. harzianum* se destacaram com mais de 50% de todas as cepas estudadas e identificadas. O trabalho realizado indica pela primeira vez uma alta diversidade de *Trichoderma* spp. da Amazônia, particularmente dos fungos endofíticos. Os dados quimiométricos derivados dos perfis químicos por espectrometria de massas, relativos a seus metabólitos intracelulares e extracelulares, mostraram várias correlações químicas e semelhanças intraespecíficas entre as linhagens e espécies de *Trichoderma*.

Conclusões: as análises polifásicas permitiram verificar a alta diversidade de espécies e a complexidade morfológica e metabólica dessa pequena amostra de linhagens do gênero *Trichoderma.* Algumas linhagens mostraram sinais de boa adaptação a suas hospedeiras, sinalizando serem endófitos verdadeiros. Também foram destacadas linhagens com potencial para a produção de peptaibois. Uma linhagem foi apontada com uma possível nova espécie. A complexidade quimiotaxonômica dos perfis químicos e respectivos dendogramas de HCA sinalizou uma relação de dependência cepa-específica com os micro-ambientes de origem das diversas linhagens.

Contribuição e impacto do estudo foram gerados novos dados sobre a diversidade genética e química de espécies do gênero *Trichoderma*, a maioria oriunda de plantas da Amazônia. Esses dados ajudarão a compreender melhor a complexidade e rica diversidade genética e química desse gênero em clima tropical. Este é o primeiro trabalho polifásico de fungos do gênero *Trichoderma* associados a ambientes da Amazônia.

Palavras-chave: Fungos endofíticos; *Trichoderma*, Análises filogenéticas, Perfis químicos.

Diversity and chemotaxonomy of Amazonian *Trichoderma* spp.

Abstract

Trichoderma is a genus of interest to researchers from various areas, due to its species diversity, biotechnological importance, and its applications. The present study aimed at the grouping and characterization of 37 *Trichoderma* spp. through morphological, molecular, and chemotaxonomic analysis of the fungus obtained in different tropical habitats. The lineages were evaluated with concatenated phylogenetic analysis through the sequences of internal transcribed spacer (ITS) regions, and portions of the translation elongation factor 1-α gene (*TEF1*-α). From the 37 strains of *Trichoderma*, nine species groups were obtained, namely: *T. asperellum* (2), *T. harzianum* (9), *T. lentiforme* (12), *T. spirale* (1), *T. koningiopsis* (2), *T. atroviride* (3), *T. asperelloides* (2), *T. afroharzianum* (4), *T. reesei* (2). A *Clonostachys rosea* strain was used as out group. *T. lentiforme* and *T. harzianum* stood out with more 50% of all strains studied and identified. The work done indicates the high diversity of *Trichoderma* spp. from the Amazon. The chemometric data showed the chemical correlations and intraspecific similarities among the species of *Trichoderma* through its intracellular and extracellular metabolites, as the main ions and in common and chemical proximity between species of the same clade.

Keywords: Endophytic fungi, *Trichoderma*, Philogenetic analysis, Chemical profile.

1 Introduction

The genus *Trichoderma* has been gaining prominence over the years, due to its high level of adaptability in different habitats, rapid growth in culture mediums, species diversity on several continents and its production of metabolites and enzymes of biotechnological interest (Harman et al., 2012; Gupta et al., 2014; Atanova, 2014; Du Plessis et al., 2018). The genus was first described by Persoon (Persoon, 1794) more than 200 years ago, according to Samuels (1996), and the first classification was proposed by Wittaker (1969). Currently, more than 300 species have been reported (Samuels, 2006; Marik et al., 2019).

Over the years, morphological and genetic features have been discussed and studied in order to describe morphologically indistinguishable species, cryptic species, frequent species, complex species, clades, and new species of *Trichoderma*, which have been revealing with the evolution and combination of techniques for diversity studies, such as morphological and molecular techniques, new markers, microculture, microscopy, mass spectrometry, and phylogeny techniques (Chaverri et al., 2004; Druzhinina and Kubicek, 2005; Druzhinina et al., 2010; Samuels et al., 2010; Krédics et al., 2014; Chaverri et al., 2015).

Some species are associated with the production of marker molecules, which is why an increasing number of studies of molecules produced by the genus have been increasing, with more than a thousand molecules already described (Hermosa et al., 2014; Zeilinger et al., 2016). The reports of production and chemical elucidation of these metabolites are generally associated with their biotechnological potential, such as in antibiotics, antifungals, anticancer treatments, plant growth stimulators, protection against pathogens in plants, among others (Shuster et al., 2010; Keswani et al., 2013; Gupta et al., 2014; Liu et al., 2016).

In addition to the use of morphological and molecular techniques, chemotaxonomy has been used as an alternative, investigative and complementary approach to differentiate *Trichoderma* species, by using secondary metabolites to distinguish and determine the genus and then group its species (Thrane et al., 2001; Daejung et al., 2011). Chemotaxonomy permits the evaluation of similarities among individuals through a sum of information, whereby the large amount of chemical data obtained from the mass spectrometry can be analyzed using hierarchical cluster analysis (HCA) (Ferreira, 2016).

Despite the availability of all of these approaches, there are few studies on the strains of *Trichoderma* isolated from habitats in the Amazon region. Chemotaxonomic studies of the genus are also few in number, and few patents have been registered, which is probably due to the lack of financial incentives for scientific research (Machado et al., 2012). As a result, the present study sought to analyze genotypic characteristics and phenotypic expressions of *Trichoderma*, together with the results of the chemical evaluation of its secondary metabolites, intracellular and extracellular of 37 strains of *Trichoderma* and one out group from different habitats and, as a result, shows the biodiversity of the genus *Trichoderma* in the Neotropical regions of Brazil.

2 Materials and Methods

Reagents

All solvents, including methanol (MeOH), ethyl acetate (EtOAc), and ethanol (EtOH), were HPLC-grade for extraction, and were purchased from Tedia (Mexico City, DF, Mexico). Formic acid (FA), (99%) was provided by Acros Organics (Morris Plains, NJ, USA). Glutaraldehyde and potassium phosphate for the analyses using scanning electron microscopy were acquired from Sigma Aldrich (San Luiz, Missouri, USA). Glycerol for the

conida suspension was from Synth (Diadema, São Paulo, Brazil). Ultrapure water (18.2 MΩ.cm) was obtained from a Milli-Q gradient system (Millipore, Milford, MA, USA).

Origin of fungal isolates

The 38 strains used in this study are deposited into the work collection of the Laboratory of Bioassays and Microorganisms of the Amazon (LABMICRA), located at the Federal University of Amazonas (UFAM), and were isolated from various tropical environments, most as endophytic strains. Brazilian SISGEN registration: A39C76B.

Lineage reactivation

The selected strains were reactivated in Petri dishes containing 20 mL potato dextrose agar culture medium (PDA) at 28° C for 2 to 5 days of growth. Viable and pure colonies were inoculated in the center of new plates with PDA under the same conditions. After eight growth days, conidia suspensions were prepared in 20% glycerol to achieve the turbidity of the McFarland No. 6 standard (Souza et al., 2004). These suspensions were used in all subsequent experiments, as follows.

Fungi morphological analyses

For macromorphological analyses: shape, texture, colors of the front and back, type of border, and pigmentation, the fungi were cultivated on PDA into Petri plates. For micromorphological analyses, they were grown by the microculture technique and their microscopic structures (conidiophore, filiade, chlamydospore, and conidia) were observed under an optical microscope (Zeiss), with a magnification of 200x and 400x. The mean values of the diameter, length, and width of the structures were described (Samuels, 1996; Chaverri and Samuels, 2011).

Analysis by scanning electron microscopy (SEM)

The fungi were cultured for 3 to 5 days at 28° C on microscope slides in Petri dishes and too isolated in Petri dishes, both containing PDA culture. The medium and slides were covered with 3% (v/v) glutaraldehyde in 0.05 M potassium phosphate buffer at pH between 7.2 and 7.4. After 24 h, the cultures were washed with 0.05 M potassium phosphate buffer 10 times and subjected to gradual dehydration for 10 minutes with EtOH (25, 50, 70, 80, and 99% v/v). Small blocks of culture medium containing fungal material were sectioned

or were used whole microscope slides containing fungal material, and left to dry naturally for 30 min. The samples were stuck to double-sided carbon tape on alluminium stubs and visualized in a scanning electron microscope (SEM) model JSM IT500HR (JEOL Ltd., Japan). High vacuum images were made at voltages of 10 and 15 kV, and with different magnifications (Martinelle et al., 2010).

DNA extraction

The fungi were grown on 50 mL of potato dextrose 0.2% yeast broth extract (PDY) for 48 to 72 h at 28° C under constant stirring at 120 rpm. The mycelium of each strain was separated from the medium by vacuum filtration and triturated with silica for cell lysis. Then, the genomic DNA was extracted with a Quick - DNA Fungal/Bacterial Microprep kit (Zymo Research).

Polymerase chain reaction (PCR) and sequencing

For each fungus-rDNA sample, the internal transcribed spacer (ITS) region was amplified using the primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATG 3') (White et al., 1990). Also, portions of the translation elongation factor 1-α gene (*TEF1*-α) were amplified with the primers EF-1αF (ATGGGTAAGGARGACAAGAC) and EF-1αR (GGARGTACCAGTSATCATGTT) (Carbone and Kohn, 1999; O'Donnell et al., 1998). For the ITS region, the amplification was performed using a total volume of 25 µL: 19 µL of sterile water, 2 µL each primer (20 pmol) and 2 µL of DNA (20 ng/µl), along with freeze-dried reagents from the kit: 200 µM of each dNTP, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, (pH 9.0 at room temperature), and 1 unit of *Taq* DNA polymerase (GE Healthcare, Ilustra™). Thermocycling (Veriti 96-well Thermal Cycler, Applied Biosystems) was used and the program for the ITS region consisted of initial denaturation at 94º C/4 min; 40 cycles of 94º C/4 min, 55º C/2 min, and 72º C/2 min; and final extension at 72º C/10 min.

For the gene *TEF1*-α, the amplification was performed using a total volume of 25 µL: 17.3 µL of sterile water, 1 µL each primer (5 pmol) and 2 µL of DNA (50 ng/µL), 1 µL of dNTPs (10 mM), 2.5 µL of $MgCl_2$ (15 mM in buffer 10 X) and 0.2 µL of *Taq* DNA polymerase (5U/µL). For thermocycling of *TEF*-1-α gene, the method consisted of initial denaturation at 96º C/3 min; 40 cycles of 94º C/30 sec, 55º C/1 min, and 72º C/1.30 min; and final extension at 72º C/10 min.

The amplification products were separated by electrophoresis on 1.5% agarose gel and stained with 0.3 µg/mL ethidium bromide in 0.5 X Tris/borate/EDTA (TBE) pH 8.0 buffer (0.1 M Tris-HCl, 0.1 M boric acid, and 2 mM ethylenediaminetetraacetic acid - EDTA). The detection of the amplification products was performed using the L-PIX program (Chemi Molecular Imaging, Image software). 1 kb Plus DNA Ladder (Invitrogen) was used to estimate the size of the amplicons.

The PCR products were purified using polyethylene glycol (PEG) 6000 precipitation according to the protocol described by Schmitz & Riesner (2006). The purified PCR products were sequenced using the dideoxy chain terminator method, using Big Dye 3.1 (Applied Biosystems) and a capillary DNA sequencer (ABI 3500 Genetic Analyzer, Applied Biosystems/Hitachi-Embrapa Amazônia Occidental, Manaus, Brazil). The sequences were assembled and edited with MEGA 7.0 (Molecular Evolutionary Analyses, Pennsylvania, USA). The sequences were deposited in GenBank.

Phylogenetic analyses

The concatenated phylogenetic analysis was done with the sequences from the transcribed internal spacer (ITS) regions and the 1-α translation elongation factor (*TEF1-α*) gene of 35 *Trichoderma* strains and the outgroup strain. The *TEF1-α* sequences of two *Trichoderma* strains were not included since they were not satisfactory. The Bayesian inference was based on the model adopted by Nylander (2004) who used PAUP*4 and Mr Model test v2. The most appropriate evolutionary model found for explaining the Bayesian inference was SYM + G for the *TEF1-α* region and GTR + I for ITS. All sequence data were considered, and the analysis was performed for ten million generations, with the first 25% of trees were discarded using the MrBayes v. 3.6 tools available from CIPRES Science Gateway. Posterior probability (PP) and tree topology were visualized with Figtree v.1.1.2, according to Rambaut (2009). The final tree was derived from the maximum likelihood (ML) analysis with maximum parsimony (MP) and posterior probability (PP) values included.

Fungal extracts of the *Trichoderma* strains

For the production of the extracts in micro-scale of the 37 *Trichoderma* strains, 2 µL of conidia suspension in concentration No. 6 on the McFarland scale were inoculated in triplicate in tubes (12 x 1.5 cm) containing 3.0 mL potato dextrose 0.2% yeast broth (PDY) (Souza et al., 2004). The cultivation of the strains was carried out in the static method at

28° C for 20 days. 50 µL of the cultivated medium of each sample was removed and stored in microtubes; the mycelium separated from the medium by filtration was extracted with 2 mL of AcOEt/MeOH (1:1): after the addition of the solvents, it was vortexed for 2 min, stored for 24 hours at room temperature, and filtered. The extracts were then transferred to penicillin flasks for evaporation, resuspended with 1 mL of MeOH, stirred for complete solubilization, re-filtered with cotton, transferred to microtubes, and stored at 4 ºC.

Analyses of the chemical profiles of the *Trichoderma* strains

The samples were analyzed using 20 µL of each stored solution (medium or mycelial extract) that was added to 500 µL of MeOH (analysis solution) by direct insertion in a mass spectrometer (Thermo Scientific) operating with an *electrospray* source (ESI) and ion-trap analyzer (model LCQ Fleet), in the range of *m/z* 100 to 2000 in the positive mode, using MeOH with 0.5% formic acid as the carrier solvent. Mass spectrometer conditions: positive ionization; *spray voltage*, 5 kV; *sheat gas*, 10 arb; *auxiliary gas*, 5 arb; *sweep gas*, 0 arb; *capillary temperature*, 200 °C; *capillary voltage*, 40 V; *tube lens* 115 V; *mass range*, *m/z* 100 a 2000*;* colision gas He; scan 3 seconds.

Statistical analysis

For the multivariate analyses, the mass spectra of the fungal extracts were converted into an Excel spreadsheet (Microsoft), containing the relative intensity of the nominal peaks of each triplicate and respective average. Ions with intensity below 5% in relation to the base peak of each sample were considered noises. All analyses were performed using the R version 3.6.3 program. The data of the average intensities of the *m/z* values were previously transformed into a two-dimensional matrix for each lineage, and were standardized by the “scale” function (“base” package) for the construction of the hierarchical cluster analysis (HCA). For the HCA, the standardized data were converted into a Euclidean distance matrix followed by clustering using the Ward’s method. The HCA analyses were calculated using the “FactoMineR” package, and the dendrograms were generated using the “Factoextra” package.

3 Results

Fungi identification and diversity

The identification of the 37 *Trichoderma* strains, 94.7% isolated from different habitats of the Amazon Region, such as plants (endophytic), soil, water, and decomposing wood (saprophytes) (Table 1), were performed through the molecular analyses. The most of this identification was performed by molecular multigene analyses, based in 1780 characters, including gaps: 708 corresponded to ITS and 1072 to *TEF1-α*, that resulted in the concatenated tree presented in the Figure 1. Only the strains T.219 and T.220 were identified as *T. atroviride* by similarity of its ITS sequences with the GenBank data. T.219 identification also used pieces of *TEF1-α*, since this gene presented fails for this fungus. The T. 531 strain presented a deletion of 30 base pairs (bp) in the large intron of the *TEF1-α* region, a fail observed for other lineages of *T. afroharzianum* (Figure 2).

Under this parameter, they were distributed in nine groups of species. These species and their quantities were: *T. asperellum* (2), *T. harzianum* (9), *T. lentiforme* (12), *T. spirale* (1), *T. koningiopsis* (2), *T. atroviride* (3) *T. asperelloides* (2), *T. afroharzianum* (4), and *T. reesei* (2). One *Clonostachys* strain selected as out group was identifyed as *C. rosea* (Table 1). The macro and micromorphological characteristics of the *Trichoderma* strains are at the Table S1 Figure S2 and S3 of the Suplementary Material.

Relationship between the fungi origin and clustering

Among the 37 *Trichoderma* strains, 25 are of endophytic origin (seven species), distributed in the Harzianum complex and Viride clade; seven strains are wood decomposers (three species) distributed in the Harzianum complex and Reesei clade; three strains originate from the soil (three species) and were distributed between the Harzianum complex and Viride clade. Finally, the *C. rosea* and *T. reesei* strains are from aquatic origin. Together with the *T. spirale* T.263 strain, the two *T. reesei* strains represent the Reesei clade in this study (Table 2).

It is possible to observe that most fungal strains from the same vegetal resource are from the same species: from *Rollinia* sp. only *T. lentiforme* was identified, as endophytic fungi; from *Scleronema micranthum*, *T. harzianum* was dominant (6 strains), but one *T. spirale* strain was also identified, as wood decomposers; from *Victoria amazonica*, 3 *T. afroharzianum* strains and one *T. asperelloides* strain were identified, as endophytic fungi;

finally, from *Murraya paniculata* two *T. koningiopsis* strains were identified as endophytic fungi. (Table 1).

In the concatenated tree, for the Harzianum complex, it can be observed the distance among strains of a determinated species obtained from the same vegetal source (Figure 1). Six *T. lentiforme* strains (T.28, T.42, T.51, T.54, T.55, and T.60) are from *Rollinia* sp. spread into the same big HCA branch, with the lineages T.54, T.55, and T.60 as the more dispersed. In contrast, six *T. harzianum* strains (T.81, T.82, T.122, T.123, T.126, and T.139), isolated as wood decomposer from *S. micranthum* are more closed in a branch containing all the nine strains in the same species. The T.81 and T.123 strains are leaves in the same terminal branch that is close to the T.126 strain. The other strains from *S. micranthum* are dispersed in an internal branch. Among the four strains of *T. afroharzianum*, three (T.69, T.70, and T.88) are from *V. amazonica* and are closed one each other.

Chemical profiles and chemotaxonomy of the fungi

The spectra of cultivated media and the mycelia of the fungi strains showed a high variety of ions and respective intensities in different mass ranges. The spectra showed high noise levels, however, it was possible to recognize coherent grouping among some strains from the same species or the same clade or complex. Are examples the two *T. reesei* strains from the Reesei clade and the *T. asperellum* T.138 and *T. asperelloides* T.136 strains from the Viride clade. The fungi of each pair produce many similarities between their spectra of both mycelium (MY) and cultivated medium (CM). *T. reesei* strains similarly present ions between *m/z* 900-1000 in the mycelium spectra (MY), and ions above *m/z* 1500 in the cultivated medium spectra (CM) (Figure 3), while *T. asperellum* and *T. asperelloides* strains present ions in common the range *m/z* 800-1000 and several signals above *m/z* 1300 (Figure 4).

On the other hand, it was also found different spectral profiles among strains from the same species, for example: the spectra of *T. lentiforme* T.19 e T.55 or the *T. harzianum* T.269 and T.123 from the Harzianum complex are not similar one each other (Figures 5 and 6).

The HCA analyses made clear the complexity of the metabolism of the strains. First of all, the groups formed by the intracellular data (INTC-Figure 7-A) are different from those by the extracellular (EXTC- Figure 7-B) metabolites. Secondly, the groups in both

metabolic trees present some coherences with the molecular identifications, but many strains have got unexpected positions in the tree.

In the intracellular dendogram (INTC-Figure 7-A), almost the Viride-clade fungi grouped in two main branches: the first (purple) was the most distant group, formed by the *T. asperelloides* and *T. asperellum* strains; the other (pink) grouped the *T.* a*troviride* strains, but also included the *T. lentiforme* T.42 strain. The two *T. reesei* strains (Reesei clade) formed a branch (green) derived from the same node of the second group above. The Harzianum complex was subdivided into three major branches: the first (blue) grouped three *T. harzianum* (T.139, T.261, and T.269) and one *T. afroharzianum* (T.70) strains; the second (red) is the most complex one, with five different species, including the *T. spirale* (Reesei clade) and *T. koningiopsis* (Viride clade) as unappropriated leaves; the third (gray) is the large group in this complex and presented three minor branches, one of them formed almost by *T. lentiforme* strains and two more mixed.

In the extracellular dendrogram (EXTC- Figure 7-B), again, almost the Viride-clade fungi grouped into two main branches on the same node separated from all the other clusters: one (pink) was formed by the *T. asperelloides* and the *T. asperellum* T.138 strains; the other (brown) grouped the *T.* a*troviride* strains. The Reesei clade did not form a discreet group. Finally, in the Harzianum complex, the T.139 strain has got a separated leaf (purple). All the other strains were divided into two main branches: the first (blue) grouped *T. harzianum*, *T. lentiforme*, and the two *T. reesei* strains; the second was divided into two more branches – one (gray) containing mainly *T. afroharzianum* strains, but also the *T. spirale* and *T. asperellum* T.140 strains. The other branch was also divided in two sub-branches: one (red) with *T. lentiforme* and the two *T. koningiopsis* strains; and another (green) principally grouping *T. harzianum* and *T. lentiforme* in discreet clusters.

4. Discussion

Fungi diversity and their complexity

The 37 strains of *Trichoderma* in this study, that were taken from our work collection, point for a great diversity of this genus in the Amazon, since they are distributed in nine species, a considerable number in a relatively small sample size (Table 1). Since almost half are endophytic strains, their distribution in seven species also stands out the remarkable diversity of the Amazonian endophytic *Trichoderma* species.

The diversity of the strains observed in this work seems to corroborate the intrinsic characteristic of the *Trichoderma* genus before reported, making necessary the use of the sequencing of the *TEF1*-α region, since the ITS sequences have been proved not enough to differentiate *Trichoderma* species (Chaverri et al., 2015). Besides, the diversity of the strains observed in the concatenated tree (Figure 1) was revealed yet more complex by the fungal macro and micromorphological characteristics (Table S1). This complexity did not permit group the fungi strains with a minimal coherence, mainly when compared with the molecular classification, a fact also observed in previous works (Chaverri et al., 2003; Druzhinina et al., 2010).

Relationship between the fungi origin and clustering

In respect to the distribution of the species strains investigated, the *T. lentiforme*, *T. afroharzianum*, and *T. harzianum* strains make up the main group with 68% (25 isolates) (Figure 8). They were associated with plants as endophytes or wood decomposers. Their grouping into the *T. harzianum* complex, as known as Harzianum complex (Figure 1), that include fourteen or more species, is in accordance with the literature that reports the geographic distribution of the fungi of this complex as neotropical, mainly as endophytic fungi (Chaverri et al., 2015).

The second more frequent fungal group is of the Viride clade, with 24% of the strains (9 isolates), also mostly obtained from plants as endophytes, with only two from soil. The two strains of *T. koningiopsis* are from São Paulo State, far 2.5-thousands miles from the Amazon, and were isolated from *M. paniculata*, an oriental plant introduced in Brazil. A large variety of species and in geographic locations of this small group is in accordance with reports pointing out Viride clade as a diversified group into the *Trichoderma* genus, with a wide geographical distribution (Samuels et al., 2006).

The last group with 8% of the fungi (3 isolates) is into the Reesei clade and presented two aquatic and one wood decomposer strains. *T. reesei* strains are generally reported with enzyme producers, used for bioremediation and production of biofuel (Martinez et al., 2008; Deshpand et al., 2009; Nazifa et al., 2018) while *T. spirale* strains are reported to have potential for biocontrol of phytopathogens (Baiyee et al., 2018).

Fungi from plants

The observation of predominant species in a same plant (Table 1) can indicate its good adaptation with the respective host, revealing it as a true endophyte. On the other side, the greater or lesser approximation among strains of a determined species isolated from a same plant, can either be derived from their evolutive adaptation into different vegetal micro-habitats or indicate own characteristics for different fungi species (Druzhinina et al., 2010). For example, the three *T. afroharzianum* strains from *V. amazonica* are closer in the concatenated tree than the six *T. lentiforme* obtained from *Rollinia* sp. (Figure 1). The better proximity among the six *T. harzianum* strains, isolated as wood decomposers from *S. micranthum*, than among the six *T. lentiforme* obtained from *Rollinia* sp. is also an issue to be considered. Were before those decomposer fungi-strains well-adapted endophytes into the *S. micranthum*?

The case of the *T. afroharzianum* T. 531

The deletion of 30 pb in the *T. afroharzianum* T. 531's *TEF1*-α region is not a novelty for this species and does not prevent its identification in the concatenated tree (Figures 1 and 2). However, the 40-measurements averages for the sizes of its conidia and phialides were somewhat different in relation to the respective averages for the other *T. afroharzianum* strains in this work, after 48 hours of growth, at 30 ºC (Table 3). It can be highlighted the height-to-width ratios of 1.12 against 1.34 for the conidia and of 5.72 against 4.15 for the phialides, observed for T.531 and the other *T. afroharzianum* strains respectively. These differences may indicate a new species for the *T. afroharzianum* T. 531, which needs other DNA data and morphological data to be confirmed.

Metabolic HCA-dendrograms complexity

The metabolic complexity observed in the mass spectra and spectral HCA dendrograms, both of the intra- and extracellular samples (Figures 3 to 7), is a reflection of the variety of substances produced by the strains. This metabolic variety reveals some apparent strain-specific metabolism, a possible influence of the respective environmental origins of the strains. Especially for strains from the same vegetal host, the micro-habitats could play an important role in activating or not determined metabolic genes or gene clusters. The greater the host-fungal strain coevolution time, the greater the strain-specific

adaptation can be expected, considering the known symbiosis of endophytic microorganisms (Rodriguez et al., 2004; Macías-Rodríguez et al., 2020).

The considerable differences between the extracellular and intracellular metabolic HCA dendrograms (Figure 7), as also in relation to the concatenated phylogenetic groupings (Figure 1), reinforce the idea of strain-specific environmental-dependent differentiation. On a side, the habitat adaptation certainly influences the extracellular metabolism, for the better survival of the strain. On another side, it also can alter the intracellular metabolism as a consequence of the use of the available food, among other factors (Vinale et al 2014; Macías-Rodríguez et al., 2020).

More specifically, the complexity of the groupings observed for the strains of the *T. harzianum*, *T. lentiforme*, and the *T. afroharzianum*, in both intra- and extracellular HCA dendrograms, are in coherence with the reported complexity of the Harzianum complex (Chaverr et al., 2015). Attributed to speciation related to different evolutionary histories of the organisms into this complex and lack of strict genetic borders among them (Druzhinina et al., 2010), such reported complexity appears to explain the plural metabolic profiles for Harzianum complex strains observed in this work.

In contrast, the better grouping between the *T. reesei* strains, from the Reesei clade, as also among strains in a same-species into the Viride clade (Figure 7) seems to point the species in these clades as more resilient to the environmental changes than species in the Harzianum complex. It is worth highlighting the good groupings formed by the *T. asperellum* and *T. asperelloides* strains, considered cryptic sister species (Samuels et al., 2010). Both species have been reported as hard to be differentiated, except by molecular multigenic comparison (Fernandes et al., 2020), which is in accordance with our findings above.

Besides, the unexpected positions for many strains in the intracellular or extracellular HCA dendrograms reveal the similarities among the metabolites produced by them with that of the lineages of different species that are together in branches of the trees. Indirectly this also reveals the coincidence of metabolic genes or gene clusters present and activated.

Metabolomic diversity

The fungi of the *Trichoderma* genus have worldwide revealed a powerful and versatile metabolism. Many molecules of different classes have already been described for various species into this genus (Keswani et al., 2013; Zeilinger et al., 2016; Macías-Rodríguez et al., 2020). In the present work, while no specific metabolite was searched for, the different signals observed in the mass spectra of the strains remember spectral ranges of substances reported for *Trichoderma* species. Since the metabolome reflects the genome and proteome related to the secondary metabolism as well as to part of the primary metabolism, those metabolic spectral ranges confirm the richness of the genetic machinery for *Trichoderma* species. They also point out strains with the potential to supply substances of interest or to produce new substances.

As examples, we highlight species whose strains remained grouped both in intra- and extracellular in the HCA dendrograms (Figure 7): *T. reesei*, *T. asperellum* together with *T. asperelloides*, and *T. atroviride*. For the two *T. reesei* strains the ions observed in the ranges of *m/z* 900-1000 and above of 1500 in both CM and MY extracts (Figure 3) are characteristic of peptaibols. Peptaibols were reported for *T. reesei* together and other species of the same clade (Van Bohemen et al., 2016). These authors reported a high production of several subfamilies of peptaibols with 11 to 20 amino acid residues, whose molecular masses are compatible with the values above. These peptaibols possibly are of the harzianins, trichobrachins, trichorozins, and trichovirins, among others peptaibols subfamilies, also reported by Ruiz and coworkers for *T. reesei* (2007).

The signal at *m/z* 800-1000 and above *m/z* 1300, observed in both CM and MY spectra of *T. asperellum* and *T. asperelloides* (Figura 4), are also of peptaibols subfamilies, mainly the asperelines and trichotoxins, reported for both species (Chutrakul et al., 2008; Ren et al., 2009; Ren et al., 2013; Chen et al., 2013; Fernandes et al., 2020).

5. Conclusion

In the present study, 37 *Trichoderma* strains were achieved belonging to nine different species, revealing for the first time a high diversity for this genus in the Amazon. Among those strains, the 25 endophytic ones are also highly diversified and showed to belong to seven species. While identified by concatenated DNA regions, the *Trichoderma* strains in this work was revealed a sample of fungi yet more complex by their morphological structures, being impossible to recognize any defined grouping. This high complexity was

also noted through the mass spectra of the extracts of the strains, mainly for that of species into the Harzianum complex. Fungi from this complex were the most abundant (68%), while the Viride and Reesei clades were also present.

Apparently, fungi into the Harzianum complex and the Viride clade are well adapted to vegetal micro-habitats, as endophytes or wood decomposers. Strains of *T. afroharzianum* and *T. lentiforme* seem to be true endophytes of *V. amazonica* and *Rollinia* sp., respectively.

The diverse metabolism revealed by the complex HCA dendrograms from the mass spectra of the strains signalizes a strain-specific environmental-dependent differentiation that enables or disables different metabolic genes or gene clusters. Environmental differentiation was more apparent among the strains into the *T. harzianum*, *T. lentiforme*, and the *T. afroharzianum* species, ratifying the complexity of the Harzianum complex. *T. reesei* strains and species into the Viride clade, especially the strains of the cryptic sister species *T. asperellum* and *T. asperelloides*, have shown more metabolic resilience to the environmental changes, according to the HCA dendrograms.

The mass spectra of many *Trichoderma* strains in this work revealed ranges of signals correspondent to different subfamilies of peptaibols, coherent with those reported for their respective species. These signals and those in the typical range for polyketides and metabolites of low molecular masses, observed for different strains, corroborate the richness of the *Trichoderma* metabolism.

Finally, our data indicate the T.531 strain as a possible new species by its deletion of 30 pb in the DNA's *TEF1*-α region and through the comparison of its morphological data with data of the other *T. afroharzianum* strains in this work. Investigation if this strain represents a new species or is suffering progressive speciation is a future task to be realized.

Author contributions

Fernandes, K. R. P.; Bianco, E. A.; Souza, A. D. L.; Souza, A. Q. L.; Oliveira, M. R.; da Silva, F. H.; Evangelista, D. E.; Pereira, J. O.; Hanada, R. E.; Filho, E. R.; Melo, L. S.; and Alencar, L. F. participated in the collect, isolation, purification, and morphological identification of the lineages and the study outline. Fernandes, K. R. P.; Queiroz, C. A.; Vasconcelos, A. S. Participated in the morphological identification of strains by SEM. Silva, G. F.; Cruz, J. C.; Fernandes, K. R. P.; Sousa, T. F. participated in molecular identification,

phylogenetic analyzes, and NCBI deposits. Fernandes, K.R.P.; da Silva, F. M.A.; Rodrigues, R. S.; Barbosa, A. N.; Couceiro, P.R.C; Souza, A. Q. L; Souza, A. D. L. Participated in the production of secondary metabolites and statistical analysis of the chemical profile data of the strains. Souza, A.Q. L.; Fernandes, K. R. P. and, Souza, A. D. L. da Silva, F. M.A. Participated in the writing of the manuscript. Souza, A. D. L. conceived the study and participated in its design and coordination. All authors read and approved the final manuscript.

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Declaration of competing interest: None.

Table 1. Origin and molecular identification of *Trichoderma* strains.

Collection number	Occurrence	Origin of isolates	Collection location	Molecular grouping	Species	GenBank accesion numbers ITS	GenBank accesion numbers *TEF1-α*
T.19	Endophytic	*Strychnos cogens*	AM	1	*T. lentiforme*	MN262491	MT681962
T.28	Endophytic	*Rollinia* sp.	AM	1	*T. lentiforme*	MN262487	MT681963
T.42	Endophytic	*Rollinia* sp.	AM	1	*T. lentiforme*	MN262512	MT681964
T.51	Endophytic	*Rollinia* sp.	AM	1	*T. lentiforme*	MN262511	MT681958
T.54	Endophytic	*Rollinia* sp.	AM	1	*T. lentiforme*	MN262495	MT681957
T.55	Endophytic	*Rollinia* sp.	AM	1	*T. lentiforme*	MN262489	MT681960
T.60	Endophytic	*Rollinia* sp.	AM	1	*T. lentiforme*	MN262481	MT681959
T.137	Wood decomposers	*Scleronema micranthum*	AM	1	*T. lentiforme*	MN262496	MT681961
T.264	Endophytic	NI	AM	1	*T. lentiforme*	MN262483	MT681953
T.265	Endophytic	NI	AM	1	*T. lentiforme*	MN262498	MT681954
T.267	Endophytic	NI	AM	1	*T. lentiforme*	MN382157	MT681955
T.364	Endophytic	*Peperomia pellucida*	AM	3	*T. lentifome*	MN262482	MT681956
T.81	Wood decomposers	*S. micranthum*	AM	2	*T. harzianum*	MN262481	MT681972
T.82	Wood decomposers	*S. micranthum*	AM	2	*T. harzianum*	MN262506	MT681965
T.122	Wood decomposers	*S. micranthum*	AM	2	*T. harzianum*	MN262505	MT681970
T.123	Wood decomposers	*S. micranthum*	AM	2	*T. harzianum*	MN262516	MT681977
T.126	Wood decomposers	*S. micranthum*	AM	2	*T. harzianum*	MN262404	MT681966
T.139	Wood decomposers	*S. micranthum*	AM	2	*T. harzianum*	MN262497	MT681969
T.269	Endophytic	NI	AM	2	*T. harzianum*	MN262484	MT681968

T.221	Endophytic	NI	AM	2	*T. harzianum*	MN262490	MT681971
T.261	Endophytic	NI	AM	2	*T. harzianum*	MN262500	MT681967
T.69	Endophytic	*Victoria amazonica*	AM	3	*T. afroharzianum*	MN262509	MT681950
T.70	Endophytic	*V. amazonica*	AM	3	*T. afroharzianum*	MN262508	MT681952
T.88	Endophytic	*V. amazonica*	AM	3	*T. afroharzianum*	MN252494	MT681951
T.531	Endophytic	NI	AM	3	*T. afroharzianum*	MN262485	MT681949
T.37	Endophytic	*Murraya paniculata*	SP	4	*T. koningiopsis*	MN262513	MT681980
T.73	Endophytic	*M. paniculata*	SP	4	*T. koningiopsis*	MN262510	MT681979
T.136	Soil	Terra preta de índio	AM	5	*T. asperelloides*	MN262503	MT681983
T.145	Endophytic	*V. amazonica*	AM	5	*T. asperelloides*	MN262514	MT281578
T.138	Soil	Terra preta de índio	AM	6	*T. asperellum*	MN262515	MT681981
T.140	Endophytic	*Hymenaea courbaril*	AM	6	*T. asperellum*	MN262502	MT681982
T.219	Endophytic	NI	AM	7	*T. atroviride*	MN262492	*
T.220	Endophytic	NI	AM	7	*T. atroviride*	MN262499	*
T.234	Endophytic	NI	AM	7	*T. atroviride*	MN262586	MT681978
T.867	Aquatic	River	AM	8	*T. reesei*	MN262493	MT681975
T.868	Aquatic	River	AM	8	*T. reesei*	MN262488	MT681976
T.263	Wood decomposers	*S. micranthum*	AM	9	*T. spirale*	MN283156	MT681974
T.869	Aquatic	River	AM	10	*Clonostachys rosea*	MN262501	MT681984

NI: Not Identified; *Not Deposited; AM: State of Amazonas, SP: State of São Paulo.

Table 2. Data composition of the thirty-seven strains.

Origin of *Trichoderma* spp.									
Isolate code	Endophytes				Wood Decomposers		Soil	Aquatic areas	
	(T.19), (T.28), (T.37), (T.42), (T.51), (T.54), (T.55), (T.60), (T.69), (T.70), (T.73), (T.88), (T.140), (T.145), (T.219), (T.220), (T.221), (T.234), (T.261), (T.264) (T.265), (T.267), (T.269), (T.364), (T.531).				(T.81), (T.82), (T.122), (T.123), (T.126), (T.137), (T.139), (T.263).		(T.136), (T.138).	(T.867), (T.868).	
Genotyping by molecular biology									
Species of *Trichoderma*	*T. harzianum*	*T. afroharzinaum*	*T. lentiforme*	*T. spirale*	*T. reesei*	*T. atroviride*	*T. koningiopsis*	*T. asperelloides*	*T. asperellum*
Isolate code	(T.81) (T.82) (T.122) (T.123) (T.126) (T.139) (T.221) (T.261) (T.269)	(T.69) (T.70) (T.88) (T.531)	(T.19) (T.28) (T.42) (T.51) (T.54) (T.55) (T.60) (T.137) (T.264) (T.265) (T.267) (T.364)	(T.263)	(T.867) (T.868)	(T.219) (T.220) (T.234)	(T.37) (T.73)	(T.136) (T.145)	(T.138) (T.140)
Phylogenetic analysis									
Species of *Trichoderma*	*T. harzianum*	*T. afroharzinaum*	*T. lentiforme*	*T. spirale*	*T. reesei*	*T. atroviride*	*T. koningiopsis*	*T. asperelloides*	*T. asperellum*
Complex/clade	Harzianum complex			Reesei clade		Viride clade			

Table 3 - Morphological characteristics of *T. afroharzianum* strains.

Strains	Conidia*			Phialides structures*		
	Width	Height	H/W	Width	Height	H/W
T.69	2.01	3.46	1.72	2.40	7.90	3.29
T.70	2.02	2.46	1.22	2.11	8.80	4.17
T.88	2.02	2.16	1.07	1.30	7.40	5.69
Average#	**2.02**	**2.69**	**1.34**	**1.94**	**8.03**	**4.15**
T.531	**1.70**	**1.90**	**1.12**	**1.70**	**9.73**	**5.72**

*Average of 40 measurements.
#Average for the three strains without deletions in this work.
All measurements were done after 48 hours of growth, at 30 ºC.

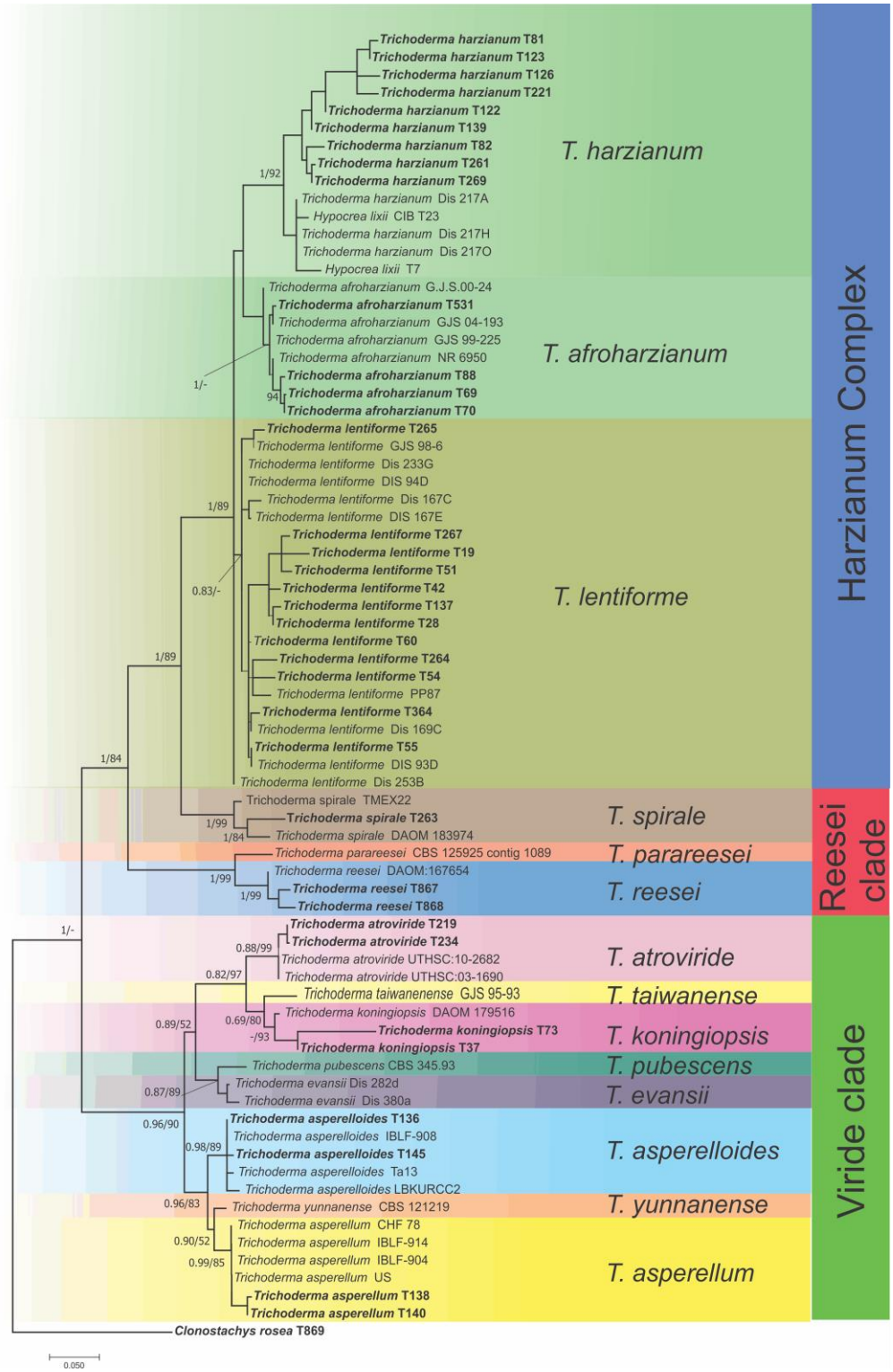

Figure 1**.** Maximum parsimony (MP) concatenated phylogenetic analysis of ITS and *TEF1-α* region relationships for *Trichoderma* strains. In bold, the lineages of this report. All others are NCBI deposits. The T.219 strain was grouped with the ITS sequences and pieces of *TEF1-α*, since this had presented fails.

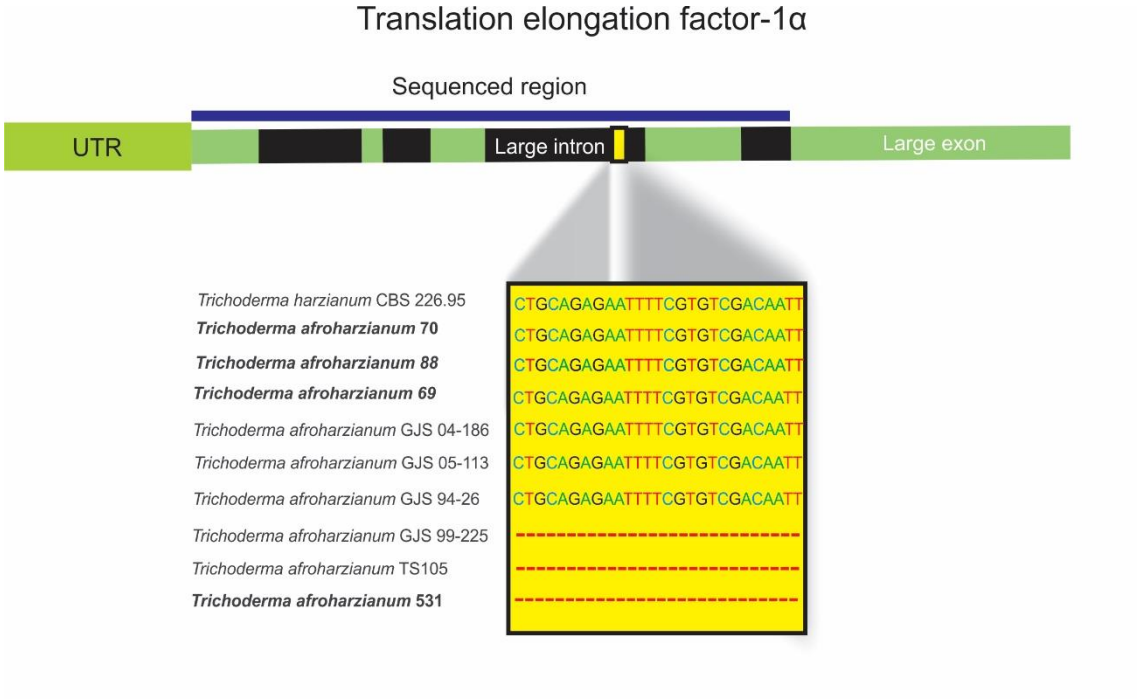

Figure 2. Schematic structure of *TEF1-α* region the showing the deletion of 30 base pairs in the large intron of of *T. afroharzianum* strains obtained in this study.

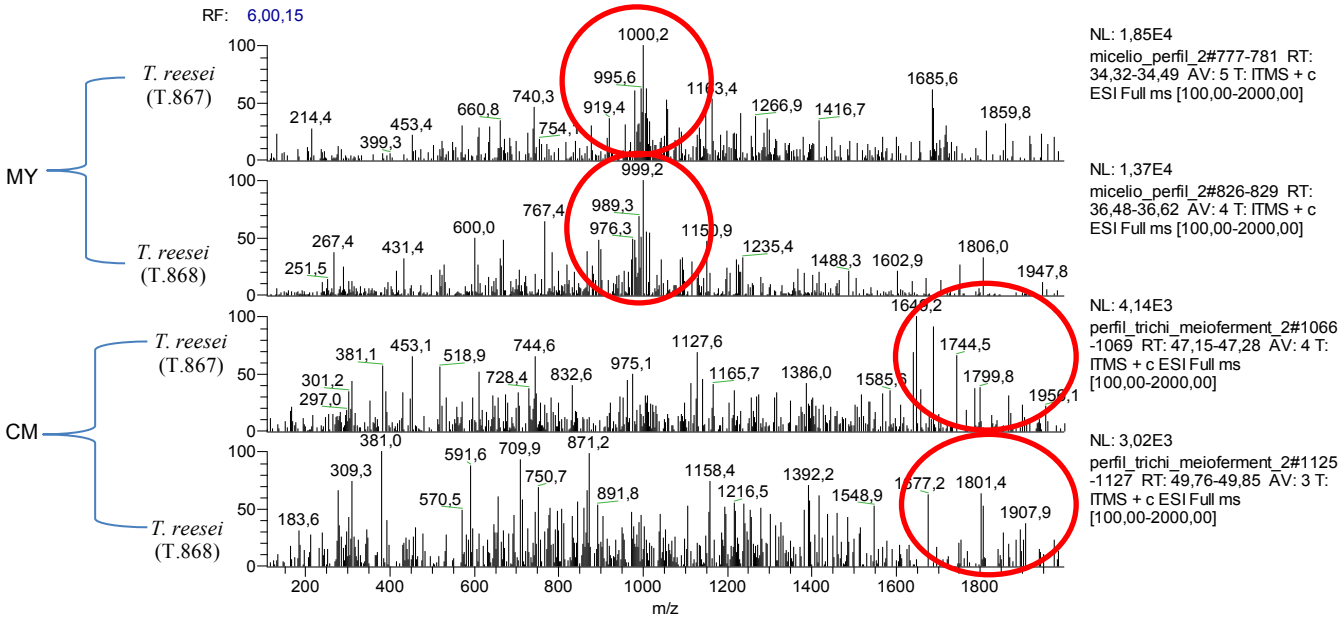

Figure 3. Coherence between spectra of *T. reesei* strains. MY: Mycelium and CM: cultivated medium.

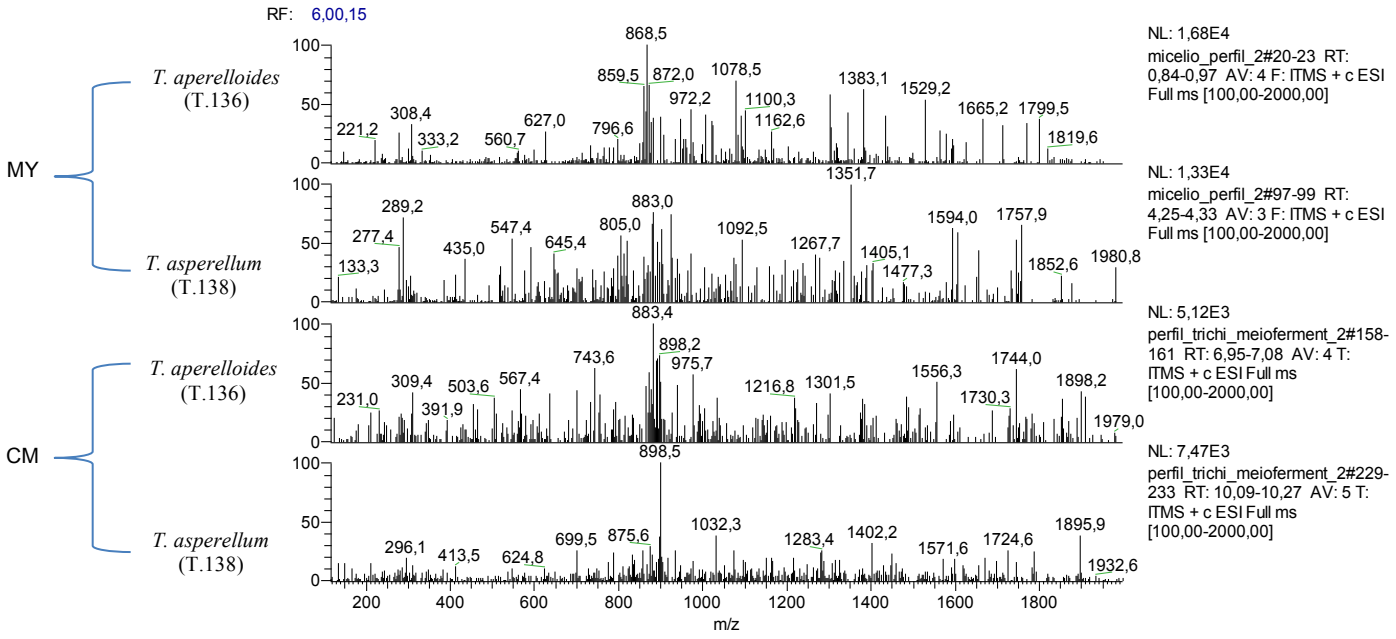

Figure 4. Coherence between spectra of *T. asperellum* and *T. asperelloides* strains.

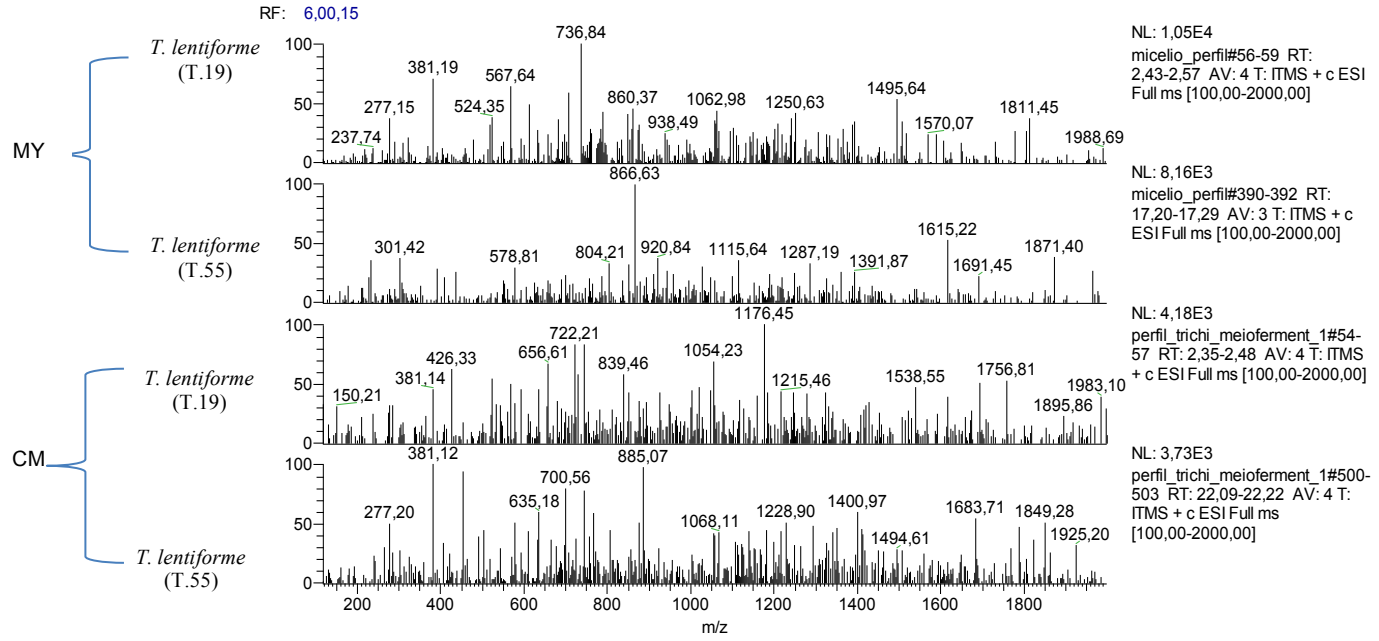

Figure 5. Spectral difference between strains of the same species of *T. lentiforme*.

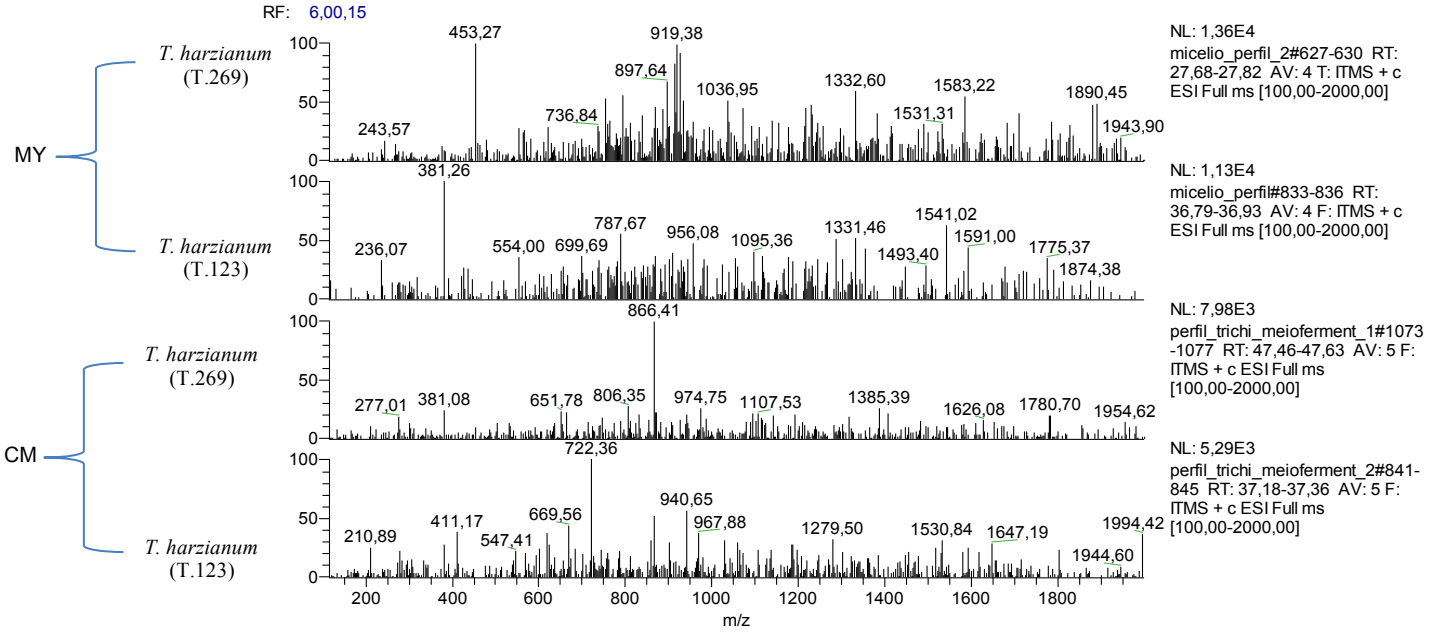

Figure 6. Spectral difference between strains of the same species of *T. harzianum*.

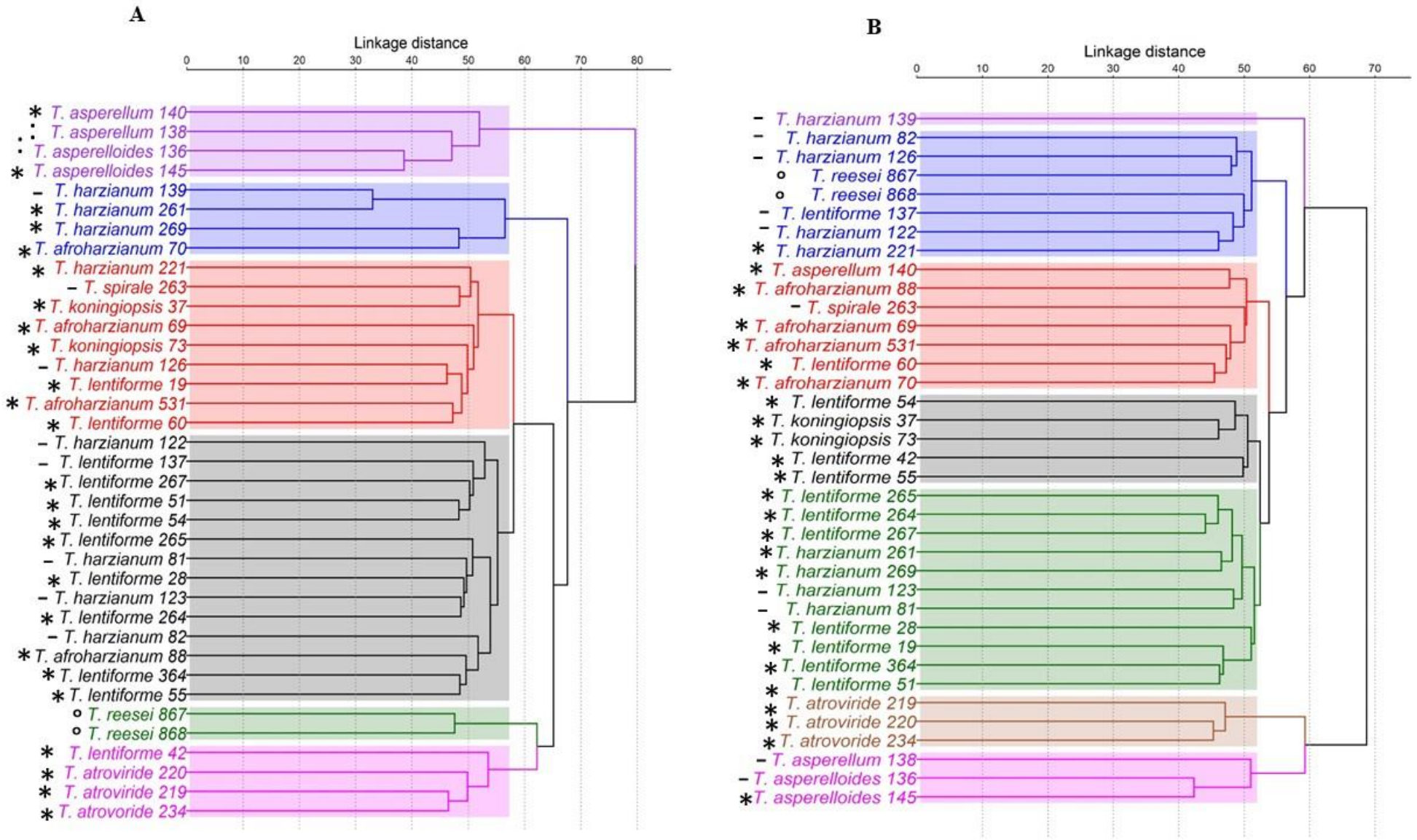

Figure 7. Hierarchical cluster analysis (HCA) of intracellular metabolites – (INTC-A) and extracellular phenotypes – (EXTC-B). Symbol legend: *endophytes; ¯wood decomposers; :soil; ° aquatic areas.

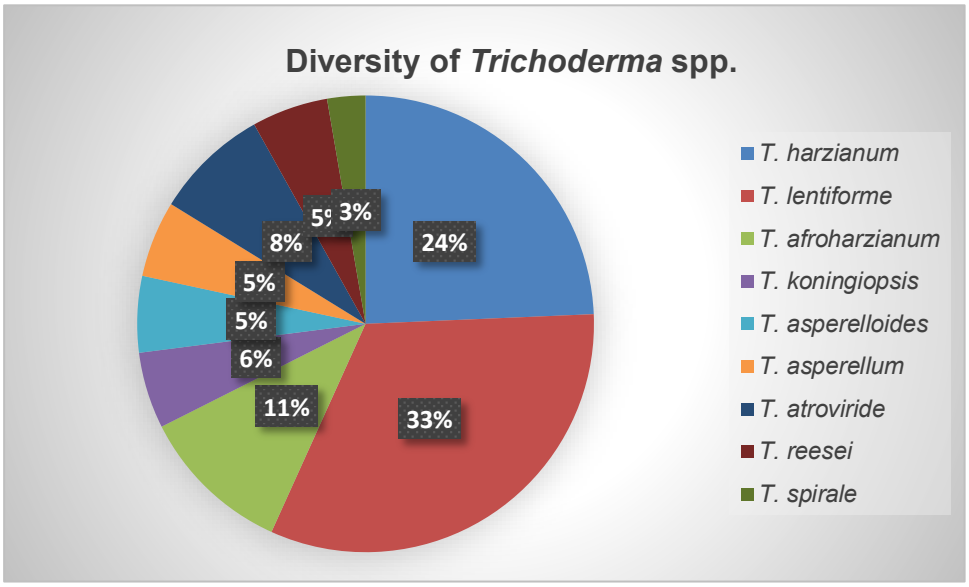

Figure 8. Percentage of *Trichoderma* species.

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7. Supplementary material

Diversity and chemotaxonomy of Amazonian *Trichoderma* spp.

Table S1. Macro and micromorphological characteristics of *Trichoderma* strains.

Figure S2. Macromorphological structures of groups 1 - 9.

Figure S3. Microscopic structures (200x) of fungi T.19, T.269, T.37, T.145, T. 140, T.219, T.868 and T.263 representing groups 1– 9.

Figure S4. Morphological analysis of *T. afroharzianum* (T.69).

Figure S5. Morphological analysis of *T. afroharzianum* (T.70).

Figure S6. Morphological analysis of *T. afroharzianum* (T.88).

Figure S7. Morphological analysis of *T. afroharzianum* (T.531).

Table S1. Macro and micromorphological characteristics of *Trichoderma* strains.

Isolate number	Species	Colony color	Reverse color	Mycelium shape	Conidiation	Conidia format	Conidia color	Conidia diameter (µm)	Conidiophores (µm)	Phialides length x width (µm)	Chlamydospore (µm)
T.19	*T. lentiforme*	Ligth green	Cream	Velvety. Moderate aerial mycelium	Concentric halos Green asparagus	Ellipsoid, globular and oval	Dark green	1.532 x 1.104	74.536	Ampuliform 9.474 x 2.141	NF
T.28	*T. lentiforme*	Dark green, olive green and white	White	Cottony. High aerial mycelium	Central zone	Globular, subglobular and ovoid	Olive green to dark green	0.855 x 0.729	17.525	Ampuliform: 2.996 x 0.948	NF
T.42	*T. lentiforme*	Yellow and olive green	Orange yellow	Vellum central zone and high aerial mycelium	Concentric halos	Oval and subglobular	Olive green	2.219 x 1.712	42.753	Ampuliform 8.573 x 2.015	NF
T.51	*T. lentiforme*	White and dark green	Light yellow/ chrome yellow	Sandy with conidiogenic pustules	Concentric halos	Oval and subglobular	Dark green	1.282 x 1.296	44.558	Ampuliform and Langeniform 3.631 x 1.113	NF
T.54	*T. lentiforme*	Army green	Brown	Cottony. Moderate aerial mycelium	Concentric halos	Ellipsoid and oval	Dark green	NM	NM	Ampuliform 32.750 x 12.628	NF
T.55	*T. lentiforme*	Dark green	Orange/ yellow	Velvety in the center and concentric halo of conidiation pustules	Distant concentric halos	Globular and sub-globular	Dark green	NM	NF	NF	NF
T.60	*T. lentiforme*	Light green and white	Green	Sandy and Arachnoid	Distant concentric halos	Ellipsoid and oval	Dark green	NM	NF	NF	NF
T.137	*T. lentiforme*	Yellow, green and white	Orange	Sandy and arachnoid. Moderate aerial mycelium	Concentric halos Dark green and white	Globular and subglobular	Dark Green and army green	1.103 x 1.049	14.677	Ampuliform: 2.364 x 1.338	NF
T.264	*T. lentiforme*	Army green and white	Cream	Cottony. High aerial mycelium	Concentric halos	Oval, globular and subglobular	Dark green	0.189 x 0.169	2.937	Ampuliform: 0.411 x 0.205	NF

T.265	*T. lentiforme*	White and olive green	Cream	Cottony with conidiogenic pustules.	Olive green concentric halos	Globular and subglobular	Dark green	0.197 x 0.184	3.590	Ampuliform: 0.486 x 0.188	NF
T.267	*T. lentiforme*	White and moss green	White	Velvety to cottony	Moss green moderate Concentric halos	Oval, globular and subglobular	Dark green	0.381 x 0.357	8.616	Ampuliform: 0.778 x 0.282	NF
T.81	*T. harzianum*	White and green	Light brown	Cottony, sandy and arachnoid	Dark green central conidiation and misshapen and sparse conidia halos	Ellipsoid and oval	Dark green	1.289 x 1.029	38.229	Ampuliform and Langeniform 3.735x1.082	NF
T.82	*T. harzianum*	Light yellow and olive green	Light yellow	Velvety and moderate cottony	Olive central conidiation zone	Oval and globular	Dark green	1.340 x 1.472	20.609	Ampuliform and moderate solitary 3,074 x 1.032	NF
T.122	*T. harzianum*	White and dark green	Cream	Velvety, sandy and arachnoid	Dark green central conidiation	Oval, globular and subglobular	Dark green	2.241 x 2.149	23.106	Ampuliform 2.683 x 1.152	NF
T.123	*T. harzianum*	White and dark green	Light brown	Cottony and velvety. Moderate aerial mycelium	Concentric ring Dark Green	Globular and subglobular	Dark green	NM	495.555	Ampuliform 40.760 x 12.940	NF
T.126	*T. harzianum*	Orange, light green and white	Orange/ yellow	Moderately velvety in the center. High airborne mycelium. Concentric halos	Concentric halos Light green with white borders	Globular and subglobular	Dark green	8.745 x 7.621	321.232	NF	NF
T.139	*T. harzianum*	Green and white	White	Velvety and Cottony with moderate aerial mycelium	Concentric halos. Central zone of high concentration of green conidia	Globular, subglobular and oval	Dark green	2.438 x 2.249	48.105	Ampuliform: 5.912 x 2.494	NF
T.269	*T. harzianum*	White ande dark green	White	Velvety. Moderate aerial mycelium	Central halo	Globular and oval	Dark green	0.204 x 0.185	6.219	Ampuliform: 0.500 x 0.198	NF

T.221	*T. harzianum*	White and moss green	White	Moderate cottony. Moderate aerial mycelium	Concentric halos	Globular and subglobular	Dark green	1.206 x 1.137	8.801	Ampuliform and oblong: 3.325 x 0.987	NF
T.261	*T. harzianum*	Moss green and white	Light brown and white	Velvety and cottony	Concentric halos. High concentration central zone	Globular, subglobular and oval	Dark green	0.104 x 0.086	1.512	Ampuliform: 0.292 x 0.090	NF
T.69	*T. afroharzianum*	Yellow	Orange yellow	Cottony. Moderate aerial mycelium	Concentric halos Light yellow (center) and orange	Ellipsoid and oval	Dark green	0.092x0.062	NF	NF	NF
T.70	*T. afroharzianum*	Moss green and light yellow	Reddish orange	Cottony. High aerial mycelium	Concentric halos Green moss and light yellow	Ellipsoid and oval (formation of curls at phialides tips)	Dark green	0.081 x 0.056	1.315	Langeniform and oblong 0.250x0.054	NF
T.88	*T.afroharzianum*	Yellow and dark green	Reddish yellow	Velvety and moderate aerial mycelium	Concentric halos Dark green and olive green	Ellipsoid and oval	Dark green to asparagus green	0.103 x 0.076	1.544	Langeniform and oblong: 97.156 x 29.741	High incidence. Interval: 0.276 x 0.178 Terminal region: 0.187 x 0.166
T.364	*T. afroharzianum*	White and green	White and moss green	Velvety to sandy	Concentric halo	Globular and subglobular	Dark green	0.261 x 0.230	4.618	Oblong ampuliforms: 0.723 x 0.143	Abundant. Intercalars: 0.592 x 0.560; Hyphae terminal region: 0.618 x 0.574
T.531	*T. afroharzianum*	Moss green	Persimmon brown	Cottony. Abundant and high aerial mycelium	Concentric halos	Ellipsoid and oval	Dark green	0.203 x 0.168	NF	NF	Abundant region terminal: 0.573
T.37	*T. koningiopsis*	Moss green and white	Moss green/ opaque	Velvety and sandy	Concentric halos Green asparagus	Ellipsoid and oval	Dark green	NF	NF	NF	NF
T.73	*T. koningiopsis*	White	White	Cottony. High aerial mycelium	Low conidiogenic pustules in the central region	Ellipsoid and oval	Dark green	1.771 x 1.162	28.207	Ampuliform and oblong Langeniform 5.229 x 1.232	NF
T.136	*T. asperelloides*	Dark green and white	Black ebony	Shaggylooking/ cottony. High aerial mycelium.	Concentric halos Dark Green	Ellipsoid and oval	Dark green	1.571 x 1.280	NF	Ampuliform and moderate langeniform 4.465 x 1.048	NF

T.145	*T. asperelloides*	Dark green and white	Black ebony	Shaggy-looking/ cottony. High aerial mycelium	High white circular zone. Moderate dark green conidiation regions	Oval, globular and subglobular	Dark green	1.785 x 1.447	10.308	Ampuliform and Langeniform 4.351 x 1.088	NF
T.138	*T. asperellum*	Dark green and white	Black coffee	Sandy and arachnoide	Concentric halos Dark Green	Oval, globular and subglobular	Dark green	3.315 x 2.847	18.823	NF	High frequency. Interim: 6.306 x 4.409 Terminal region: 5.242 x 4.438
T.140	*T. asperellum*	Dark green and white	Black coffee	Sandy and arachnoide	Concentric halos Dark green	Dark green concentric halo	Dark green	3.229 x 2.792	NF	NF	High frequency. Interstitials: 8.506 x 6.061 Terminal region: 8.156 x 6.409
T.219	*T. atrovoride*	White and dark green	Persimmon brown	Sandy and arachnoid	Concentric halos	Ellipsoid, oval, globular and subglobular	Dark green	1.588 x 1.435	NF	Ampuliform and oblong: 4.606 x 1.065	Abundant. Terminal region: 4.016 x 3.755
T.220	*T. atrovoride*	White and dark green	Persimmon brown	Sandy and arachnoide	Concentric halos	Oval and globular	Dark green	1.803 x 1.584	42.001	Ampuliform and oblong: 9.581 x 2.078	Moderate Interval (length x width): 9.952 x 7.631 Terminal region: 8.830 x 8.683
T.234	*T. atrovoride*	White, moss green and army green	Brown	Sandy and arachnoid	Concentric halos	Globular and subglobular	Dark green	0.149 x 0.129	NF	NO	Abundant. Terminal region: 0.325 x 0.323
T.867	*T. reesei*	Yellow, white and, olive green	Yellow	Velvety and moderately cottony	Central zone of conidiation	Ellipsoid and oval	Olive green	0.150 x 0.122	3.804	Ampuliform and Langeniform: 0.320 x 0.114	NF
T.868	*T. reesei*	Yellow, white, and dark green	Yellow	Velvety and moderately cottony	Sparse conidiogenic pustules	Ellipsoid and oval	Dark green	0.584 x 0.343	NF	NF	NF
T.263	*T. spirale*	Bright yellow and white	Yellow	Moderate membrane. Moderate aerial mycelium	Concentric halo White	Ellipsoid and oval	Dark green (in the form of grape clusters)	0.120 x 0.074	1.096	Langeniform and oblong: 0.288 x 0.081	NF

869*	*Clonostachys rosea*	White and dark green	White	Velvety in the center and concentric halo	Concentric halos	Ellipsoid and oval	Olive green	0.573 x 0.321	1.074	NF	NF

NF: Not Found; NM: Not Mensured. *Out-group strain.

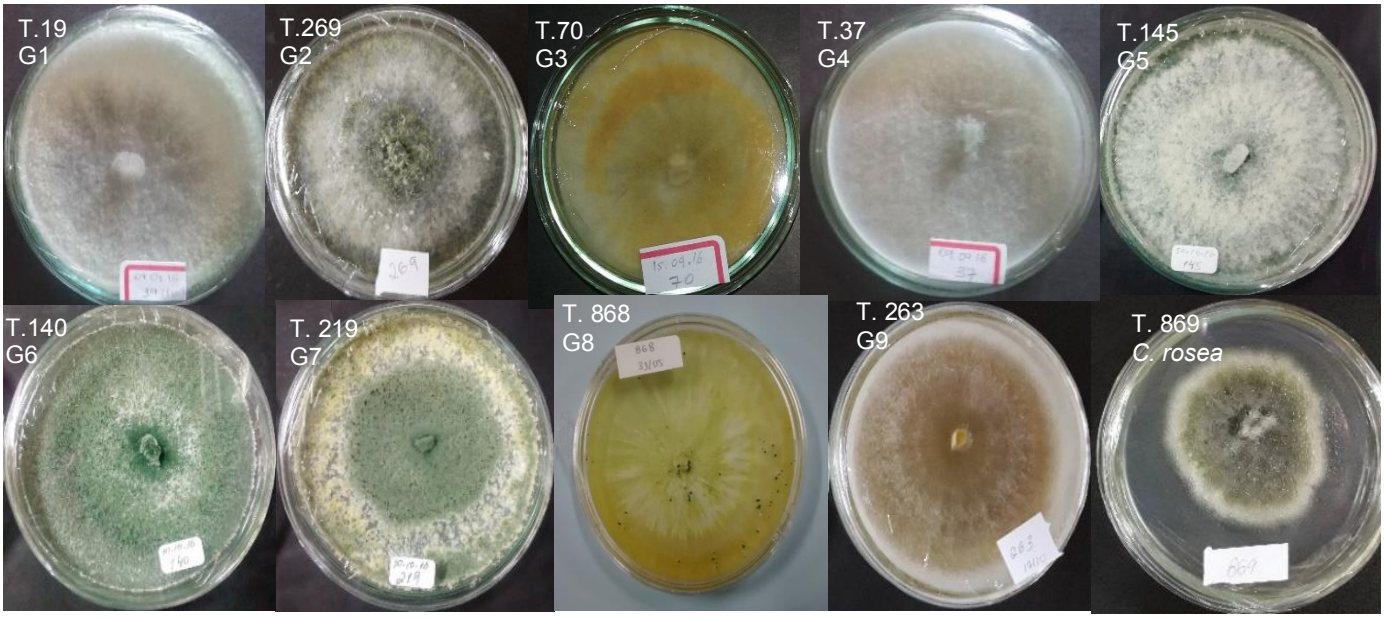
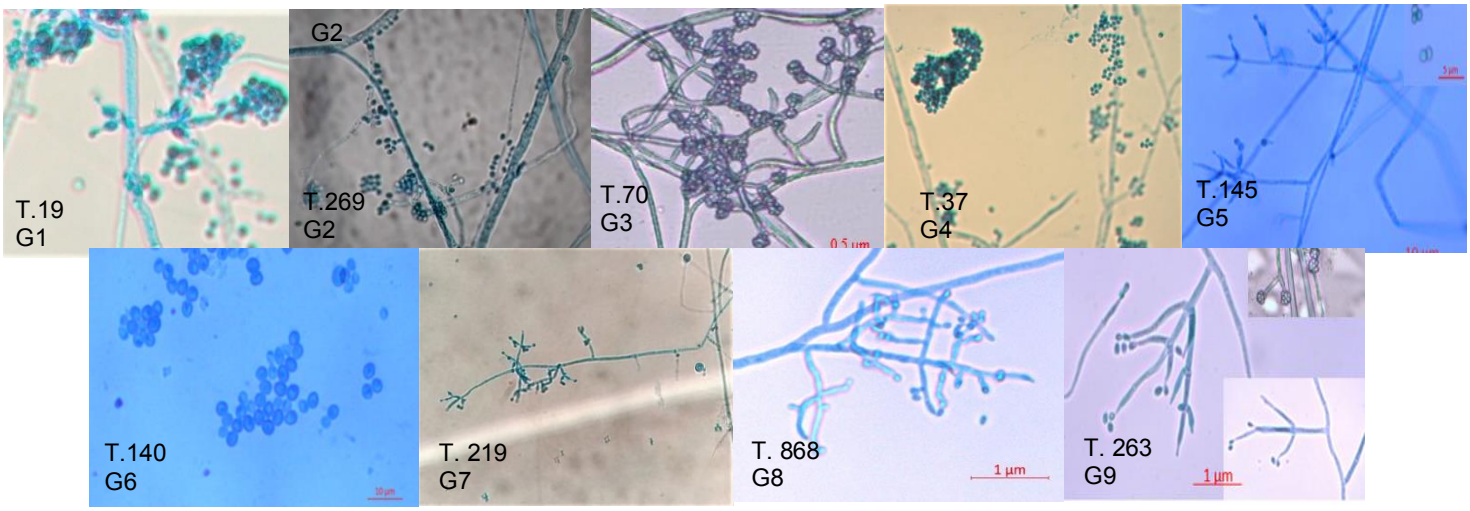


Figure S2. Macromorphological structures of groups 1 - 9.



Figures S3. Microscopic structures (200x) of fungi T.19, T.269, T.37, T.145, T.140, T.219, T.868 and T.263 representing groups 1-9.

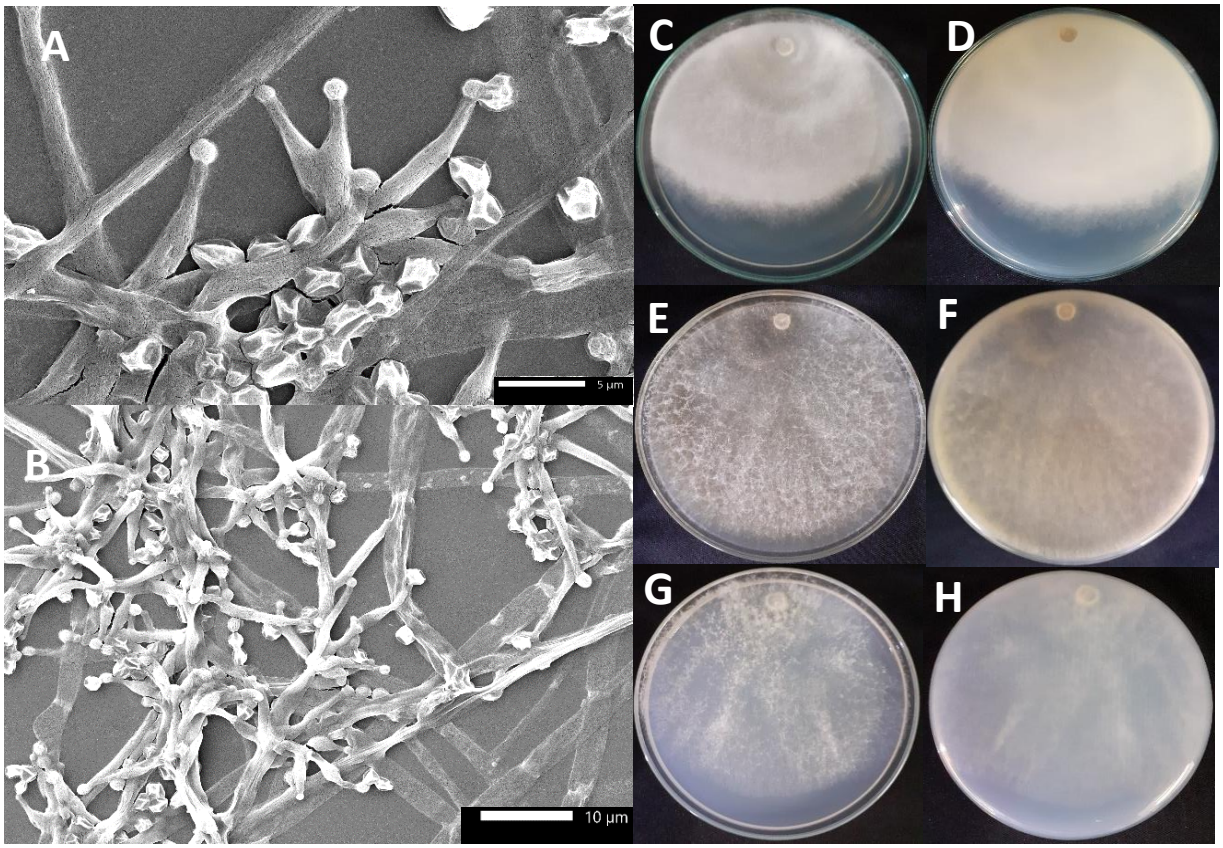


Figure S4. Morphological analysis of *T. afroharzianum* (T.69) by scanning electron microscopy (SEM) model JSM IT500HR (JEOL Ltd., Japan) - Colonies after five days of cultivation: A- phialides (5 µm); B- conidiophores (10 µm); C-D colony in PDA culture medium; E-F colony in malt medium; G-H colony in SNA medium. Conidia: Width: 1.5 - 2.3 µm (mean: 2.01); Height: 1.95 - 3.62 µm (mean: 3.46 um). Phialides structure: Width: 1.7 - 3.6 µm (mean: 2.4); Height: 4.2 - 9.4 µm (mean: 7.9 µm).

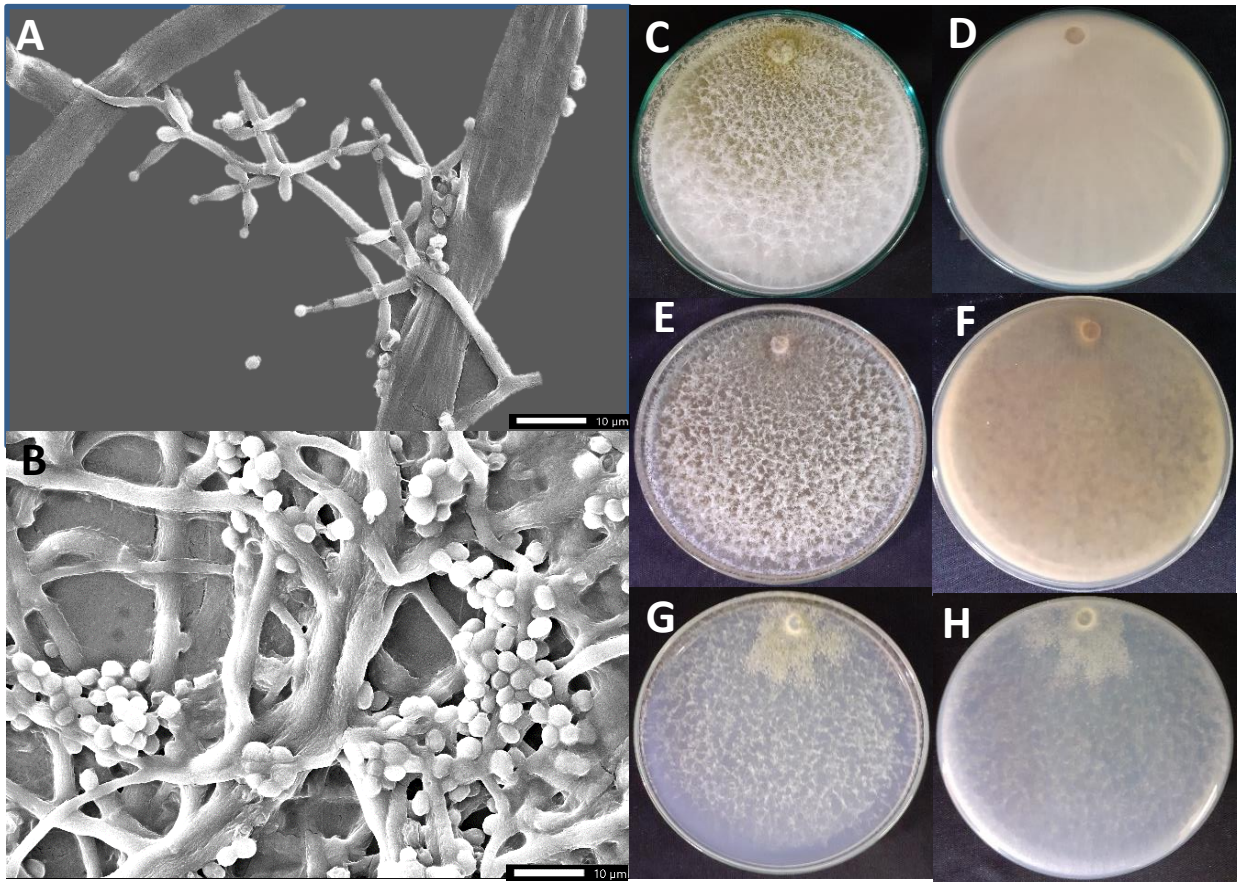


Figure S5. Morphological analysis of *T. afroharzianum* (T.70) by SEM – Colonies after five days of cultivation: A- phialides (10 µm); B- conidia (10 µm); C-D colony in PDA culture medium; E-F colony in malt medium; G-H colony in SNA medium. Conidia: Width: 1.5 - 2.4 µm (mean: 2.02 µm); Height: 2.0 - 3.0 µm (mean: 2.46 µm). Phialides structure: Width: 1.21 - 3.0 µm (mean: 2.11 µm); Height: 4.0 - 10.9 µm (mean: 8.8 µm).

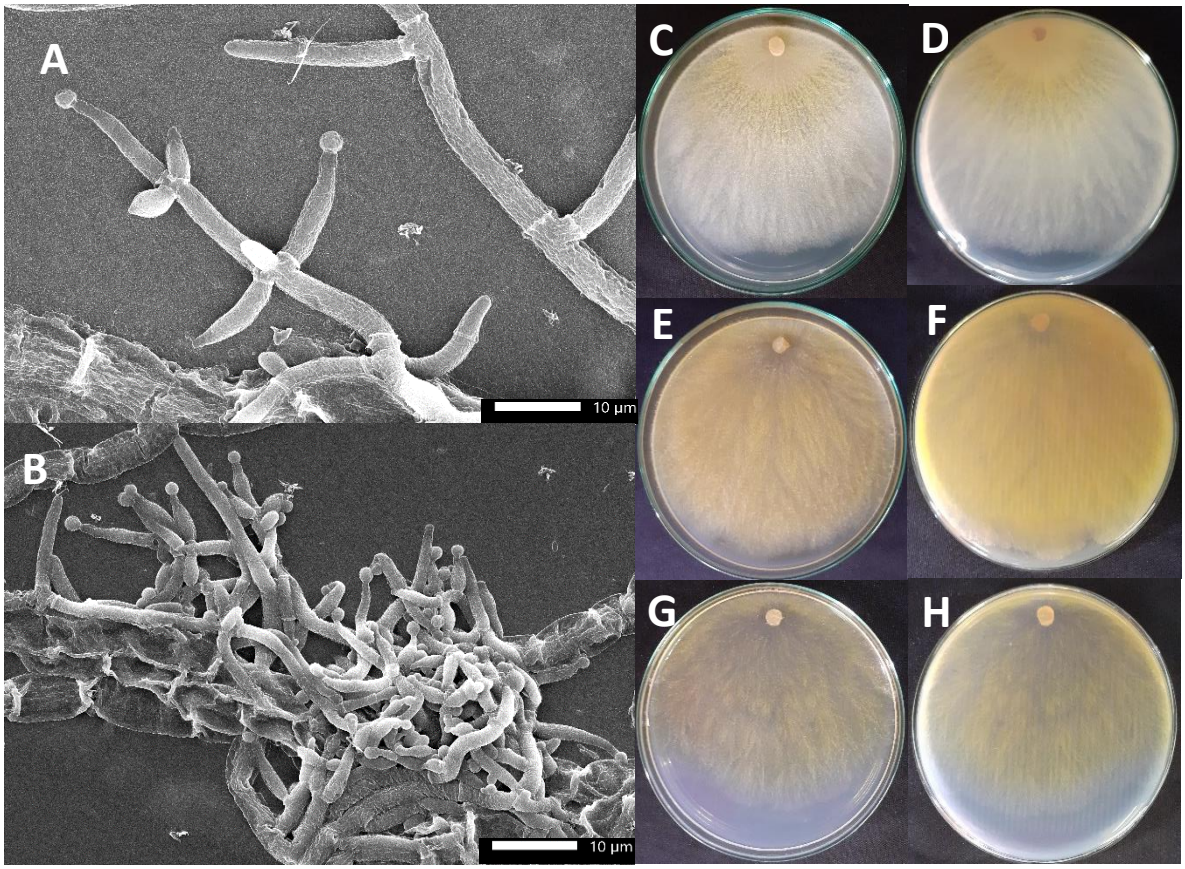


Figure S6. Morphological analysis of *T. afroharzianum* (T.88) by SEM - Colonies after five days of cultivation: A- phialides (10 µm); B- conidiophores (10 µm); C-D colony in PDA culture medium; E-F colony in malt medium; G-H colony in SNA medium. Conidia: Width: 1.7– 2.5 µm (mean = 2.02 µm); Height: 1.5 - 2.6 µm (mean: 2.16 µm). Phialides structure: Width: 1.0 - 1.5 µm (mean: 1.3 µm); Height: 3.1 - 7.8 µm (mean: 7.4 µm).

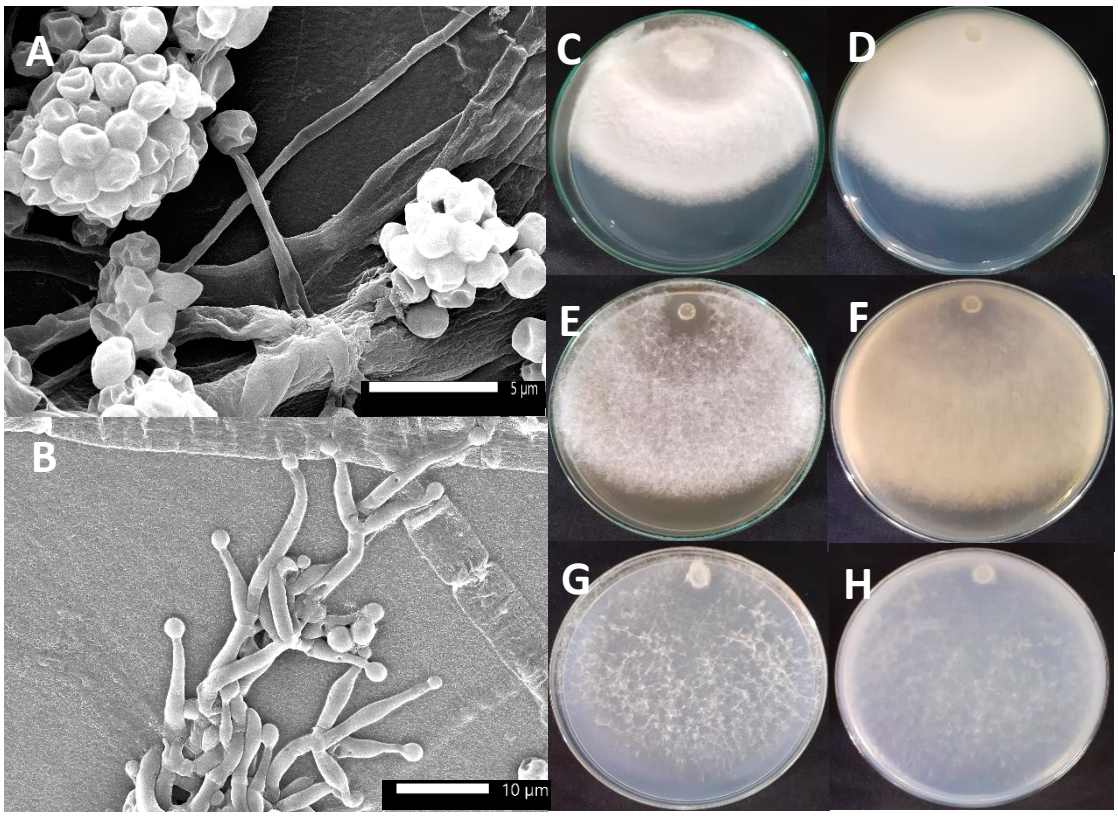


Figure S7. Morphological analysis of *T. afroharzianum* (T.531) by SEM - Colonies after five days of cultivation: A- conidia (5 µm); B-phialides (10 µm); C-D colony in PDA culture medium; E-F colony in malt medium; G-H colony in SNA medium. Conidia: Width: 1.4 - 2.3 µm (mean: 1.7 µm); Height: 1.6 - 2.61 µm (mean: 1.9 µm). Phialides structure: Width: 1.35 - 2.28 µm (mean: 1.70 µm); Height: 6.14 - 14.1 µm (mean: 9.73 µm).

CAPÍTULO 2

Novas asperelinas produzidas por um fungo endofítico *Trichoderma asperelloides* da planta aquática Amazônica *Victoria amazonica*

O presente capítulo refere-se aos objetivos seis e sete da tese e foi elaborado como manuscrito a ser publicado ao periódico Fungal Biology, um jornal internacional de pesquisa aplicada envolvendo fungos e organismos semelhantes a fungos (https://www.elsevier.com/journals/fungal-biology/1878-6146/guide-for-authors). Na sequência, apresentamos um resumo em língua portuguesa, o manuscrito em língua inglesa e os materiais suplementares de forma a viabilizar uma análise mais completa do que será publicado.

Resumo

Objetivo: identificar peptaibois produzidos por *Trichoderma asperelloides*, uma cepa endofitica de *Victoria amazonica*. Adicionalmente, avaliar o potencial antimicrobiano e citotóxico das frações contendo peptaibois.

Metodologia: as substâncias foram identificadas por LC-HRMS/MS em um modo de aquisição positivo, usando HCD para fragmentação. O potencial antimicrobiano foi avaliado por método de difusão em ágar e determinado por ensaios de concentração mínima inibitória, os ensaios de atividade citotóxica foram realizados por técnica Alamar Blue em células do tipo VERO.

Resultados e discussão: quinze asperelinas de dez resíduos de aminoácidos foram identificadas, oito dessas asperelinas são peptaibois do modelo Ac-Aib-xxx-Val-Aib-xxx-Aib-xxx-xxx-Aib-Prolinol. As outras sete têm o tipo raro de sequência com terminação em prolina: Ac-Aib-xxx-Val-Aib-xxx-Aib-Aib-xxx-Aib-Prolina. Nove sequencias de asperelinas são novas e foram identificadas juntamente com as conhecidas A, D, E, O, U e Z_{11}. A fração Fr19 (contendo asperelinas) apresentou alta atividade contra *L. monocytogenes* com o mesmo potencial do padrão usado, TIENAM, bem como apresentou alta viabilidade celular com baixa viabilidade de morte contra células do tipo VERO.

Conclusões: a linhagem T.145 foi capaz de produzir não apenas asperelinas com C-terminal prolinol (mais comuns) como também seis novas asperelinas com C-terminal prolina, provavelmente precursoras dos peptaibois estruturalmente semelhantes encontrados. A produção de asperelinas por *T. asperelloides* pode ter um significado quimiotaxonômico na relação de proximidade com *T. asperellum*, confirmando-a.

Contribuição do estudo: este é o primeiro relato de asperelinas produzidas por uma cepa de *T. asperelloides* e o primeiro relato de peptaibois para *Trichoderma* da região.

Palavras-chave: Fungo Amazônico; *Trichoderma*; Peptaibois; Peptaibes; Asperelinas; Espectrometria de massas.

Novel asperelines produced by an endophytic fungus *Trichoderma asperelloides* from the Amazonian aquatic plant *Victoria amazonica*

Abstract

In the present work, we report the first identification of asperelines produced by *Trichoderma asperelloides*, an endophytic strain from *Victoria amazonica*. Fifteen asperelines with ten amino acid residues were identified by LC-HRMS/MS in a positive acquisition mode, using HCD for fragmentation. Eight of these asperelines are peptaibols of the model Ac-Aib-xxx-Val-Aib-xxx-Aib-xxx-xxx-Aib-Prolinol. The other seven have the rare proline-ending sequence type: Ac-Aib-xxx-Val-Aib-xxx-Aib-Aib-xxx-Aib-Proline. Nine new asperelines were identified, together with the known asperelines A, D, E, O, U, and Z_{11}. A chemotaxonomic significance of the findings and a name “peptaibes” to the aspereline types ending in amino acids are suggested. The asperelines-containing fraction Fr19 showed activity against *Streptococcus mutans* and *Staphylococcus aureus*, with a minimal inhibitory concentration of 1000 $\mu g.mL^{-1}$ for both pathogens. Fr19 also presented high activity against *Listeria monocytogenes,* with the same potential of the standard TIENAM, as well low mortality against VERO-type cells.

Keywords: Amazonian fungus; *Trichoderma*; Peptaibols; Peptaibes; Asperelines; Mass spectrometry.

1 Introduction

The genus *Trichoderma* has been reported in different parts of the world. New species, functions, and metabolites have been discovered for it. More than 300*Trichoderma* species have been listed and cataloged (Kredics et al., 2012; Samuels, 2006). They can parasitize, antagonize, and kill other fungi in plants and are used as biocontrol agents of plant diseases (Bettiol, 2011; Fipke, 2015; Kubicek et al., 2011). They are producers of various volatile and nonvolatile compounds and enzymes (V. Gupta, M. SCHMOLL, A. HERRERA-ESTRELLA, R. UPDHAYAY, I. DRUZHININA, 2014).

T. asperelloides and *T. asperellum* are cryptic sister species, almost morphologically and chemotaxonomically indistinguishable. However, they can be differentiated one each other by molecular biology (Samuels et al., 2010). Their close resemblance also suggests a similar metabolism between them (Samuels et al., 2010; Sumida et al., 2018). They are relevant biocontrol fungi, also used to promote plant growth and to treat soil diseases caused by several microorganisms. *T. asperelloides* is already commercialized as TRICHO PLUS Biofungicide® in Africa, aiming to improve the development of the crops (dos Santos et al., 2017).

Various *Trichoderma* species are capable of producing peptaibols, a family of linear peptides contained residues of diverse non-proteinogenic amino acid, mainly the most common residue, the α-aminobutyric acid (Aib) (Ren et al., 2013). Aib is biogenetically depicted as derived from 1-alanine in a methyltransferase reaction, using adenosyl-methionine as a Me donor (Kubicek et al., 2007). Peptaibols have N-terminal alkyl groups, normally the acetyl one, and C-terminal amino alcohol groups, including valinol or prolinol (Brito et al., 2014; Daniel and Rodrigues Filho, 2007; Marinas et al., 2010). While there is no report of molecules characterized for *T. asperelloides*, for *T. asperellum*, subfamilies of peptaibols trichotoxins and asperelines have already been described (Chutrakul et al., 2008; Chutrakul and Peberdy, 2005; Ren et al., 2013, 2009; Tamandegani et al., 2016).

Peptaibols are described with significant antibacterial, antifungal, antiparasitic, and antiviral bioactivities. However, they have high toxicity to human cells ("Bessler et al, Ecology 2009.pdf," n.d., "Yun et al 2003 eds1," 2003; Peltola et al., 2004; Rebuffat et al., 1995), difficulting their application as drugs. These properties may change according to the number, types, and sequence of the amino-acids, that is, analogs can have different properties (Oh et al., 2005).

Peptaibols are frequently produced with high diversity and are molecules very hard to isolate since they occur as complex mixtures of analogs with high structural and chemical-behavior similarities. The difficulty for isolate peptaibols is as greater as minors are their sequences (Ren et al., 2013). For their structural determination, it is necessary to associate chemical techniques, mostly the combination of HPLC and mass spectrometry (Delmar et al., 2008). Besides, for unambiguous structural determination, a high-resolution mass spectrometer is required for precursor and product ions detection, promoting accurate sequencing (Daniel and Rodrigues Filho, 2007). The use of mass spectrometers with orbitrap analyzers is advantageous because they are capable of generating spectra with high mass precision and resolution. The use of the hybrid mass spectrometers, such as the quadrupole and orbitrap present in the Q Exactive, permits high-speed performance in the acquisition of MS/MS facilitating the interpretation and elucidation of amino acid residues (Delmar et al., 2008).

Asperelines are reported as peptaibols produced by *T. asperellum.* They have nine or ten amino acid residues and, as expected, almost all with a C-terminal amino alcohol. Only the aspereline Z_{11} presents a C-terminal does not reduced to alcohol, the amino acid proline

(Ren et al., 2013). In the present work, we report the determination of fifteen aspereline sequences produced by *T. asperelloides*, an Amazonian endophytic fungal strain isolated from the aquatic plant *Victoria amazonica* (Poepp. J.C. Sowerby, 1787-1871). We also evaluated the antimicrobial and cytotoxic potential of the fungus asperelines-containing fraction Fr19.

2. Materials and Methods

2.1 Chemicals

All solvents, including methanol (MeOH), ethyl acetate (EtOAc), 2-propanol (2-PrOH), hexane (Hex), ethanol (EtOH), and acetonitrile (ACN) were HPLC-grade for chromatography, purchased from Tedia (Mexico City, DF, Mexico). Formic acid (99%) was provided by Acros Organics (NJ, USA). Ammonium acetate (98%) was provided by Biotecnologia (PR, BRA). Ultrapure water (18.2 MΩ.cm) was obtained from a Milli-Q gradient system (Millipore, Milford, MA, USA).

2.2 Endophytic fungus strain

The fungal strain (T.145) was isolated from the amazon aquatic plant *Victoria amazonica*, collected in Careiro city, Amazon State, Brazil. Brazilian SISGEN registration: A39C76B. Concatenated phylogenetic analysis using sequences of Internal Transcribed Spacer (ITS) regions, portions of the translation elongation factor 1-α gene (*TEF-1-α*), and RNA Polymerase II subunit (RPB2) was carried out with MrBayes via the CIPRES Science Gateway. The strain T.145 was identified as *T. asperelloides*, and the sequences are deposited in Genbank, accession numbers MT210100, MT281578, and MT282579.

2.3 Culture

The fungal strain was cultured in 27 L potato dextrose 0.2% yeast extract broth (PDY) (Souza et al., 2004). 50 µL conidia suspension (McFarland No. 6) were inoculated in 1000 mL conical flasks containing the liquid medium (300 mL/flask) and incubated for 20 days at 28 °C under static conditions. After cultivation, the culture broth was sieved in a laboratory cap for separation from mycelium.

2.4 Obtainment and fractionation of the cultured broth extract

The cultured broth was partitioned with EtOAc/2-PrOH (9:1, v/v), and the organic phase was evaporated under reduced pressure to remove the solvents. The extract was dried, weighed (3.800 mg), and stored at 4 °C. The extract (145CB) was chromatographed on a 1.8 cm x 7 cm open column (silica gel, 200-400 mesh, Sigma Aldrich), eluted under vacuum with Hex/EtOAc/MeOH/H_2O gradient to yield 24 fractions.

The 100% MeOH fraction (Fr19, 484.6 mg) was subjected to a 0.63 cm x 1 cm open column (C18 SPE, 55 µm, Strata E, Phenomenex), eluted under vacuum with H_2O/MeOH mixtures (1:1, 3:7, 2:8, 1:9, and 0:1, v/v) to obtain five fractions (A–E). The peptaibols-containing fraction D (154.6 mg), according to analyses by mass spectrometry (TSQ Quantum Acess, Thermo Scientific) and ^{1}H NMR (500.13 MHz, DMSO, Avance III HD, Bruker), was further fractionated on a 0.38 cm x 1.3 cm open column (C18 SPE, 33 µm, Strata X, Phenomenex), eluted with H_2O/MeOH (2:8, 1:9, 0.5:9.5, and 0:1, v/v), to yield four subfractions (D1-D4).

2.5 HPLC semi-purification

Fraction D3 (58 mg) was chromatographed in a semi-preparative HPLC equipment (LC 6AD, Shimadzu), equipped with a 10 mm x 250 mm column (C18, 5 µm, Luna, Phenomenex), eluted with 54% EtOH/H_2O with 0.1% ammonium acetate to yield 10 fractions (D3.1-D3.10).

2.6 LC-ESI-MS/MS analysis

The peptaibols-containing fractions D3.1, according to preliminary ^{1}H NMR and ESI-MS analyses, were analyzed by nLC-nESI-MS/MS system, in an Easy-nLC 1000 nano-LC system coupled to a quadrupole-orbitrap (Q-Exactive Plus) mass spectrometer equipped with a nanoelectrospray ion source (Thermo Scientific). The sample at 0.5 µg/µL was dissolved in 100 µL of methanol with 0.1% FA, loaded onto a homemade trap column 2 cm x 150 µm (C18 ReprosilPur, 5 µm) with a flow rate of 200 µL/min, and separated on a homemade analytical column 25 cm x 75 µm (C18 ReprosilPur, 3 µm), with a constant flow rate of 200 nL/min and gradient of B (95% ACN, 0.1% FA) and A (5% ACN, 0.1% FA). B was changed as follows: 10-50% in 12 min, 50-90% in 18 min, 90-98% in 10 min, and 98%

for 10 min. Mass spectrometer conditions: positive ionization; *capillary voltage:* 3 kV; *sheat gas flow rate:* 0; *auxiliary gas flow rate:* 0; *sweep gas flow rate:* 0; *capillary temperature:* 320 °C; *s-lens RF level*: 65.0; *auxiliary gas heater level:* 0 °C; *mass range: m/z* 200 to 2500. It was used data-dependent analysis (DDA), with dynamic exclusion of 45 ms and resolution of 70.000, where the 20 most intense ions were selected to be fragmented with high energy collisional dissociation (HCD), working at normalized collision energies (NCE) of 10, 20, and 40, with a resolution of 17.500 in MS/MS scans.

2.7 Bacterial pathogens and inoculum standardization

The human bacterial pathogens *Staphylococcus aureus* (ATCC 33591), *Streptococcus mutans* (ATCC 25175), and *Listeria monocytogenes* (ATCC 15313) were from the library of the Biotechnological Bioassay Platform (RPT11H), of the Leônidas and Maria Deane Institute (ILMD), Oswaldo Cruz Foundation (FIOCRUZ). These bacterial strains were cultivated in Brain Heart Infusion (BHI) broth (HIMEDIA, Mumbai, India) and diluted in the same culture broth to 0.5 on the McFarland scale (1×10^8 CFU.mL^{-1}). All tests were performed in triplicate.

2.8 Agar diffusion bioassay

The agar-diffusion bioassay was carried out according to Balouiri and coworkers (Balouiri et al., 2016) with modifications. Briefly, the bacteria were diffused onto the culture medium Müeller Hinton Agar (MHA) (HIMEDIA) in Petri dishes containing 6 mm diameter wells, 50 µL of the sample at 1000 µg.mL^{-1} in 10% (v/v) DMSO were added to each well, and the dishes were incubated at 37 °C for 24 h. Subsequently, 10 mL of the 0.01% dye solution of triphenyl tetrazolium chloride (CTT) and 0.1% of bacteriological agar mixture was added. After 30 minutes, the inhibition halos were measured. 500 µg.mL^{-1} TIENAM (imipenem and cilastatin sodium) solution was the positive, and 10% (v/v) DMSO, the negative control.

2.9 Minimal Inhibitory Concentration assay

The MICs were determined using a stock 10% (v/v) DMSO solution of the sample Fr19 at 2000 $\mu g.mL^{-1}$, diluted in series to obtain the concentration range of 1000 to 7.81 $\mu g.mL^{-1}$ in 96-well plates: 100 µL of Mueller Hinton Broth (MHB) was added into each well and 100 µL of the sample solution at the first well; after to homogenize, 100 µL from this well were transferred to the next, following this procedure until the last well; 100 µL from this well were discarded. After, 20 µL of the bacterial suspensions (1×10^8 $UFC.mL^{-1}$), 20 µL of 0.01% resazurin, and 60 µL of MHB were added to each well. After incubating the plates at 37 °C for 24 h, the bioactive in the wells were recorded as blue. The negative control was the 2.5% DMSO and the positive control was the TIENAM in the same conditions of the samples (Koolen et al., 2012).

2.10 Cytotoxic assay

The VERO strain (ATCC® CCL-81™) was acquired and grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco), supplemented with 10% inactivated fetal bovine serum (Gibco) and penicillin (50 µg/mL). The test was determined by the Alamar Blue method (Ansar Ahmed et al., 1994). Briefly, 100 µL DMEM with 1.0×10^4 cells/well were plated in 96-well plates, samples were added in serial dilution to achieve the concentration range of 1000 to 125 $\mu g.ml^{-1}$, and the plates were kept in a CO_2 incubator for 24 h at 5% CO_2 at 37 ºC. After, 10 µL of 0.4% resazurin (1:20 v/v) were added to each well and, after 2 h the fluorescence was monitored in a microplate reader (GloMax Explorer), at 580-640 nm and excitation at 520 nm. Cell growth was used as a positive control and 0.1% DMSO as a negative control. The percentage of cell viability was calculated: % Viability = Ft x 100/Fb, Where: Ft = (cell fluorescence + medium + substance + resazurin); Fb = (cell fluorescence + medium + resazurin) (Ansar Ahmed et al., 1994).

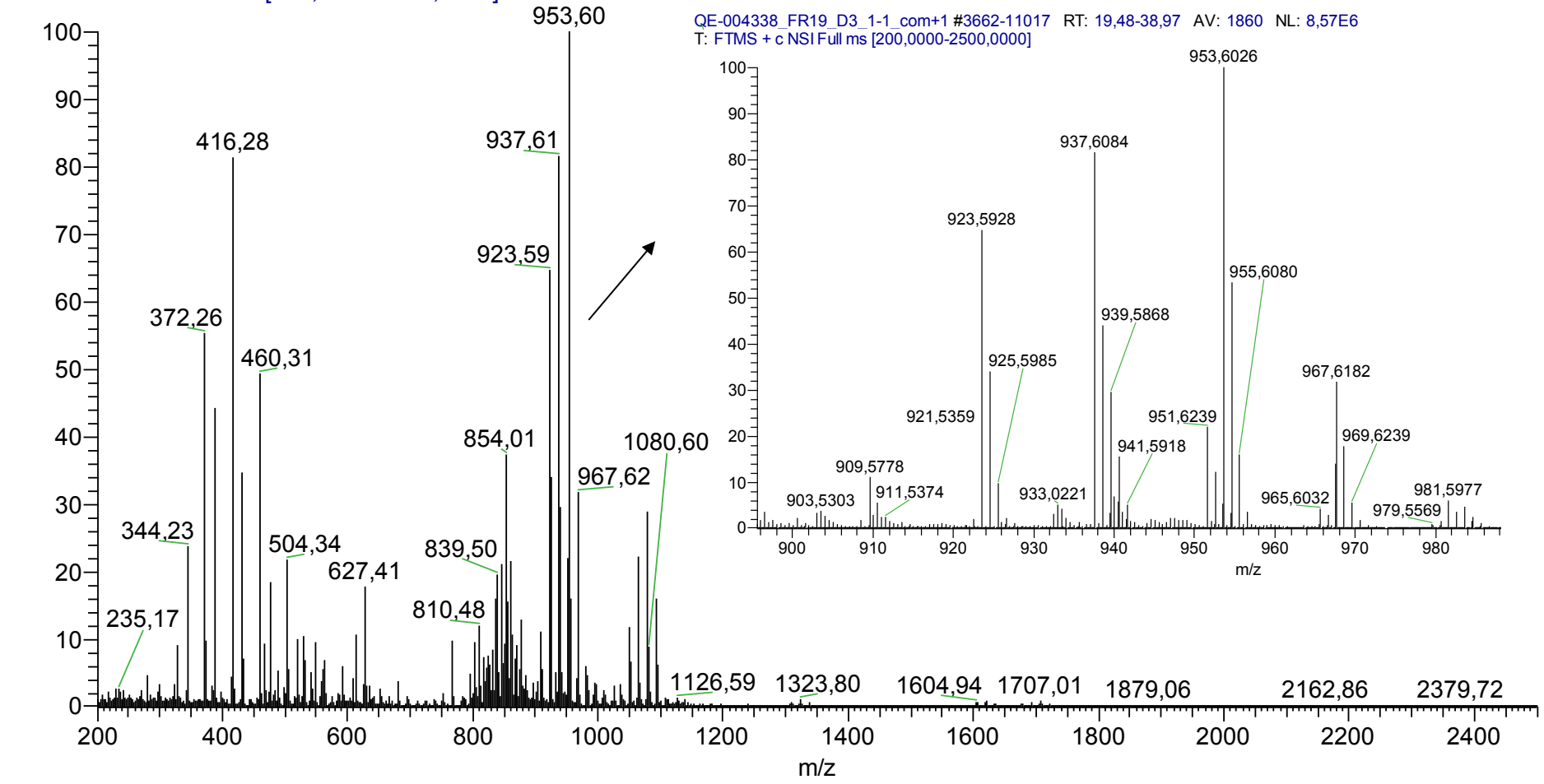


Fig 1. Full-scan at *m/z* 200 to 2500 of the peptaibols-containing fraction D3-1 in positive ion mode. Highlighted, the range of the asperelines.

3. Results

The preliminary analysis of the full-scan spectrum of the peptaibols-containing fraction D3-1 permitted to detect six main potential ion clusters of asperelines at *m/z* 909.57, 923.59, 937.60, 953.60, and 967.61 (Fig. 1). The MS^2 analyses of these ion clusters and their larger fragment ions, and the consideration of the exact mass of each molecular ion and respective fragment ions permitted to identified fifteen aspereline molecules, nine for the first time described. For the exact mass was taking into account an error tolerance of 5 ppm. The fragmentation spectra of the fragment ions were especially useful for analyzing minority components in isobaric coelutions.

As examples of this methodology, we describe the interpretations of the aspereline peptides (D, U, and Z_{16}) with a mass peak around 923.6. The MS/MS fragmentation of the ($[M+H]^+$) *m/z* 923.5928 ion (34.36 min; molecular formula $C_{44}H_{78}N_{10}O_{11}$; calculated molecular mass: 922.5852 u; mass error: 0.1) provided a group of b-type fragment ions, which included a 213.1234 Da ion, characteristic to the acetylated N-terminal residue containing two Aib units and an 822.5088 Da ion, corresponding a loss of a prolinol residue as C-terminal. Further, the analysis of the other fragment ions permitted to identify the complete sequence as Ac-Aib-Aib-Val-Aib-Val-Aib-Aib-Ala-Aib-Prolinol (Table 1; Figure 6 in the Supplementary Material). The choice for valine instead of the isovaline residue was done after observing the ^1H NMR of the fraction D3-1 compared to other studies reported with asperelines (Chen et al., 2013; Ren et al., 2009). This sequence was identified as aspereline D (Ren et al., 2009).

An isobaric ion of aspereline D (34.36 min; mass error: 0.2) presented the same acetylated N-terminal residue containing two Aib units (*m/z* 213.1235 Da) and also prolinol as C-terminal. However, they are different in the fifth and eighth positions. While aspereline D has valine and alanine, its isobaric molecule has isoleucine or leucine (Lxx) and glycine respectively. The choice between isoleucine and leucine residues was not possible in the ^1H NMR of the fraction D3-1 due superposition of signals. Therefore, the complete sequence of this isobaric molecule of aspereline D is Ac-Aib-Aib-Val-Aib-Lxx-Aib-Aib-Gly-Aib-Prolinol (Table 1). This sequence is novel and was named as aspereline Z_{16}.

Another isobaric ion of aspereline D (32.84 min; mass error: 0.2) was identified as aspereline U, which differs to aspereline D into the second (Ala instead Aib) and fifth positions (Lxx instead Val). Its sequence already reported is Ac-Aib-Ala-Val-Aib-Lxx-Aib-Aib-Ala-Aib-Prolinol (Table 1) (Ren et al., 2013). All the other aspereline were similarly characterized.

Fr19 presented moderate inhibition against *Streptococcus mutans* and *Staphylococcus aureus* in the agar diffusion assay. Its inhibitory halos were 10.3 and 11.7 mm, respectively. This moderate activity was also confirmed in the minimal inhibitory concentration assay with MIC of 1000 $\mu g.mL^{-1}$ for both strains.

On the other hand, Fr19 presented for *L. monocytogenes* a MIC of 125 $\mu g.mL^{-1}$, the same as the reference drug TIENAM. In the cytotoxic activity assay, this fraction showed IC50 of 0.39 $\mu g.mL^{-1}$.

4. Discussion

In this study, fifteen asperelines were characterized in a micro heterogeneous mixture produced by the strain *T. asperelloides* (T.145). As expected, spectra overlap was observed, revealing more than one sequence in a single mass spectrum, hindering the elucidation process and hardly precluded their purification.

Most of the sequences were consistent with our inclusion criteria present above in the results part. However, the aspereline sequences Z_{11}, Z_{17}, Z_{20}, and Z_{22} presented the mass errors > 5 ppm. Aspereline Z_{11} (mass error 37.3 ppm) was considered because is already known (Ren et al., 2013). Aspereline Z_{17} (mass error 37.8 ppm) and aspereline Z_{20} (mass error 6.0 ppm) were considered due to their high similarities with the asperelines Z_{14} and D, differing only in the C-terminus by change proline by prolinol. It can be to infer that asperelines Z_{17} and Z_{20} are precursors of the asperelines Z_{14} and D, respectively, also identified in the sample. Finally, aspereline Z_{22} (mass error 32.4 ppm) was considered because, despite its mass error, like the previous ones, its fragments were consistent. The higher mass errors of the asperelines Z_{11}, Z_{17}, Z_{20}, and Z_{22} were attributed to their low concentrations in the mixture and overlap with other isobaric isoforms.

The structures of the entire aspereline sequences are shown in table 2, all with ten amino acid residues. In common they present the first, third, fourth, sixth, and ninth positions, with Aib, Val, Aib, Aib, and Aib residues, respectively. In the second position, some present Ala residue although most asperelines has Aib residue. In the fifth position, the Lxx residue is present more frequently while some present the Val residue. In the eighth position Gly, Ala, and Ser occur. Finally, only the aspereline O, instead of Aib residue, presents the Ala residue in the seventh position. The conservation or not of the amino acid residues above observed is in general determined by the binding domains of the nonribosomal peptide synthetases (NRPSs) that produce the peptaibols and other nonribosomal peptides (Mukherjee et al., 2011; Stoppacher et

al., 2013 (Stachelhaus et al., 1999). Preferentially, amino acids that occupy not conserved positions are close in size and result in a mixture of peptides and so homolog sequences. For example, the pocket for Aib can also accept Iva, Val, or Ala (Mukherjee et al., 2011; Stoppacher et al., 2013), as in the second and seventh positions in this work (Table 2).

The asperelines found in this work forme two groups: one ending in prolinol and the other ending in proline at C-terminus (Table 2). The first group, with 53.33% of the sequences identified, has eight asperelines: Z_{14}, Z_{15}, Z_{16}, O, U, D, A, and E. The second group, 46.67%, has seven asperelines: Z_{11}, Z_{17}, Z_{18}, Z_{19}, Z_{20}, Z_{21}, and Z_{22}. The asperelines Z_{14}-Z_{22} are novel. While C-terminal prolinol is common, it was reported before only one aspereline with C-terminal proline, the aspereline Z_{11} [10]. The identification of six new asperelines ending in amino acid instead of amino alcohol suggests that other similar compounds may be found in the future. Since the term peptaibol sounds inappropriate, we propose to name “peptaibes” Aib-containing peptides that end in amino acids, like the above second group of asperelines.

The bacterial tests revealed low activity of the asperelines-containing fraction Fr19 against the pathogenic strains of *Streptococcus mutans* and *Staphylococcus aureus*. *Streptococcus mutans* has been detected in the caries process, affecting the majority of the world population (Eriksson et al., 2018). *Staphylococcus aureus* has been found with high frequency in the skin and in the nasal passages of healthy people. However this bacterium can cause as simple as serious infections (Santos et al., 2007). On the other hand, the Fr19 activity was promising against *L. monocytogenes*, considered a threat to public health because it causes outbreaks of listeriosis in humans and animals (Tourdjman et al., 2014). In addition, at the same concentration that inhibits *L. monocytogenes*, the Fr19 fraction showed reasonable cytotoxicity, revealing a potential to be confirmed for asperelines.

Table 1. Asperelines produced by *Trichoderma asperelloides*.

N°	Asperelines	Observed ($[M+H]^+$)	Calculated ion mass	LC-MS/MS Rt (min)	Molecular formula	Mass error (ppm)	Sequence
1	Z_{14}	909.5771	909.5773	(31.23)	$C_{43}H_{76}N_{10}O_{11}$	0.2	Ac-Aib-Ala-Val-Aib-Lxx-Aib-Aib-Gly-Aib-Prolinol
2	O	909.5773	909.5773	(31.99)	$C_{43}H_{76}N_{10}O_{11}$	0.0	Ac-Aib-Aib-Val-Aib-Val-Aib-Ala-Ala-Aib-Prolinol
3	Z_{15}	909.5780	909.5773	(32.76)	$C_{43}H_{76}N_{10}O_{11}$	0.8	Ac-Aib-Aib-Val-Aib-Val-Aib-Aib-Gly-Aib-Prolinol
4	U	923.5927	923.5929	(32.84)	$C_{44}H_{78}N_{10}O_{11}$	0.2	Ac-Aib-Ala-Val-Aib-Lxx-Aib-Aib-Ala-Aib-Prolinol
5	Z_{16}	923.5927	923.5929	(32.84)	$C_{44}H_{78}N_{10}O_{11}$	0.2	Ac-Aib-Aib-Val-Aib-Lxx-Aib-Aib-Gly-Aib-Prolinol
6	D	923.5928	923.5929	(34.36)	$C_{44}H_{78}N_{10}O_{11}$	0.1	Ac-Aib-Aib-Val-Aib-Val-Aib-Aib-Ala-Aib-Prolinol
7	A	937.6085	937.6086	(34.06)	$C_{45}H_{80}N_{10}O_{11}$	0.1	Ac-Aib-Aib-Val-Aib-Ile-Aib-Aib-Ala-Aib-Prolinol
8	Z_{17}	937.6076	937.5722	(32.50)	$C_{44}H_{76}N_{10}O_{12}$	37.8	Ac-Aib-Aib-Val-Aib-Lxx-Aib-Aib-Gly-Aib-Proline
9	Z_{18}	937.5739	937.5722	(31.42)	$C_{44}H_{76}N_{10}O_{12}$	1.8	Ac-Aib-Ala-Val-Aib-Lxx-Aib-Aib-Ala-Aib-Proline
10	Z_{29}	937.5751	937.5722	(30.66)	$C_{44}H_{76}N_{10}O_{12}$	3.1	Ac-Aib-Ala-Val-Aib-Lxx-Aib-Aib-Ala-Aib-Proline
11	Z_{20}	937.5778	937.5722	(32.18)	$C_{44}H_{76}N_{10}O_{12}$	6.0	Ac-Aib-Aib-Val-Aib-Val-Aib-Aib-Ala-Aib-Proline
12	Z_{11}	951.6233	951.5878	(34.02)	$C_{45}H_{78}N_{10}O_{12}$	37.3	Ac-Aib-Aib-Val-Aib-Lxx-Aib-Aib-Ala-Aib-Proline
13	Z_{21}	953.5723	953.5671	(31.59)	$C_{44}H_{76}N_{10}O_{13}$	5.5	Ac-Aib Ala-Val-Aib-Lxx-Aib-Aib-Ser-Aib-Proline
14	Z_{22}	953.5980	953.5671	(32.60)	$C_{45}H_{76}N_{10}O_{13}$	32.4	Ac-Aib Aib-Val-Aib-Val-Aib-Aib-Ser-Aib-Proline
15	E	953.6032	953.6035	(33.35)	$C_{45}H_{80}N_{10}O_{12}$	0.3	Ac-Aib-Aib-Val-Aib-Ile-Aib-Aib-Ser-Aib-Prolinol

Table 2. Groupping of the aspereline sequences by similarities.

	N°	Asperelines	Ac	Position 1	2	3	4	5	6	7	8	9	10
1° group - Peptaibols	1	Z_{14}	Ac	Aib	Ala	Val	Aib	Lxx	Aib	Aib	Gly	Aib	Prolinol
	2	O	Ac	Aib	Aib	Val	Aib	Val	Aib	Ala	Ala	Aib	Prolinol
	3	Z_{15}	Ac	Aib	Aib	Val	Aib	Val	Aib	Aib	Gly	Aib	Prolinol
	4	U	Ac	Aib	Ala	Val	Aib	Lxx	Aib	Aib	Ala	Aib	Prolinol
	5	Z_{16}	Ac	Aib	Aib	Val	Aib	Lxx	Aib	Aib	Gly	Aib	Prolinol
	6	D	Ac	Aib	Aib	Val	Aib	Val	Aib	Aib	Ala	Aib	Prolinol
	7	A	Ac	Aib	Aib	Val	Aib	Ile	Aib	Aib	Ala	Aib	Prolinol
	15	E	Ac	Aib	Aib	Val	Aib	Ile	Aib	Aib	Ser	Aib	Prolinol
2° group - Peptaibes													
	8	Z_{17}	Ac	Aib	Aib	Val	Aib	Lxx	Aib	Aib	Gly	Aib	Proline
	9	Z_{18}	Ac	Aib	Ala	Val	Aib	Lxx	Aib	Aib	Ala	Aib	Proline
	10	Z_{19}	Ac	Aib	Ala	Val	Aib	Lxx	Aib	Aib	Ala	Aib	Proline
	11	Z_{20}	Ac	Aib	Aib	Val	Aib	Val	Aib	Aib	Ala	Aib	Proline
	12	Z_{11}	Ac	Aib	Aib	Val	Aib	Lxx	Aib	Aib	Ala	Aib	Proline
	13	Z_{21}	Ac	Aib	Ala	Val	Aib	Lxx	Aib	Aib	Ser	Aib	Proline
	14	Z_{22}	Ac	Aib	Aib	Val	Aib	Val	Aib	Aib	Ser	Aib	Proline

5. Conclusions

This report is the first on asperelines produced by a *T. asperelloides* strain, which was isolated as an endophytic fungus from the Amazonian aquatic plant *V. amazonica*. It was identified a total of fifteen aspereline sequences with ten residues. Three new and five known sequences have the most common C-terminal prolinol. However, we identified together to aspereline Z_{11} six novel asperelines with C-terminal proline, which are probably precursors to their correspondent peptaibols. The production by *T. asperelloides* of the same aspereline types reported for *T. asperellum* has chemotaxonomic significance, ratifying the proximity between these *Trichoderma* species. Besides, for the first time, possible applications were observed for an asperelines-contend fraction, since it was active against *Streptococcus mutans*, associated with the caries process, and *L. monocytogenes*, that causes listeriosis. This potential is highlighted by the low cytotoxicity of the asperelines-containing fraction Fr19 against Vero cells.

CRediT authorship contribution statement

Fernandes, K.R.P.: Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing. De Souza, A.Q.L.: Conceptualization, Formal analysis, Writing - original draft, Writing – review & editing. dos Santos, L.A.: Formal analysis. Nogueira, F.C.S.: Formal analysis, Writing – review & editing. Evaristo, J.A.M.: Formal analysis, Writing – review & editing. Carneiro, G.R.A.: Formal analysis. Silva, G.F.: Formal analysis, Writing – review & editing. Cruz, J.C.: Formal analysis. Sousa, T.F.; Formal analysis. Silva, S.R.S.; Formal analysis. Bastos, I.S.; Formal analysis. Orlandi, P.P.; Formal analysis. Souza, A.D.L; Conceptualization, Formal analysis, Funding acquisition, Supervision, Writing - original draft, Writing – review & editing.

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Declaration of competing interest: None.

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Yun et al 2003 eds1, 2003.

7. Supplementary material

Novel asperelines produced by an endophytic fungus *Trichoderma asperelloides* from the Amazonian aquatic plant *Victoria amazonica*

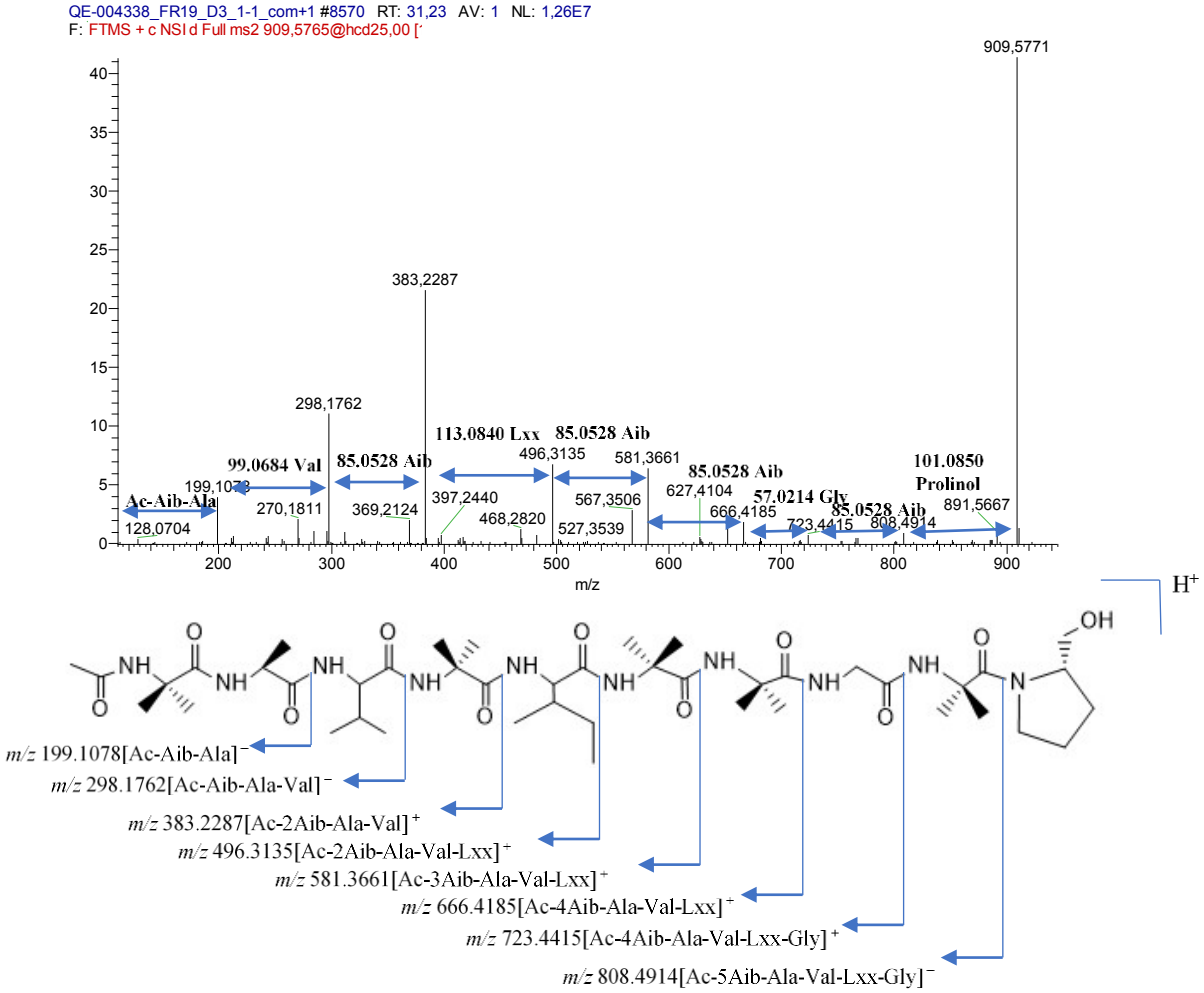
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FIGURE 1.

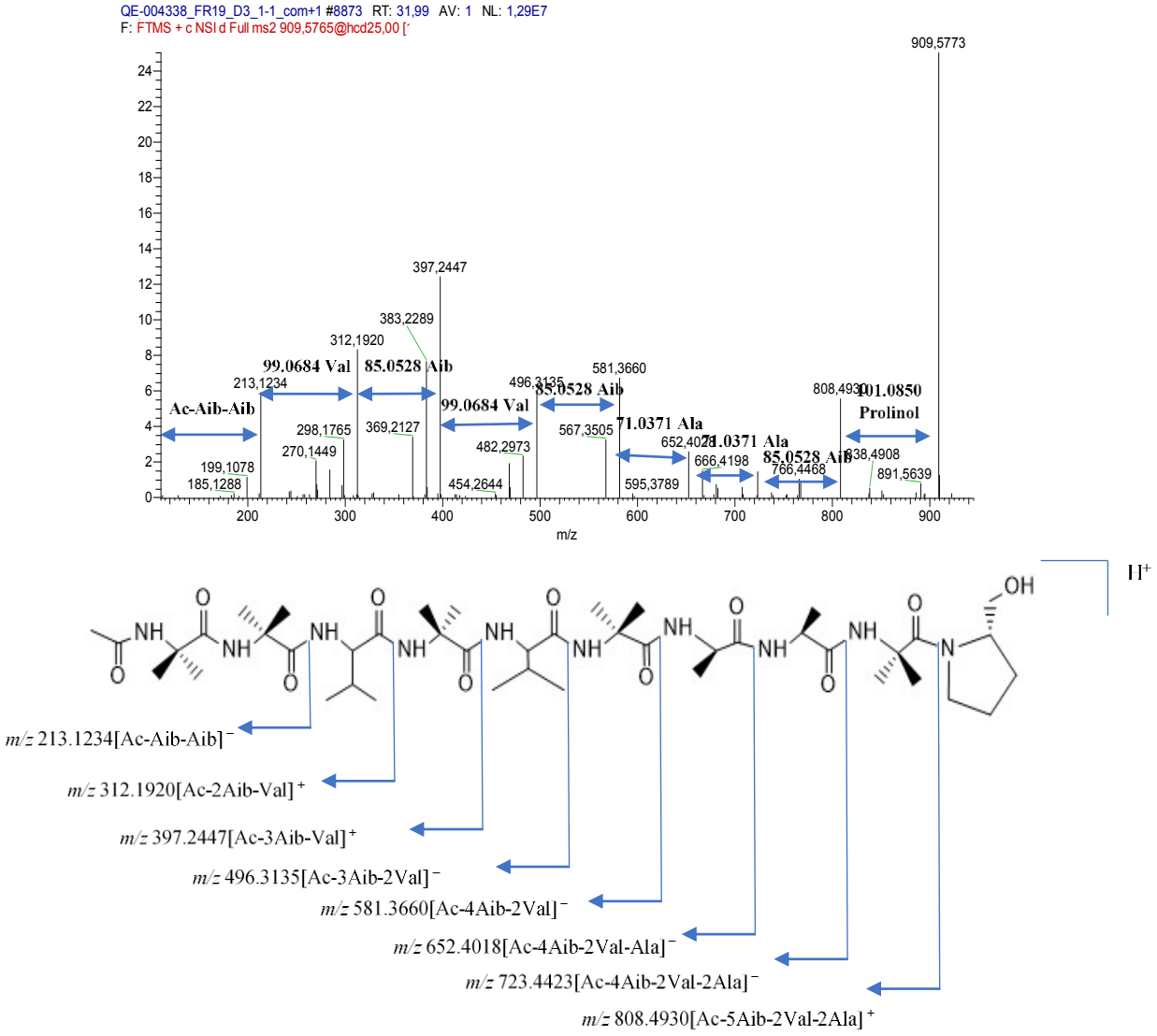
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FIGURE 2.

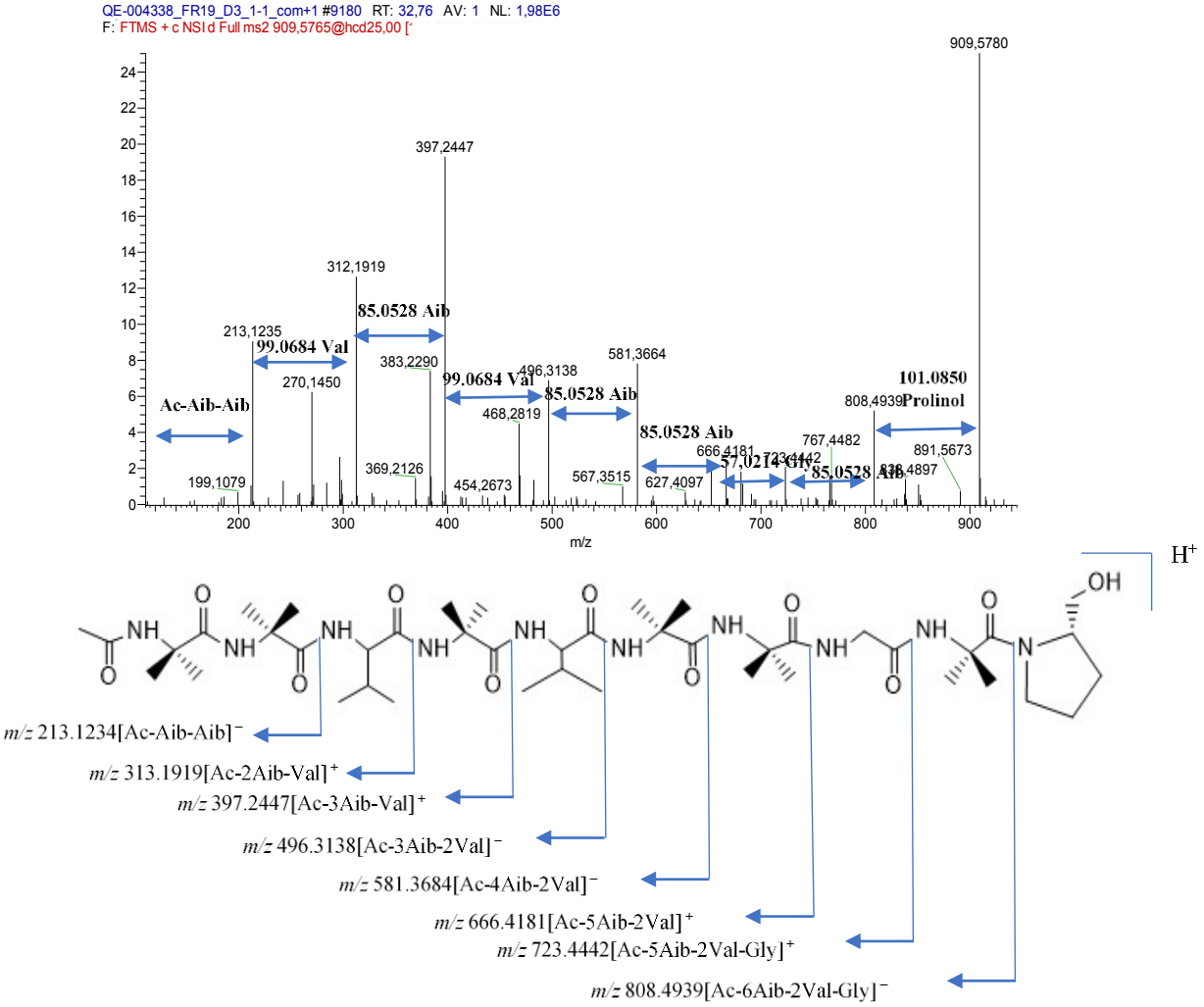
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FIGURE 3.

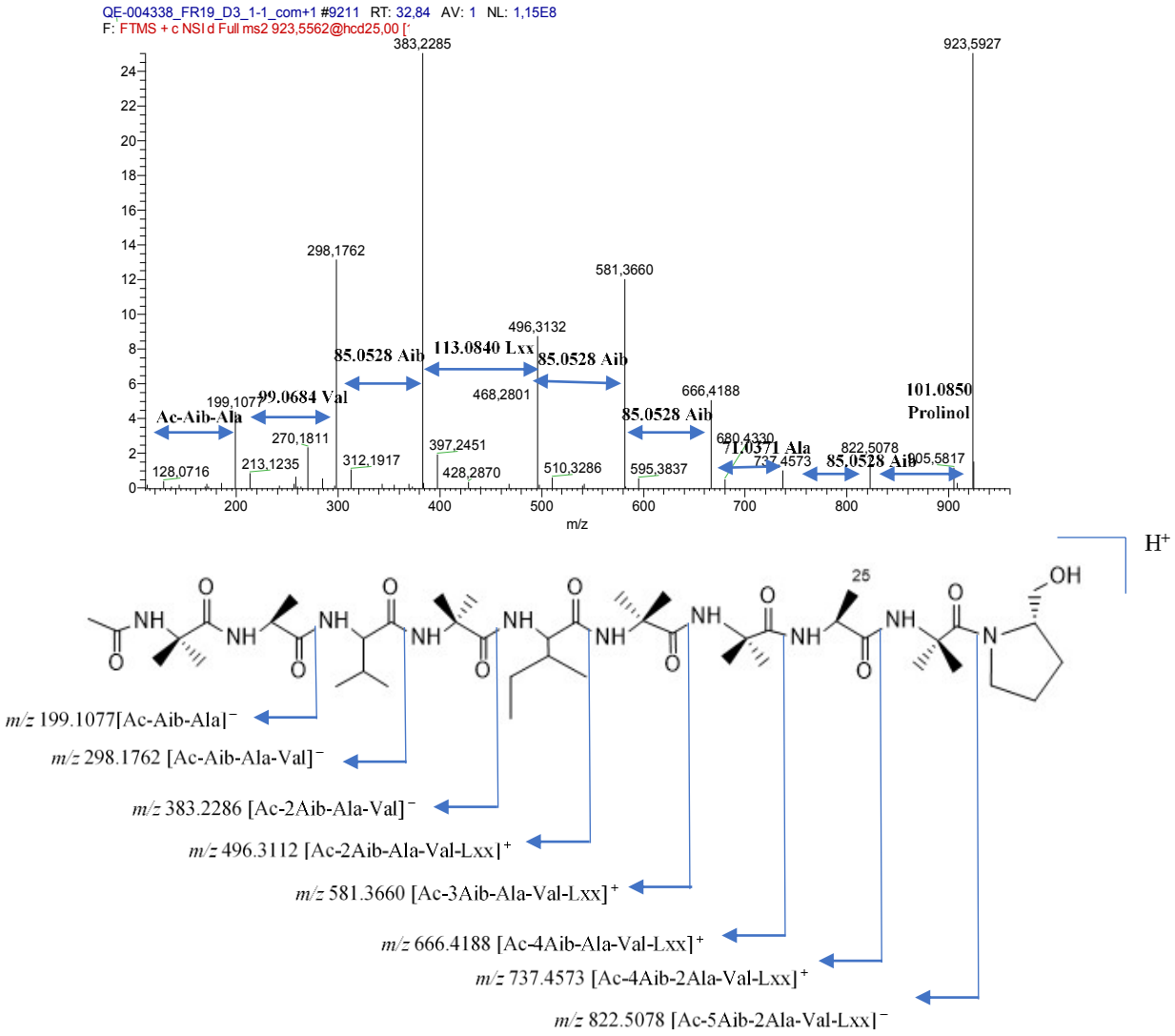
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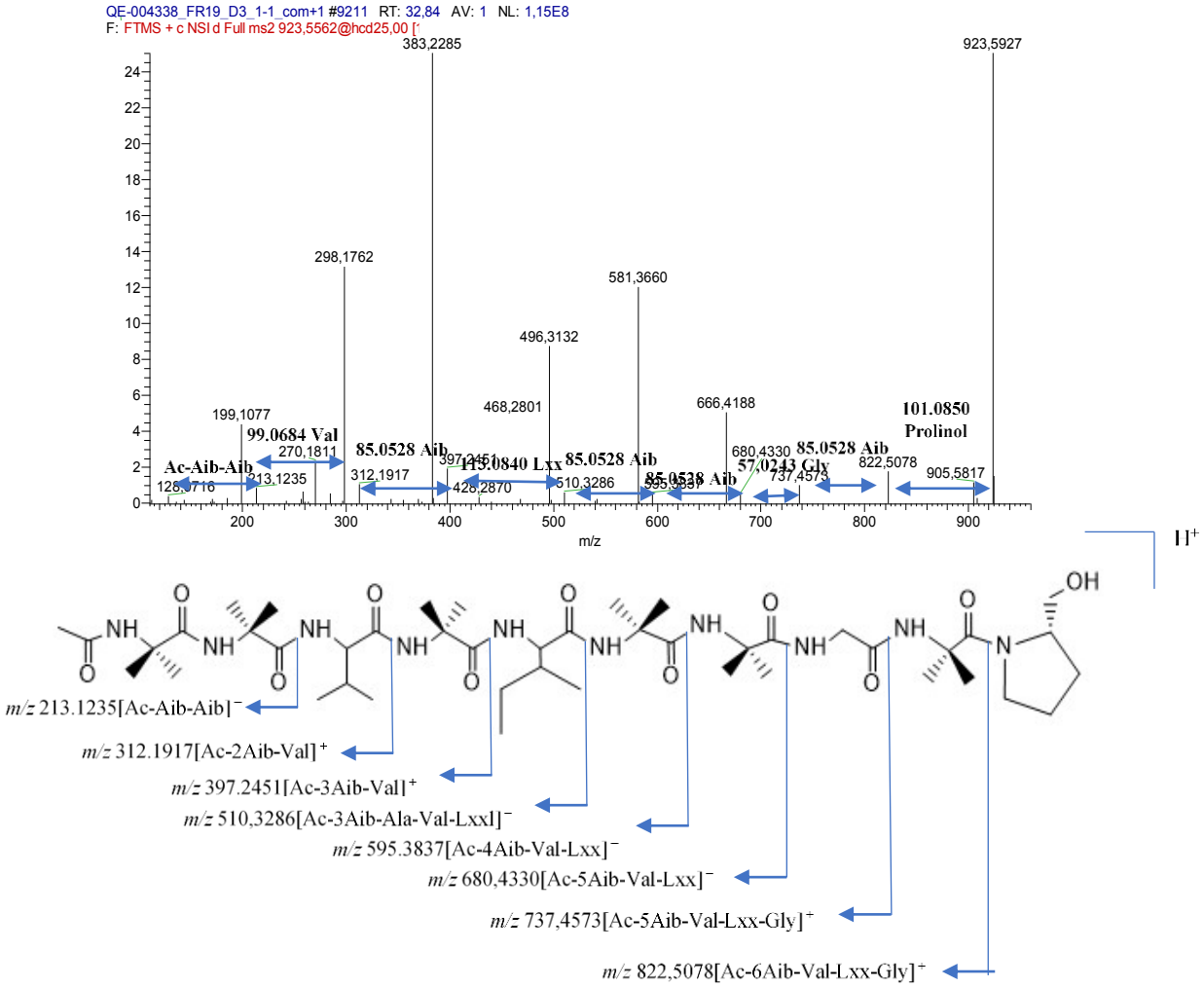
FIGURE 4.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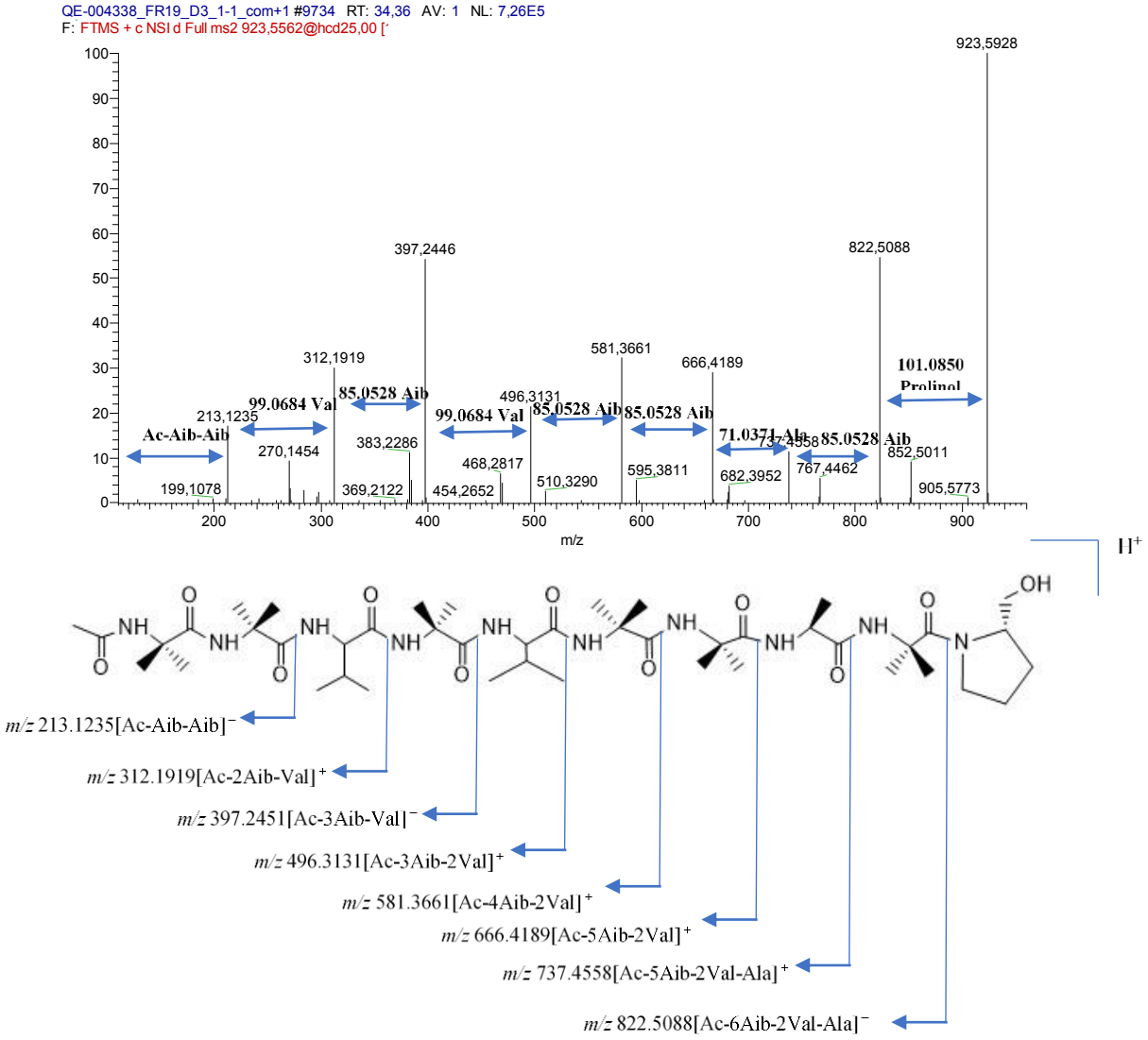
298,1762
581,3660
496,3132
468,2801
666,4188
199,1077
99.0684 Val
270,1811
85.0528 Aib
Ac-Aib-Aib
213,1235
312,1917
113.0840 Lxx
85.0528 Aib
510,3286
85.0528 Aib
57.0243 Gly
680,4330
737,4573
85.0528 Aib
822,5078
101.0850
Prolinol
905,5817
m/z
II+
m/z 213.1235[Ac-Aib-Aib]−
m/z 312.1917[Ac-2Aib-Val]+
m/z 397.2451[Ac-3Aib-Val]+
m/z 510,3286[Ac-3Aib-Ala-Val-Lxxl]−
m/z 595.3837[Ac-4Aib-Val-Lxx]−
m/z 680,4330[Ac-5Aib-Val-Lxx]−
m/z 737,4573[Ac-5Aib-Val-Lxx-Gly]+
m/z 822,5078[Ac-6Aib-Val-Lxx-Gly]+

FIGURE 5.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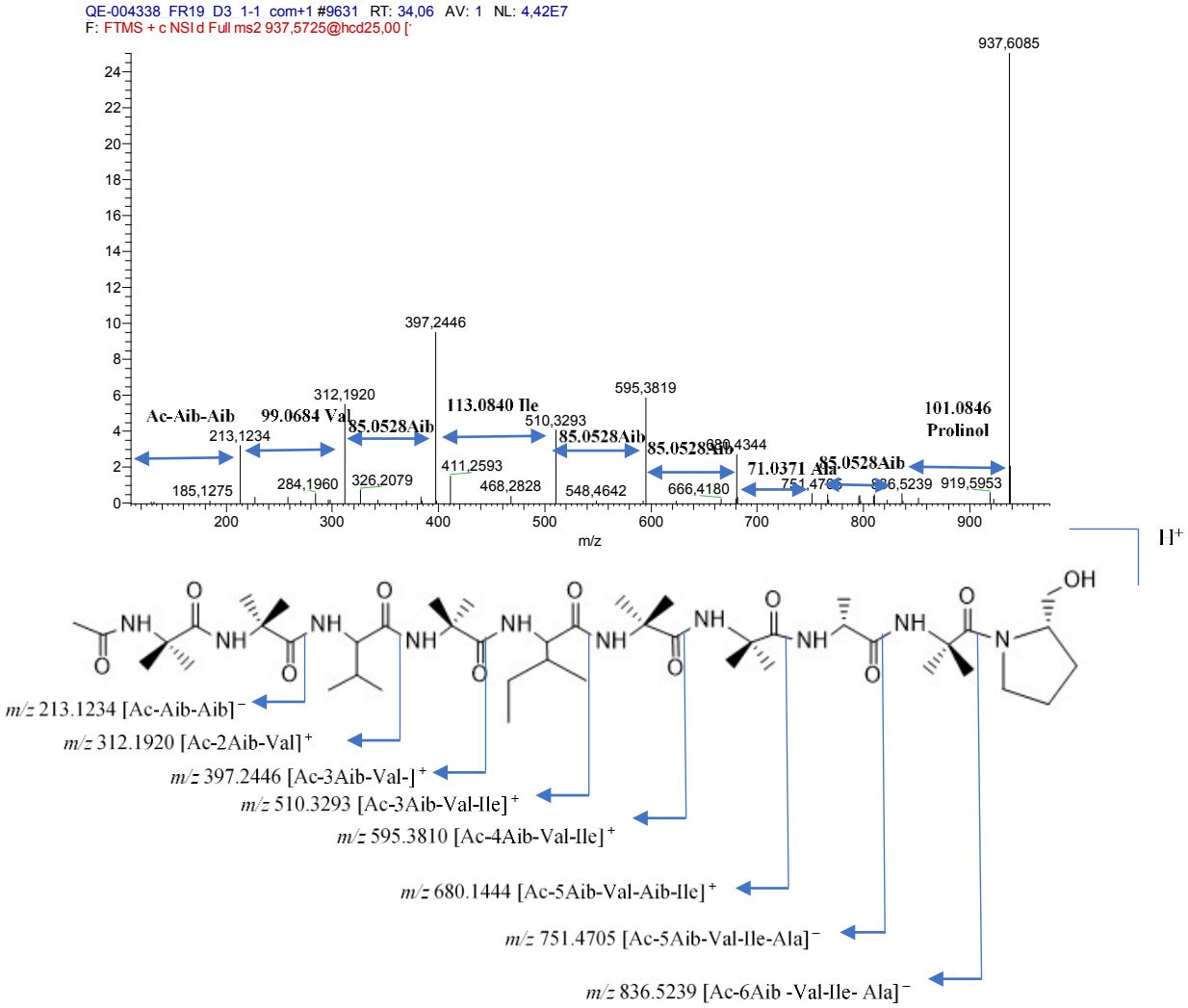
397,2446
581,3661
666,4189
312,1919
496,3131
213,1235
270,1454
383,2286
468,2817
510,3290
595,3811
682,3952
737,4558
767,4462
852,5011
905,5773
199,1078
369,2122
454,2652
Ac-Aib-Aib
99.0684 Val
85.0528 Aib
99.0684 Val
85.0528 Aib
85.0528 Aib
71.0371 Ala
85.0528 Aib
101.0850
Prolinol
m/z
H+
OH
m/z 213.1235[Ac-Aib-Aib]−
m/z 312.1919[Ac-2Aib-Val]+
m/z 397.2451[Ac-3Aib-Val]−
m/z 496.3131[Ac-3Aib-2Val]+
m/z 581.3661[Ac-4Aib-2Val]+
m/z 666.4189[Ac-5Aib-2Val]+
m/z 737.4558[Ac-5Aib-2Val-Ala]+
m/z 822.5088[Ac-6Aib-2Val-Ala]−

FIGURE 6.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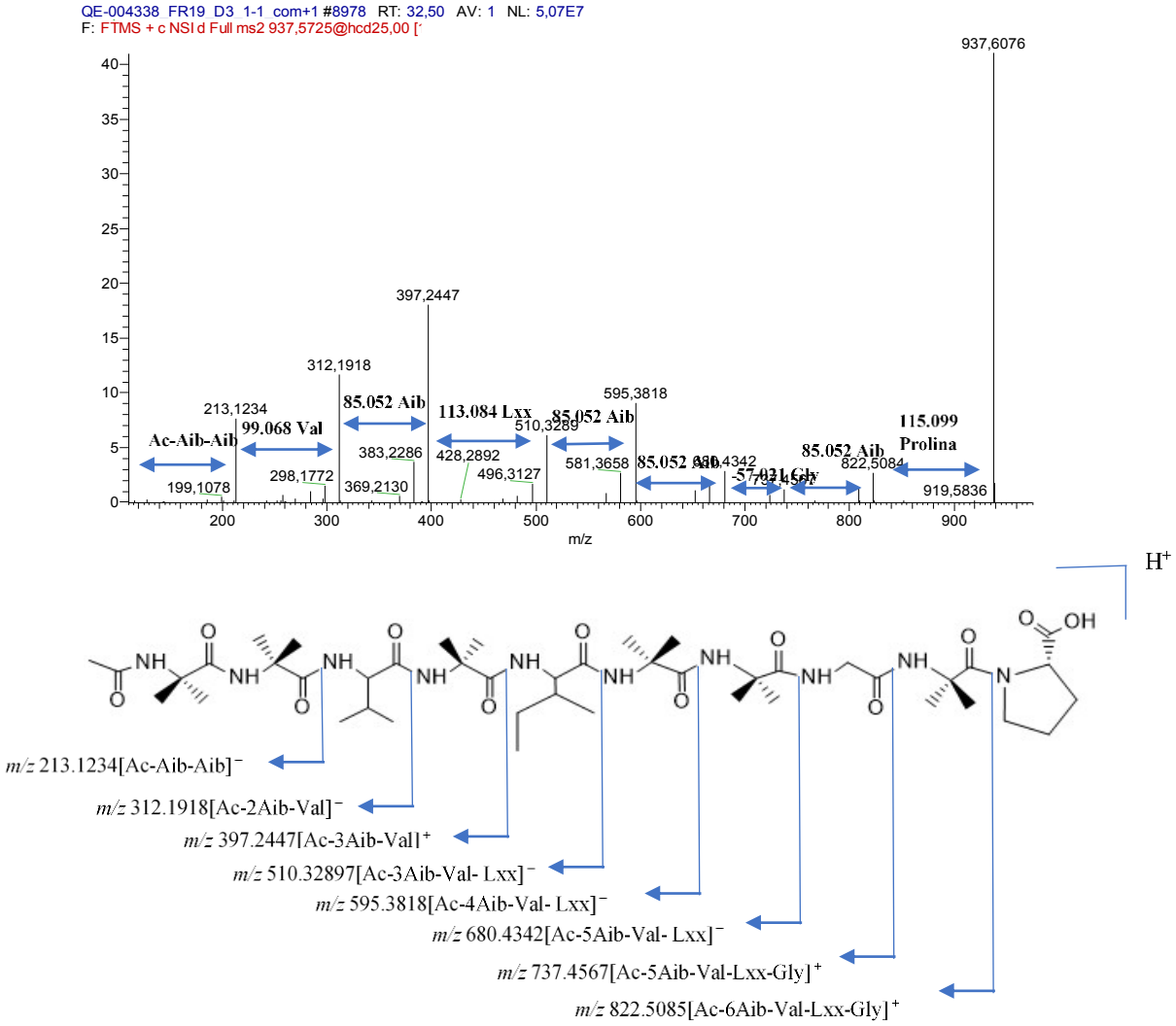
937,6085
397,2446
312,1920
595,3819
Ac-Aib-Aib
99.0684 Val
85.0528Aib
113.0840 Ile
85.0528Aib
85.0528Aib
71.0371 Ala
85.0528Aib
101.0846
Prolinol
213,1234
510,3293
411,2593
185,1275
284,1960
326,2079
468,2828
548,4642
666,4180
919,5953
m/z
H+
m/z 213.1234 [Ac-Aib-Aib]−
m/z 312.1920 [Ac-2Aib-Val]+
m/z 397.2446 [Ac-3Aib-Val-]+
m/z 510.3293 [Ac-3Aib-Val-Ile]+
m/z 595.3810 [Ac-4Aib-Val-Ile]+
m/z 680.1444 [Ac-5Aib-Val-Aib-Ile]+
m/z 751.4705 [Ac-5Aib-Val-Ile-Ala]−
m/z 836.5239 [Ac-6Aib -Val-Ile- Ala]−

FIGURE 7.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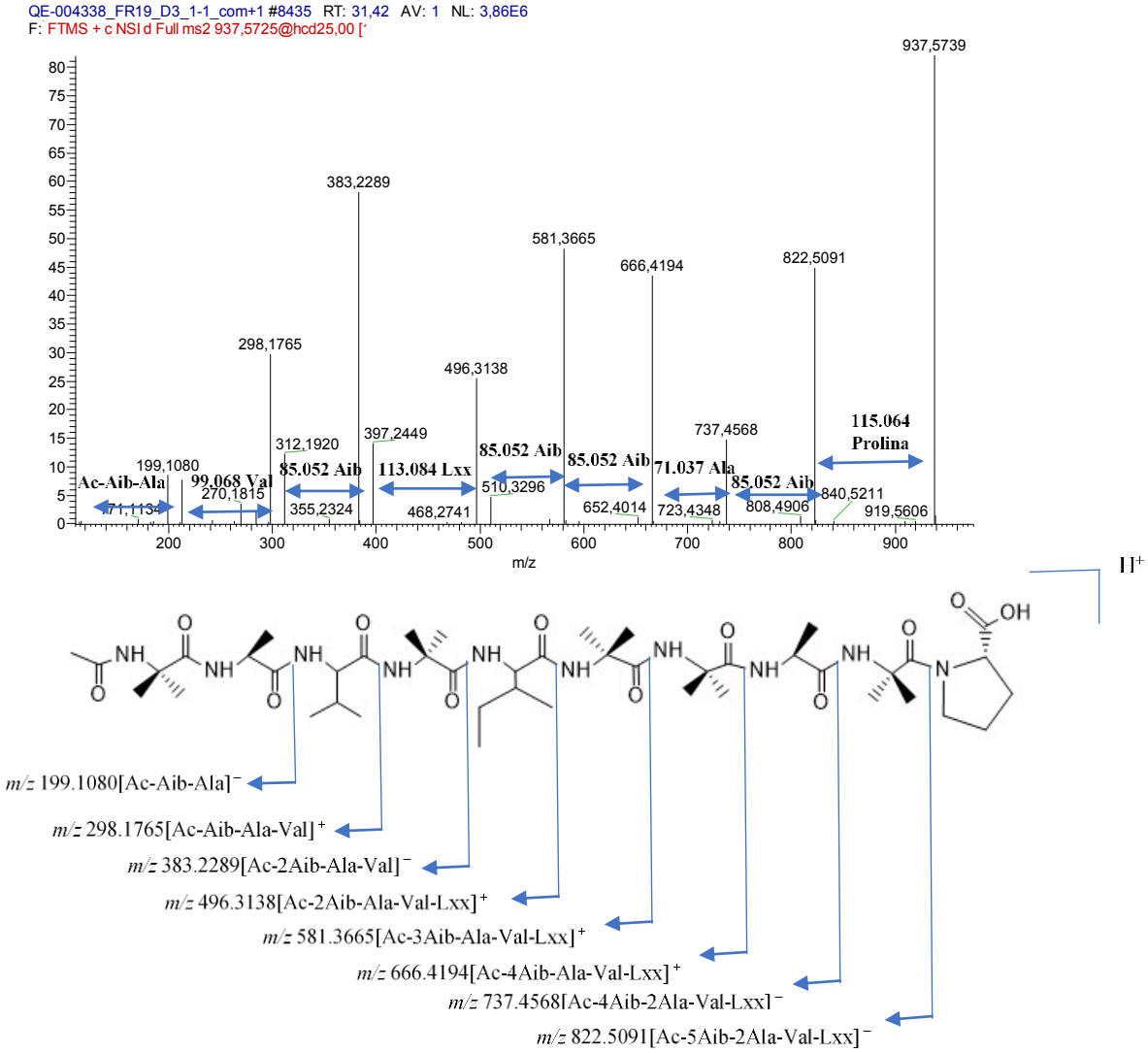
397,2447
312,1918
595,3818
213,1234
85.052 Aib
113.084 Lxx
85.052 Aib
99.068 Val
Ac-Aib-Aib
510,3289
115.099
Prolina
383,2286
428,2892
581,3658
85.052 Aib
822,5084
199,1078
298,1772
369,2130
496,3127
919,5836
m/z
H+
OH
m/z 213.1234[Ac-Aib-Aib]−
m/z 312.1918[Ac-2Aib-Val]−
m/z 397.2447[Ac-3Aib-Val]+
m/z 510.32897[Ac-3Aib-Val- Lxx]−
m/z 595.3818[Ac-4Aib-Val- Lxx]−
m/z 680.4342[Ac-5Aib-Val- Lxx]−
m/z 737.4567[Ac-5Aib-Val-Lxx-Gly]+
m/z 822.5085[Ac-6Aib-Val-Lxx-Gly]+

FIGURE 8.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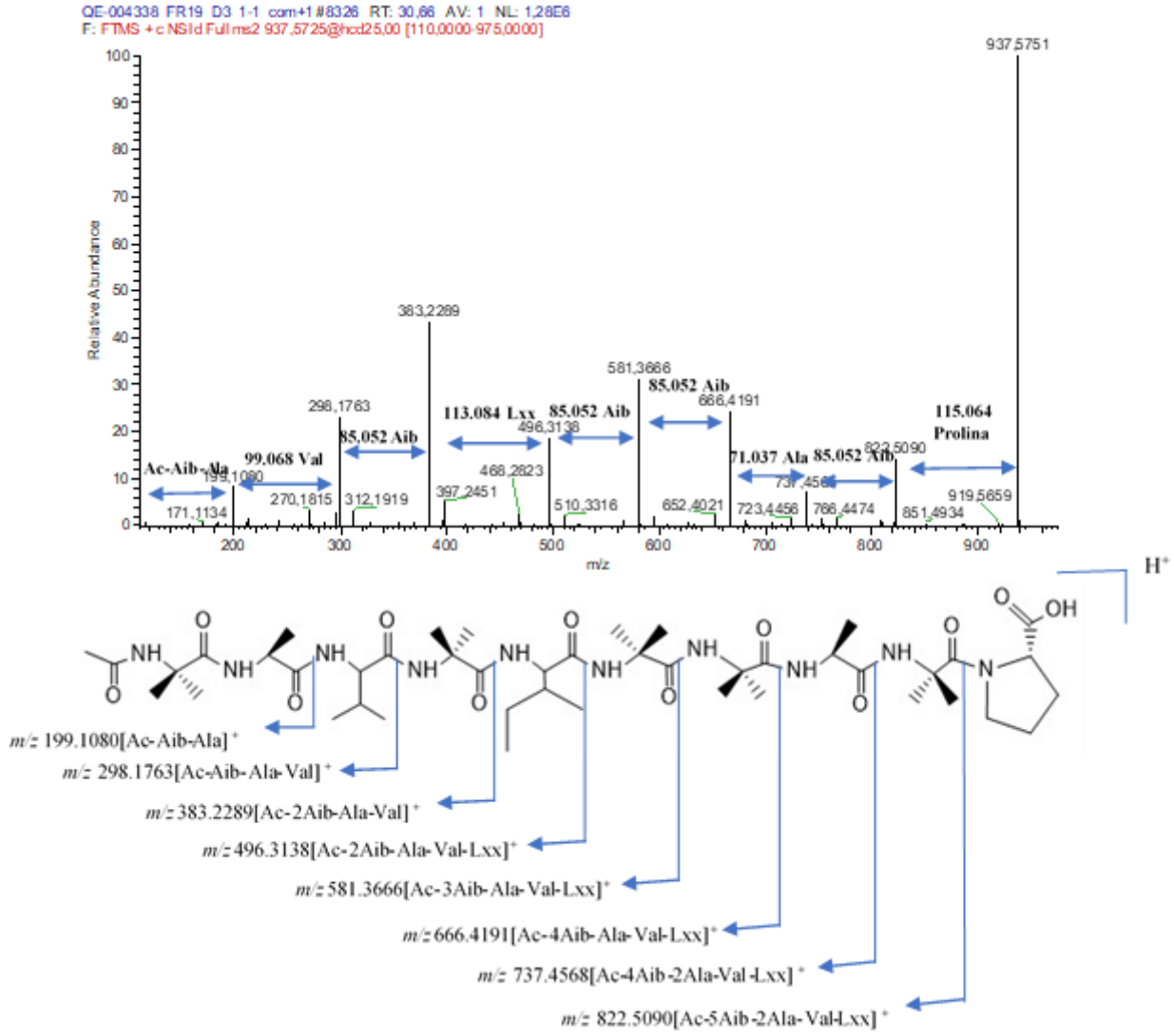
383,2289
581,3665
666,4194
822,5091
298,1765
496,3138
737,4568
115.064
Prolina
312,1920
397,2449
85.052 Aib
85.052 Aib
71.037 Ala
85.052 Aib
199,1080
Ac-Aib-Ala
99.068 Val
270,1815
85.052 Aib
113.084 Lxx
510,3296
840,5211
71,1134
355,2324
468,2741
652,4014
723,4348
808,4906
919,5606
m/z
II+
m/z 199.1080[Ac-Aib-Ala]−
m/z 298.1765[Ac-Aib-Ala-Val]+
m/z 383.2289[Ac-2Aib-Ala-Val]−
m/z 496.3138[Ac-2Aib-Ala-Val-Lxx]+
m/z 581.3665[Ac-3Aib-Ala-Val-Lxx]+
m/z 666.4194[Ac-4Aib-Ala-Val-Lxx]+
m/z 737.4568[Ac-4Aib-2Ala-Val-Lxx]−
m/z 822.5091[Ac-5Aib-2Ala-Val-Lxx]−

FIGURE 9.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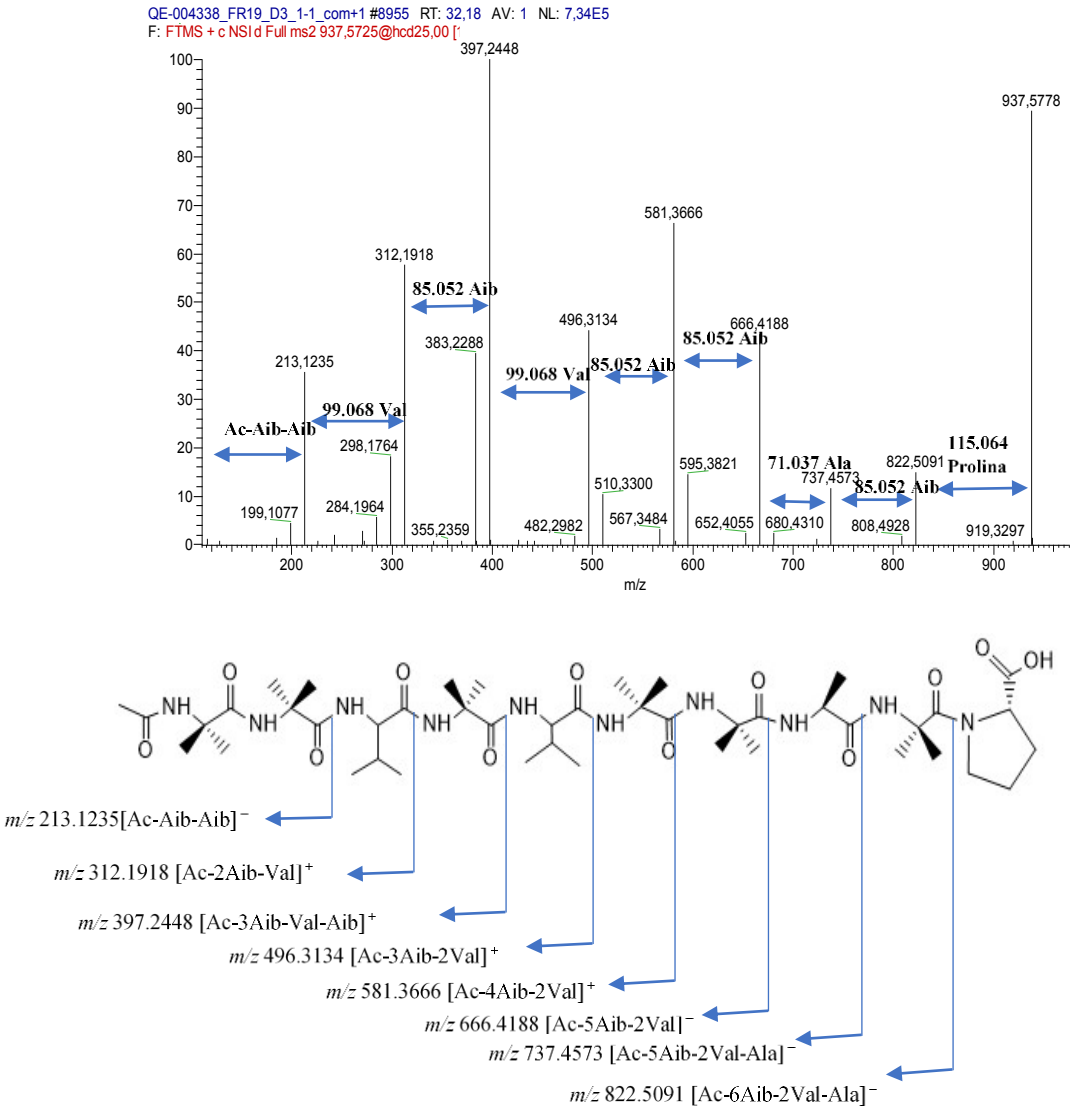
Relative Abundance
m/z
937,5751
383,2289
581,3666
85,052 Aib
666,4191
298,1763
113,084 Lxx
85,052 Aib
496,3138
115,064
Prolina
85,052 Aib
99,068 Val
Ac-Aib-Ala
71,037 Ala
85,052 Aib
822,5090
199,1080
468,2823
397,2451
270,1815
312,1919
510,3316
652,4021
723,4456
786,4474
851,4934
919,5659
171,1134
H+
m/z 199.1080[Ac-Aib-Ala]+
m/z 298.1763[Ac-Aib-Ala-Val]+
m/z 383.2289[Ac-2Aib-Ala-Val]+
m/z 496.3138[Ac-2Aib-Ala-Val-Lxx]+
m/z 581.3666[Ac-3Aib-Ala-Val-Lxx]+
m/z 666.4191[Ac-4Aib-Ala-Val-Lxx]+
m/z 737.4568[Ac-4Aib-2Ala-Val-Lxx]+
m/z 822.5090[Ac-5Aib-2Ala-Val-Lxx]+

FIGURE 10.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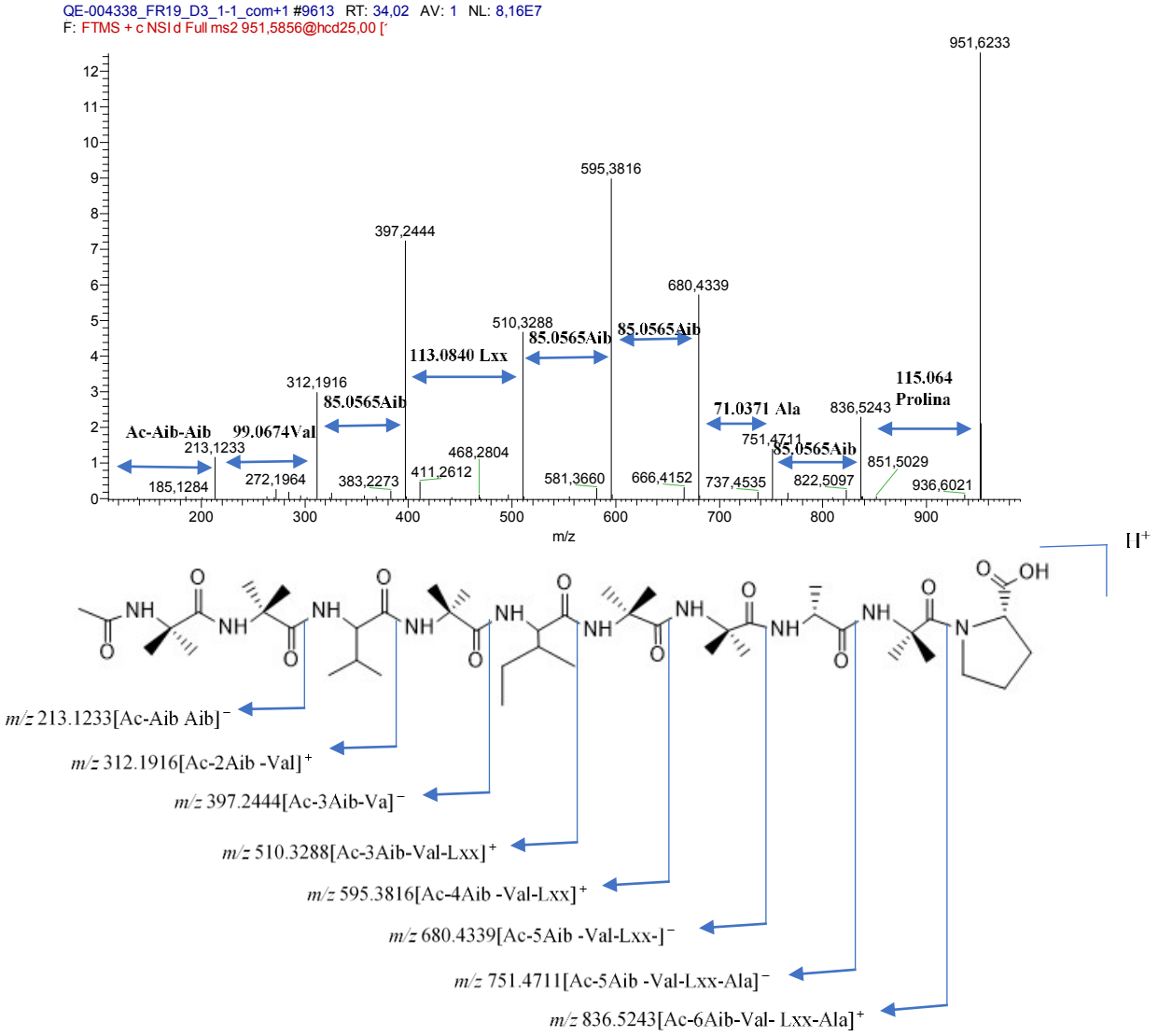
397,2448
937,5778
581,3666
312,1918
85.052 Aib
496,3134
666,4188
85.052 Aib
383,2288
213,1235
99.068 Val
85.052 Aib
99.068 Val
Ac-Aib-Aib
298,1764
595,3821
71.037 Ala
822,5091
115.064
Prolina
737,4573
85.052 Aib
510,3300
199,1077
284,1964
355,2359
482,2982
567,3484
652,4055
680,4310
808,4928
919,3297
m/z
m/z 213.1235[Ac-Aib-Aib]−
m/z 312.1918 [Ac-2Aib-Val]+
m/z 397.2448 [Ac-3Aib-Val-Aib]+
m/z 496.3134 [Ac-3Aib-2Val]+
m/z 581.3666 [Ac-4Aib-2Val]+
m/z 666.4188 [Ac-5Aib-2Val]−
m/z 737.4573 [Ac-5Aib-2Val-Ala]−
m/z 822.5091 [Ac-6Aib-2Val-Ala]−

FIGURE 11.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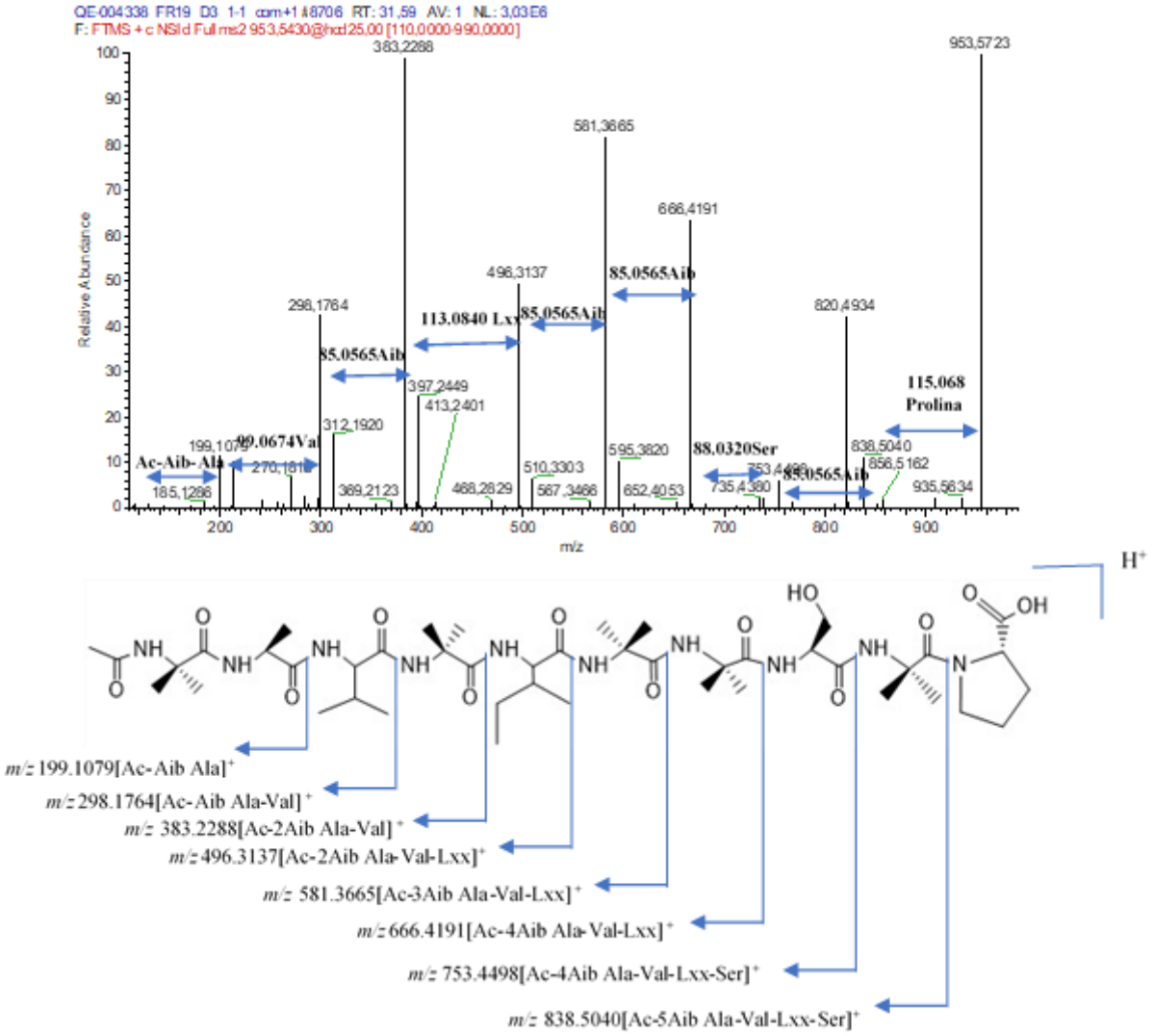
595,3816
397,2444
680,4339
510,3288
312,1916
213,1233
185,1284
272,1964
383,2273
411,2612
468,2804
581,3660
666,4152
737,4535
751,4711
822,5097
836,5243
851,5029
936,6021
Ac-Aib-Aib
99.0674Val
85.0565Aib
113.0840 Lxx
85.0565Aib
85.0565Aib
71.0371 Ala
85.0565Aib
115.064
Prolina
m/z
H+
m/z 213.1233[Ac-Aib Aib]−
m/z 312.1916[Ac-2Aib -Val]+
m/z 397.2444[Ac-3Aib-Va]−
m/z 510.3288[Ac-3Aib-Val-Lxx]+
m/z 595.3816[Ac-4Aib -Val-Lxx]+
m/z 680.4339[Ac-5Aib -Val-Lxx-]−
m/z 751.4711[Ac-5Aib -Val-Lxx-Ala]−
m/z 836.5243[Ac-6Aib-Val- Lxx-Ala]+

FIGURE 12.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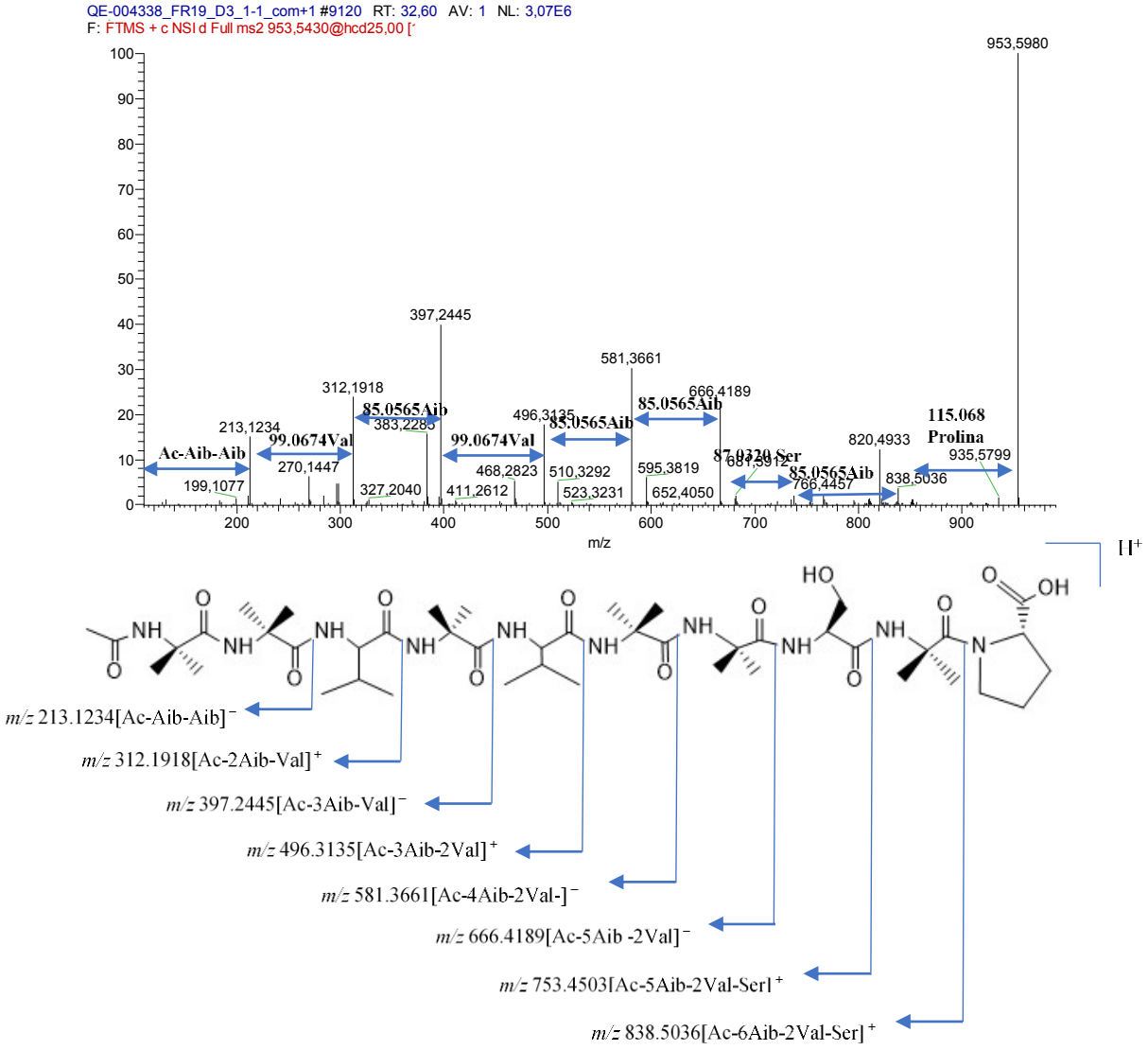
Relative Abundance
m/z
383,2288
953,5723
581,3665
666,4191
496,3137
298,1764
820,4934
113.0840 Lxx
85.0565Aib
85.0565Aib
85.0565Aib
397,2449
413,2401
312,1920
115.068
Prolina
99.0674Val
Ac-Aib-Ala
199,1079
270,1810
185,1286
369,2123
468,2829
510,3303
587,3486
595,3820
652,4053
88.0320Ser
735,4380
838,5040
856,5162
935,5634
H+
m/z 199.1079[Ac-Aib Ala]+
m/z 298.1764[Ac-Aib Ala-Val]+
m/z 383.2288[Ac-2Aib Ala-Val]+
m/z 496.3137[Ac-2Aib Ala-Val-Lxx]+
m/z 581.3665[Ac-3Aib Ala-Val-Lxx]+
m/z 666.4191[Ac-4Aib Ala-Val-Lxx]+
m/z 753.4498[Ac-4Aib Ala-Val-Lxx-Ser]+
m/z 838.5040[Ac-5Aib Ala-Val-Lxx-Ser]+

FIGURE 13.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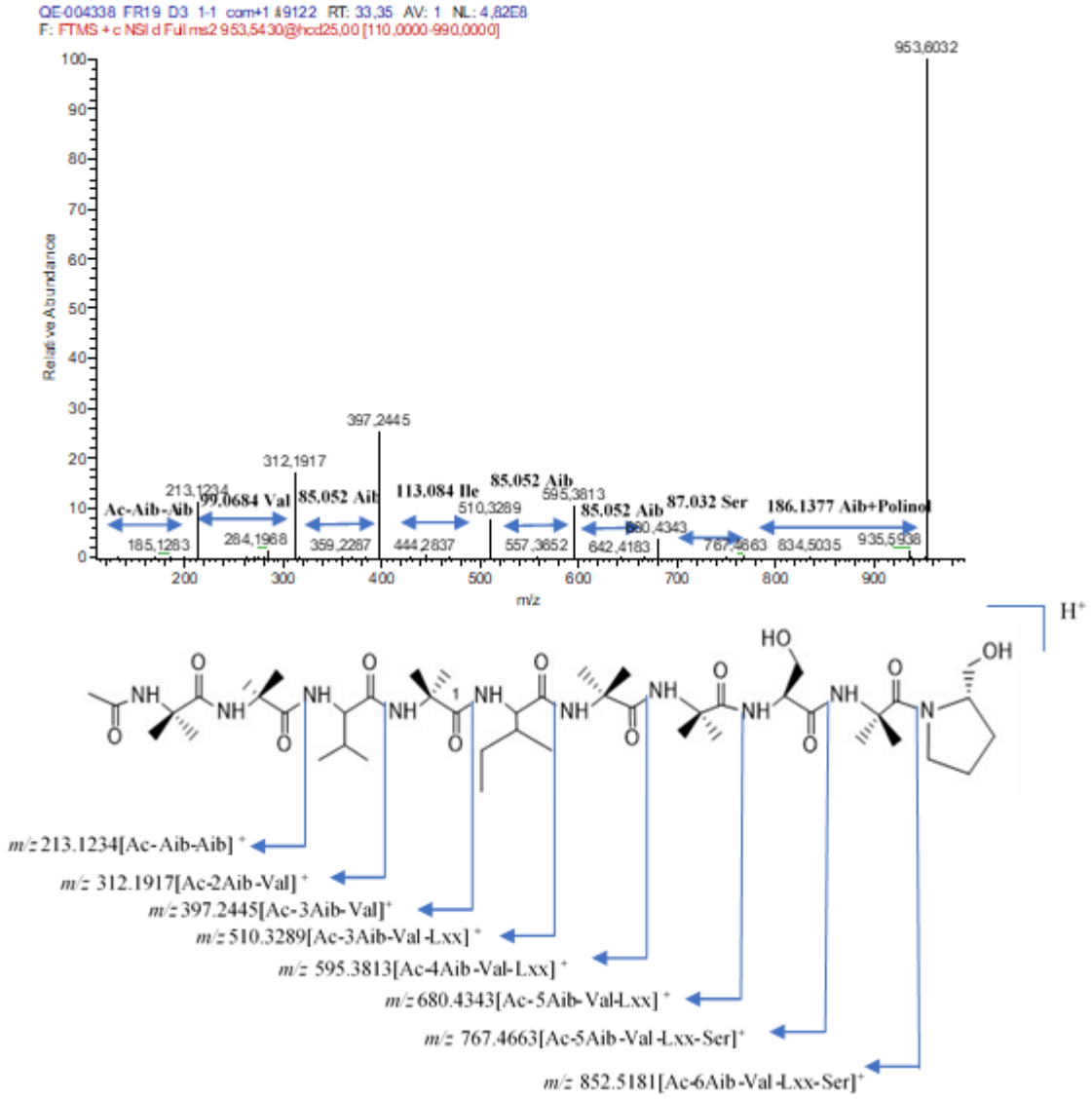
953,5980
397,2445
581,3661
312,1918
666,4189
213,1234
85.0565Aib
85.0565Aib
85.0565Aib
496,3135
115.068
Prolina
383,2285
99.0674Val
99.0674Val
820,4933
Ac-Aib-Aib
935,5799
270,1447
468,2823
510,3292
595,3819
87,0320 Ser
85.0565Aib
199,1077
327,2040
411,2612
523,3231
652,4050
838,5036
m/z
II+
m/z 213.1234[Ac-Aib-Aib]−
m/z 312.1918[Ac-2Aib-Val]+
m/z 397.2445[Ac-3Aib-Val]−
m/z 496.3135[Ac-3Aib-2Val]+
m/z 581.3661[Ac-4Aib-2Val-]−
m/z 666.4189[Ac-5Aib -2Val]−
m/z 753.4503[Ac-5Aib-2Val-Serl+
m/z 838.5036[Ac-6Aib-2Val-Ser]+

FIGURE 14.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Relative Abundance
m/z
953,6032
397,2445
312,1917
213,1234
Ac-Aib-Aib
99,0684 Val
85,052 Aib
113,084 Ile
510,3289
85,052 Aib
595,3813
85,052 Aib
87,032 Ser
186,1377 Aib+Polinol
185,1283
284,1968
359,2287
444,2837
557,3652
642,4183
680,4343
767,4663
834,5035
935,5938
H+
m/z 213.1234[Ac-Aib-Aib]+
m/z 312.1917[Ac-2Aib-Val]+
m/z 397.2445[Ac-3Aib-Val]+
m/z 510.3289[Ac-3Aib-Val-Lxx]+
m/z 595.3813[Ac-4Aib-Val-Lxx]+
m/z 680.4343[Ac-5Aib-Val-Lxx]+
m/z 767.4663[Ac-5Aib-Val-Lxx-Ser]+
m/z 852.5181[Ac-6Aib-Val-Lxx-Ser]+

FIGURE 15.

CAPÍTULO 3

Cinéticas de produção de extratos e metabólitos secundários de duas espécies de *Trichoderma* cultivadas nos modos estáticos e agitados

O presente capítulo refere-se ao quarto objetivo desta tese e foi elaborado como manuscrito a ser submetido ao periódico Microbial Biotechnology (https://sfamjournals.onlinelibrary.wiley.com/hub/journal/17517915/homepage/forauthors.html). Na sequência, apresentamos um resumo em língua portuguesa, o manuscrito em língua inglesa e os materiais suplementares, para viabilizar uma análise mais completa do que será publicado.

Resumo

Objetivo: investigar a cinética de produção de extratos e metabólitos secundários extra e intracelulares de duas espécies de *Trichoderma* de origem amazônica, cultivadas nos modos estático e agitado.

Metodologia: as linhagens de fungos foram cultivadas em tubos de ensaio contendo BDL, nos modos estático e agitado. Periodicamente, foram retiradas amostras em triplicata e registrados os valores de pH e dos níveis de glicose do meio. Após serem produzidos os respectivos extratos das amostras, foram obtidas suas massas e perfis químicos por espectrometria de massas.

Resultados e discussão: as curvas de produção de ambas as espécies foram semelhantes. No modo agitado, a produção de metabólitos foi maior e seus estágios bioquímicos foram notavelmente mais rápidos. Para ambas as espécies, houve variação no número de íons moleculares ao longo dos dias e sinais significativos foram observados em regiões específicas. Os períodos de consumo total de glicose e mudanças no pH tornaram-se evidentes.

Conclusões: os resultados permitiram observar e avaliar as condições de cultivo para a produção de metabólitos secundários nos modos agitado e estático, respectivamente.

Contribuição e impacto do estudo: foram geradas informações e diretrizes para estabelecer as melhores condições de cultivo para espécies do complexo Harzianum do gênero *Trichoderma*.

Palavras-chaves: *Trichoderma lentiforme*; *T. harzianum*; Espectrometria de massas; Perfis químicos; Cinética de produção de metabólitos.

Extracts and metabolites-production kinetics of two *Trichoderma* species cultivated under static and shaking conditions

Summary

The genus *Trichoderma* has been highlighted in the scientific field for its advantages in agricultural use and, as such, has generated an increase in research in regards to new species, its biodiversity, as well as the production of enzymes, secondary metabolites, and their biological properties. The discovery of several classes of molecules with biotechnological potential produced by species of the genus motivated the investigation here in of the production of secondary metabolites from two species of *Trichoderma* of amazonian origin. The production of extra and intracellular metabolites in different culture conditions was evaluated using mass spectrometry to analyze the chemical profiles of the extracts via the monitoring of pH levels, glucose consumption, and the masses from extracts produced over time. The results showed that the curves of both species were similar. In the shaking method, the production of metabolites and their biochemical stages was noticeably faster. For the static method, the biochemical phases were slower. However, for both species, there was a variation in the number of molecular ions over the days and significant signs were observed in specific regions. It was possible to observe the greater production of extracellular extracts in both species. The periods of total glucose consumption and changes in pH were evident. Via analysis of the correlated results, it was possible to understand the metabolic behavior of *Trichoderma* species and establish specific and favorable conditions for the production of secondary metabolites.

Keywords: *Trichoderma lentiforme*; *T. harzianum*; Mass spectrometry; Chemical profile; Metabolite production kinetics.

Introduction

The genus *Trichoderma* has gained prominence around the world over the years due to its species diversity (Krédics et al., 2014), the discovery of new species (Du Plessis et al., 2018; Zhang & Zhuang, 2018), the variety of molecules with different biological activities (Keswani et al., 2013), the diversity in enzyme production (Toth et al., 2012; Li et al., 2019), and its diverse uses in the agricultural area (Woo et al., 2014). In the context of the latter, patented products are available on the market and generate high profitability (Gupta et al., 2014; Dos Santos et al., 2017).

The increase in publications regarding the production and isolation of molecules from *Trichoderma* strains is evident, as well as reports on their different biological activities. Various of these reports are associated with production processes or to inter and intraspecific metabolites-production mechanisms by species of the genus, which have been revealing some species to be producers of specific target molecules (Keswani et al., 2013; Zeilinger et al., 2016, Shi et al., 2018).

The investigation of cultivation parameters generally occurs under laboratory conditions, and can also simulate environmental conditions (Ruiz et al., 2007; Ren et al., 2013). This type of investigation allows different cultivation conditions, whether on small or large scales, which generate intra or extracellular metabolites through their excretion or extraction, through the use of different metabolic pathways in production (Hardy et al., 2017; Mohammadi et al., 2018 Hamrouni et al., 2019).

The advantages of studies into cultivation conditions are diverse, such as establishing a favorable, fast, cheap, and practical condition for a given species, or establishing an effective condition in the production of specific metabolites. These may include the use of inducing reagents, specific culture media, monitoring and changes in pH, consumption of glucose and nutrients, such as nitrogen, phosphorus, among others (Diko et al., 2020; Gelain et al., 2020).

Such experiments demand time and accumulation of information, which must be carefully evaluated, as well as the association of biochemical results with chemical and biological ones, to detect changes and possible improvements in the production of secondary metabolites. The demand for this type of study has been increasing over the years since it provides the greatest chance of achieving the desired objective, which tends to be the discovery of new molecules (Holker et al., 2004; Li et al., 2020).

In this study, we propose to investigate the metabolic and chemical behavior of *T. lentiforme* (T.19) and *T. harzianum* (T.122), both from the Harzianum complex, under shaking and static cultivation methods, while evaluating pH, glucose consumption, the mass of extracts and the chemical profile of its intra and extracellular extracts in a microscale experiment, in order to understand the ideal conditions for the production of their secondary metabolites.

Results

Production curve - general aspects

Compared under the same static or shaking growth method, along the culture time, the *T. lentiforme* T.19 and *T. harzianum* T.122 strains have shown the same pH and glucose level variations, as well similar changes of the extract masses (Figures 1 to 4). The glucose level dropped down to almost zero in the 4th and became null in the 8th culture day in the shaking method, while in the static method it dropped down in two stages, with a ramp between the 3rd and 6th day, and reaching zero only in the 12th culture day; the pH grew up in three stages and achieved three ramps on both methods, naturally with different periods

– in the shaking method, the pH ramps were at 7.0, 2nd - 4th days, 8.0, 6th - 18th days, and 9.0 in the final culture period, while in the static method, they were at 6.0, 3rd - 6th days, 7.0, 12th - 15th days, and 9.0 in the final culture period; finally, the masses of all the extracts typically achieved their maximum ranges between the depletion of the carbon source (when glucose achieved zero) and the final period (death phase) – more specifically in the 12-14th days for the shaking and in the 15-20th days in the static method.

Chemical profiles of *Trichoderma lentiforme* T.19

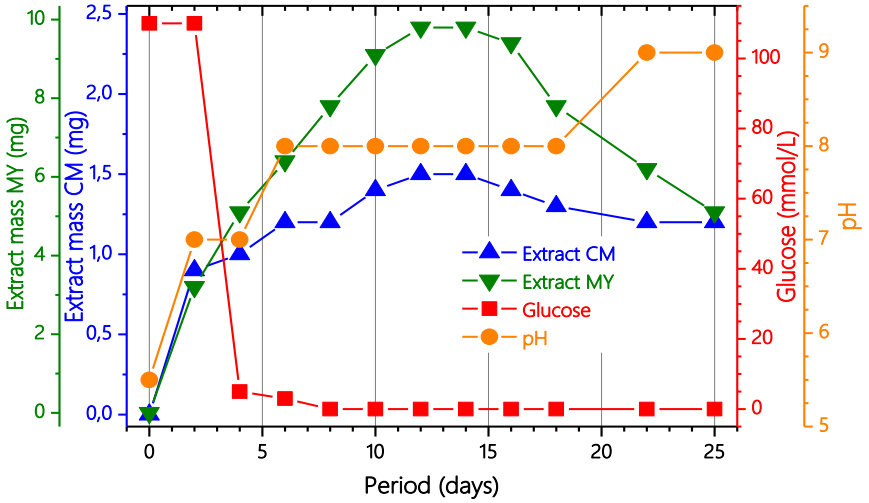
The chemical-profiles spectra obtained by mass spectrometry of the extracts of the *T. lentiforme* T.19 strain, in the static method of growth, presented along all the time high noise levels (Figure S1 and S3). For this method, different major signals were observed in the mycelium spectra from the 9th to the 15th days in the range at *m/z* 700-800, followed by decay onward the 20th day (Figure S1). In contrast, the mycelium spectra in the shaking method, show greater clarity and lesser noise signals, with strong signals from the 2nd until the 16th day in the range at *m/z* 250-350, which achieve almost total decay onward the 18th day (Figure S2). From this day, a cluster around *m/z* 919 could be observed.

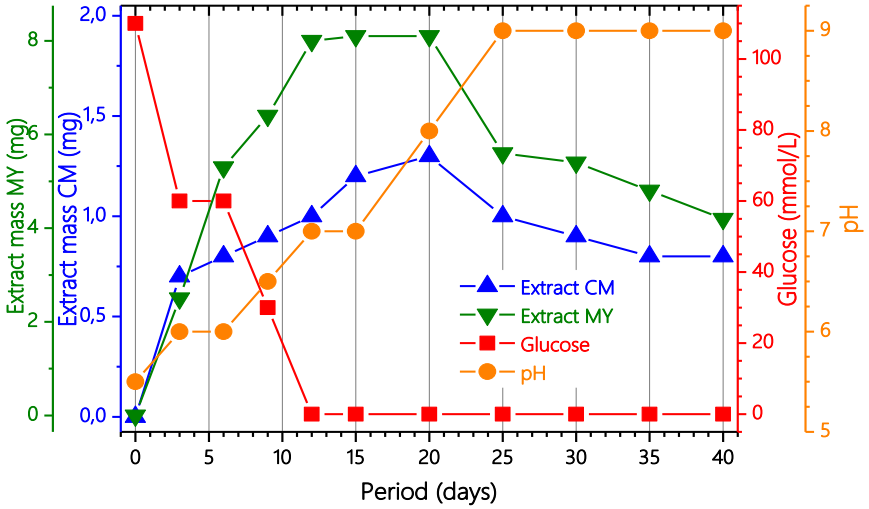
The spectra of *T. lentiforme* of the cultivated medium in the static method were so loaded with noise signals that it was impossible to observe the signals of the samples (Figure S3). However, the spectra of the cultivated medium (Figure S4) in the shaking method presented evident signals in the range at *m/z* 200-400 from the 2nd day onwards, with changes of some peaks from the 12th day and drop down after the 16th day. Discreet peaks around *m/z* 870 appear between the 18th and 25th day.

Chemical profile of *Trichoderma harzianum* T.122

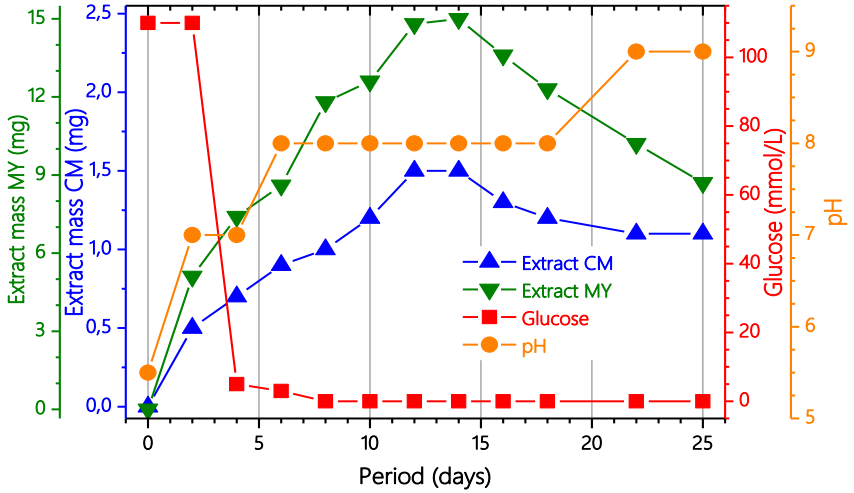
The chemical profiles by mass spectrometry of the extracts of the *T. harzianum* T.122 strain were sensitively different from those of the *T. lentiforme* T.19 strain, revealing more ion peaks above *m/z* 700. In the static method of cultivation, mycelial-extract profiles showed gradual changes in the positions and intensities of the signals, mainly in the range at *m/z* 750-950, but also above, from the 3rd to the 9th day; minor changes occurred until the 15th day; and from the 20th day onward the main changes were observed above *m/z* 900 (Figure S5). The chemical profiles of the mycelia in the shaking method were not at all so different from the static culture method. Signals in the range at *m/z* 750-950 also grew up from the 6th day onward. Signals were also observed in the range at *m/z* 1150-1250 between the 18th and 25th days (Figure S6).

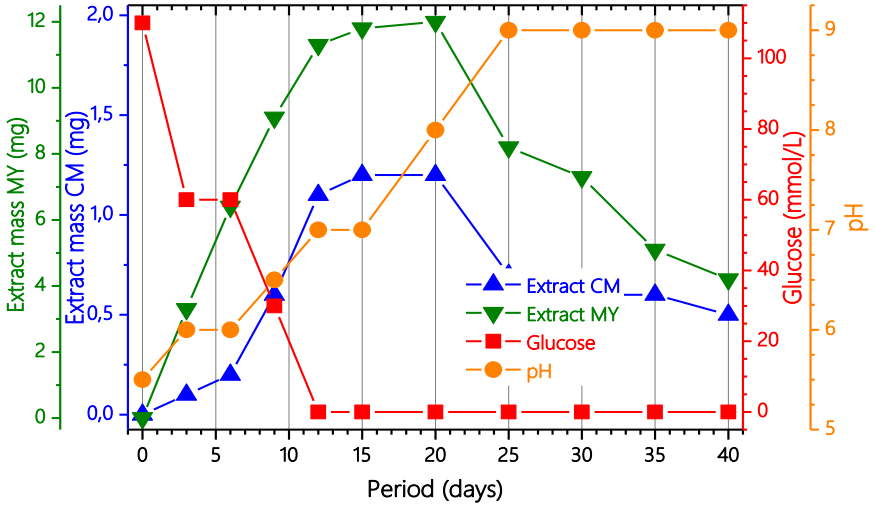
The cultured broth extracts from the static method presented significant signals from the 9th day onward, in the range at *m/z* 700-900, with a discreet change between the 30th and 40th days (Figure S7). The cultured broth extracts from the shaking method presented signals in the range at *m/z* 750-950 from the 8th day onward. Signals were also observed in the range at *m/z* 1150-1250 in the 22nd and 25th days (Figure S8).





Figures 1 and 2. Curves of the pH, glucose, and extract masses of the cultivated medium (CM) and mycelium (MY) of the of *T. lentiforme* (T.19) in shaking and static method.





Figures 3 and 4. Curves of the pH, glucose, and extract masses of the cultivated medium (CM) and mycelium (MY) of the of *T. harzianum* (T.122) in shaking and static method.

Discussion

Kinetics of the extracts-production

The similar kinetics revealed in the metabolic curves for the *T. lentiforme* T.19 and *T. harzianum* T.122 strains can be explained by the common ancestrality of these *Trichoderma* species, very close phylogenetically into the same Harzianum complex (Chaverri et al., 2015). Since the methodology of extraction by organic solvents targets mainly secondary metabolites, our method makes it possible to suggest a practical procedure to find the maximum-yield periods of the metabolic extracts for *Trichoderma* species. Initially, someone can monitor the culture medium until the pH becomes constant and glucose runs out. Then, she or he can measure every two days the masses of extract samples until a maximum value repeats two times for the shaking method, or three times for the static method, indicating the greatest yield, therefore the better time to stop the experiment. Besides, in parallel, it is also possible to accomplish the chemical profiles of the samples by mass spectrometry.

For the *T. lentiforme* and *T. harzianum* species, under the culture conditions in this present work, our data (Figures 1 to 4) point out the 12^{th}-14^{th} days, in the shaking, and the 15^{th}-20^{th} days, in the static method, as the maximum-yield periods. After this, the pH grows up to 9.0 and the masses of both extra- and intracellular extracts grow down, indicating the catabolic stage. The fastest metabolism in the shaking method for both strains is certainly due to the greater availability of oxygen and the more effective contact of the components of the culture medium with the fungal cells, since agitation better disaggregates the mycelium providing a faster exchange of nutrients on its surface (Ibrahim et al., 2015). Better contact with the nutrients also explains the better income under shaking. Finally, for both the shaking and static methods, pH control of the culture medium, using a buffer, seems a good suggestion to promote better extract yields (Mantzouridou et al., 2002; Costa et al., 2017; Vieira et al., 2010).

Indirect observation of the growth phases

The traditionally known fungal growth phases were indirectly observed. Briefly, the reduction of glucose to zero indicated the depletion of the carbon source and the end of the exponential growth phase, as also the time when secondary metabolites begin to be produced in greater quantities. From this point, the increase of the extract masses to a

maximum, at pH 8, revealed the stationary phase (Calvo et al., 2002; Vrabl et al., 2019). Finally, the rapid loss of the extract masses indicated the catabolism of the secondary metabolites, signalizing the phase of decline. In this last phase, the pH alkaline is probably necessary for the action of the enzymes that are responsible for homeostasis or catabolism (Stancik et al., 2002; Padan et al., 2005).

Chemical-profiles kinetics of the T.19 strain

The high noise levels in the mass spectra of the extracts of the *T. lentiforme* T.19 strain cultivated by the static method reflect the slow metabolism of this fungus under low oxygenation and poor delivery of nutrients. For the mycelium spectra in this method, the few signals observed (*m/z* 700-800), between the 9th to and the 20th day (Figures S1), were present in the period of the maximum mycelial income (Figure 2). In contrast, the better oxygenation and faster delivery of nutrients in the shaking method (Buchs, 2001) permitted to analyze the kinetics of the chemical profiles of the mass spectra. The signals observed between the 2nd and the 16th reveals metabolic stability (peaks at *m/z* 250-350) of the mycelium since its initial growth, throughout the maximum mycelial income period, until the start of the decline phase (Figure 1). After this, as expected, the catabolism changed the chemical profiles (peaks at *m/z* 250-350 changed to peaks around *m/z* 919) of the mass spectra while the extract mass dropped down fastly. The peaks envelope presents at *m/z* 500-850 in all mass spectra was considered as residual soap or possible surfactant molecules since this species has been suggested as a producer of potential industrial detergent (Wang et al., 2018).

While it was impossible to explore the mass spectra of the cultivated media in the static method of growing, in the shaking method the peaks observed (*m/z* 200-400) (Figure S4) since the 2nd day reveal that the secondary metabolism of this fungus begins to work before the stationary phase, the phase of the maximum yield of the extract masses. The fast decline of those peaks after the 16th day and discreet arising of others (around *m/z* 870) between the 18th and 25th day coincided with the dropped down of the extract masses, the same phenomena observed for the mycelial kinetic (Figure 1).

The peaks observed at *m/z* 280-370 of the mass spectra of the fungus cultivated medium in the shaking method are of possible polyketides, as koninginins, for example. In fact, koninginins A, D, E, and F have been isolated and characterized as extracellular

metabolites from this *T. lentiforme* T.19 strain (Souza et al., 2008). They showed the ability to inhibit edema-inducing, as well as myotoxic and enzymatic activities of the total venom of *Bothrops jararacussu* snake.

The intensity of the signals in the mass ranges of koninginins in the shaking method possibly occurred by favoring oxygenation, thus making it more available for cellular respiration and consequently for the production of acetyl-CoA, a precursor molecule of these polyketides. Therefore, the shaking method seems to provide the best conditions for the production of this class.

Chemical-profiles kinetics of the T.122 strain

The mycelial-extract profiles of *T. harzianum* T.122 strain in the static method presented apparent metabolic stability until the last day of the period of maximum yield of the extracts. The gradual change in the intensities and positions of signals at *m/z* 750-950 between the 3rd and 9th day, considering the pH ramp (Figure 4) between the 3rd and 6th day, may signalize a period of adaptation while the glucose was fastly declining. The changes above *m/z* 900 after the 20th day seem to signalize the death phase (Figure S5) when the masses of extracts dropped down by the catabolic process (Figure 4). The metabolic stability of the fungus mycelial-extract profiles was confirmed in the shaking method, as also few changes in the catabolic phase. Together, the metabolic data of both fungi strains in this work suggest that the intracellular metabolisms of the correspondent species are constant until they start the catabolic process. This regularity highlights the mycelial chemical profiles of *Trichoderma* species as reliable for chemotaxonomy studies.

The similarity of the chemical profiles of the medium cultivated in the agitated and static growth methods of the T.122 strain reveals a fungus with a regular metabolism. More surprisingly is the similarity with the mycelial extract profiles, different from the T.19 strain. Apparently, this *T. harzianum* T.122 strain produces metabolites of the same type, if not the same, intra- and extracellularly. Regarding the nature of its metabolites, the ions envelope at *m/z* 700-900 may be associated with peptaibols since peptaibols with ions of mass ranges above *m/z* 800 have been isolated from *T. harzianum* (Peltola et al., 2004; Van Bohemem et al., 2016). Usually, researchers look for these molecules only in extracellular media, but the data in this work suggest that the mycelium can be a source of them as well. The ions envelope at *m/z* 1150-1250 observed in the catabolic phase in both mycelial and medium extracts, for the culture methods, also remember peptaibols.

The comparisons of the changes in mass, pH, and glucose rate with changes of the chemical profiles, allowed us to monitor and understand their relationship during the growth of *T. lentiforme* (T.19) and *T. harzianum* (T.122). The combination of techniques and the results helped to determine the periods and conditions for the best production of metabolites. The shaking method of growth was the best in extract yields as also, of course, in time consumption. The investigation and monitoring of the cultivation parameters can make the research process for specific ions and molecules more efficient, and give information for the improvement of the production of extracts and metabolites. The control of the pH during the maximum yield phase seems a good suggestion. Both the evaluated methods (shaking and static) can serve for the production of different molecules, monitored through chemical profiles that will determine the peak of production of secondary metabolites.

Experimental procedures

Trichoderma strains

The ITS and *TEF-1α* deposit numbers of the *Trichoderma* strains in the Genbank are MN262491 and MT681962 for *Trichoderma lentiforme* T.19 and MN262505 and MT681979 for *T. harzianum* T.122. They are registered in Brazil under the SISGEN number A39C76B.

Secondary metabolite production curve

Two species of *Trichoderma* were utilized, *T. lentiforme* (T.19) and *T. harzianum* (T.122). Small-scale production was carried out in two experiments with different culture conditions, which are as follows: 1 – shaking method at 120 rpm and maintained at 28 °C for 25 days; and 2 – without agitation, and maintained at 28 °C, for 40 days. Figure 9 shows the dynamics used to obtain the results of the experiment in question. The metabolic production curves were made using OriginPro software version 2016.

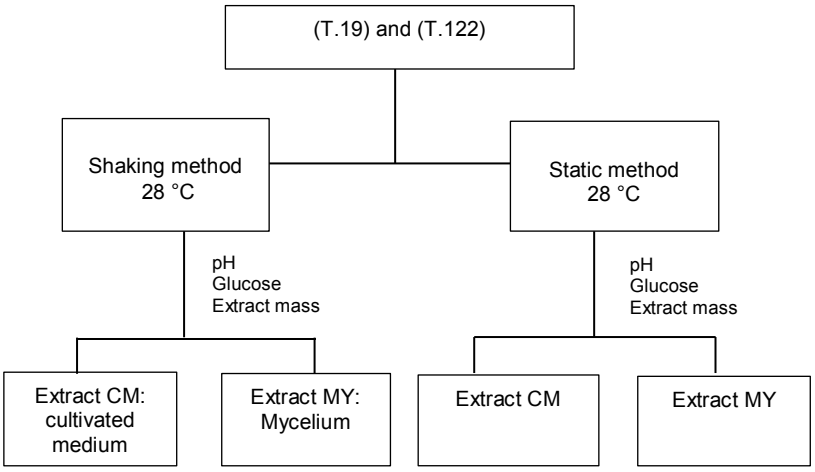


Figure 9. Flow chart for obtaining the metabolic production curve.

Obtaining extracts from T.19 and T.122

For both experiments, 3.0 µL of the conidia suspensions at the concentration of No. 6 on the McFarland scale were inoculated in test tubes (12 cm long x 1.5 cm in diameter), which contained 3.0 mL of PDY medium (potato dextrose and yeast extract,

2%), and were performed in triplicate (Souza et al., 2004; Souza, 2005 - with adaptations). For the shaking method experiment, twelve sampling points were established (0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 22 and 25 days), and in the static method experiment, eleven sampling points were established (0, 3, 6, 9, 12, 15, 20, 25, 30, 35, and 40 days). The samples were obtained after separation of the cultivated medium and the mycelium via filtration with sterile cotton. The cultivated medium was partitioned with AcOEt/2-PrOH (9:1), vortexed for 2 min and maintained for 24 h in a freezer at -20 °C to freeze the aqueous phase (which was discarded). The organic phase was filtered with cotton and transferred to penicillin flasks, then followed by evaporation of the solvent and weighing of the extracts.

In the test tubes containing mycelium, 2 mL of AcOEt/MeOH (1:1) was vortexed for 2 min, stored for 24 hours at room temperature, filtered with cotton wool, and then transferred to penicillin flasks for solvent evaporation and analysis of the mass. The extracts of the mycelium and the cultivated medium, after being dried and weighed, were resuspended with 1 mL of MeOH, stirred for complete solubilization, re-filtered with cotton, transferred to 2 mL microtubes (Eppendorf) (stock solution) and stored at –20 °C for further analysis.

Analysis of extracts by mass spectrometry

The samples were analyzed using 20 µL of the stock solution added to 500 µL of MeOH (analysis solution) directly inserted in a mass spectrometer (Thermo Scientific) operating with an electrospray source (ESI) and ion-trap analyzer (model LCQ Fleet), in the range of *m/z* 100 to 2000 in positive mode, and using MeOH with 0.5% formic acid as the carrier solvent. The analytical conditions were adjusted to reduce fragmentations and reactions at the source and optimize the sensitivity, with the following parameters being set: spray voltage, 5 kV; sheath gas, 10 arb; auxiliary gas, 5 arb; sweep gas, 0 arb; capillary temperature, 200 °C; capillary voltage, 40 V; tube lens, 115 V; mass range, *m/z* 100 to 2000; collision gas, He; scan, 3 seconds.

Evaluation of glucose concentration, pH and mass of extracts

Samples of the culture media corresponding to the number of days of growth were evaluated for pH and glucose concentration, with the use of urinalysis reagent strips, Uriclin 10 (Laborclin). The results obtained were analyzed regarding the following aspects: presence of ions indicating possible substances of pharmacological interest according to the literature, as well as through an analysis of the set of results for pH, glucose concentration, cultivation time and mass of extracts in milligrams.

Authorship contribution statement

Fernandes, K.R.P.: Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review, and editing. De Souza, A.Q.L.: Conceptualization, Formal analysis, Writing - original draft, Writing - review, and editing. Dos Santos, L. A.: Formal analysis. Couceiro, P.R.C; Formal analysis, Souza, A.D.L; Conceptualization, Formal analysis, Financing acquisition, supervision, writing - original draft, writing - review, and editing.

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Supporting information

Extracts and metabolites-production kinetics by two *Trichoderma* species cultivated under static and shaking conditions

Figure S1. Chemical profile of extracts of mycelium in the static method by *T. lentiforme* (T.19) in 3rd, 6th, 9th, 12th, 15th, 20th, 25th, 30th, 35th, 40th days respectively.

Figure S2. Chemical profile of extracts of mycelium in the shaking method by *T. lentiforme* (T.19) in 2nd, 4th, 6th, 8th, 10th, 12th, 14th, 16th, 18th, 22th, 25th days respectively.

Figure S3. Chemical profile of extracts of cultivated medium in the static method by *T. lentiforme* (T.19) in 3rd, 6th, 9th, 12th, 15th, 20th, 25th, 30th, 35th, 40th days respectively.

Figure S4. Chemical profile of extracts of the cultivated medium in the shaking method by *T. lentiforme* (T.19) in 2nd, 4th, 6th, 8th, 10th, 12th, 14th, 16th, 18th, 22th, 25th days respectively.

Figure S5. Chemical profile of extracts of mycelium in the static method by *T. harzianum* (T.122) in 3rd, 6th, 9th, 12th, 15th, 20th, 25th, 30th, 35th, 40th days respectively.

Figure S6. Chemical profile of extracts of mycelium of the shaking method in by *T. harzianum* (T.122) in 2nd, 4th, 6th, 8th, 10th, 12th, 14th, 16th, 18th, 22th, 25th days respectively.

Figure S7. Chemical profile of extracts of the cultivated medium in the static method by *T. harzianum* (T.122) in 3rd, 6th, 9th, 12th, 15th, 20th, 25th, 30th, 35th, 40th days respectively.

Figure S8. Chemical profile of extracts of the cultivated medium in the shaking method by *T. harzianum* (T.122) in 2nd, 4 th, 6th, 8th, 10th, 12th, 14th, 16th, 18th, 22th, 25th days respectively.

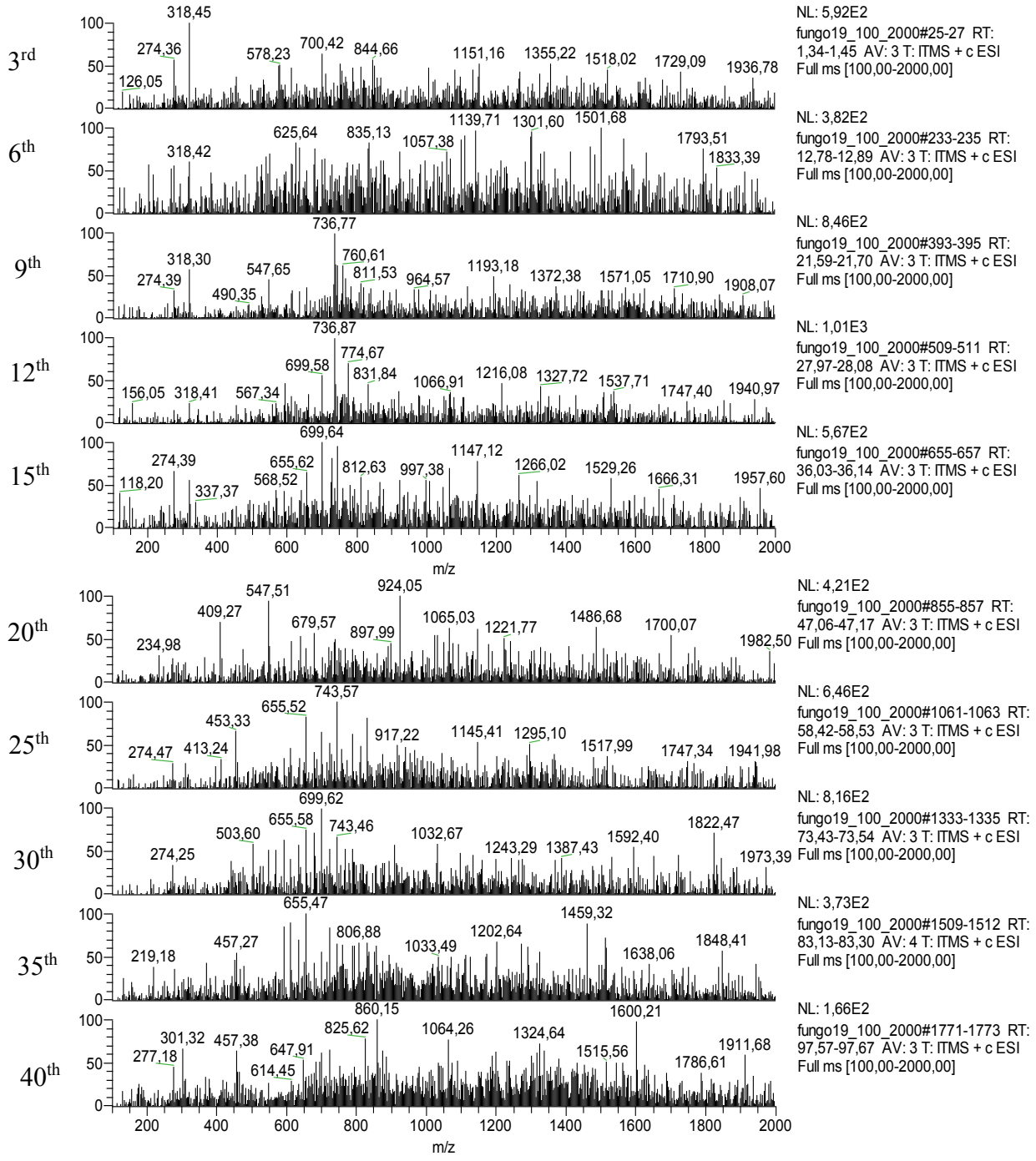

Figure S1. Chemical profile of extracts of mycelium in the static method by *T. lentiforme* (T. 19) in 3rd, 6th, 9th, 12th, 15th, 20th, 25th, 30th, 35th, 40th days respectively.

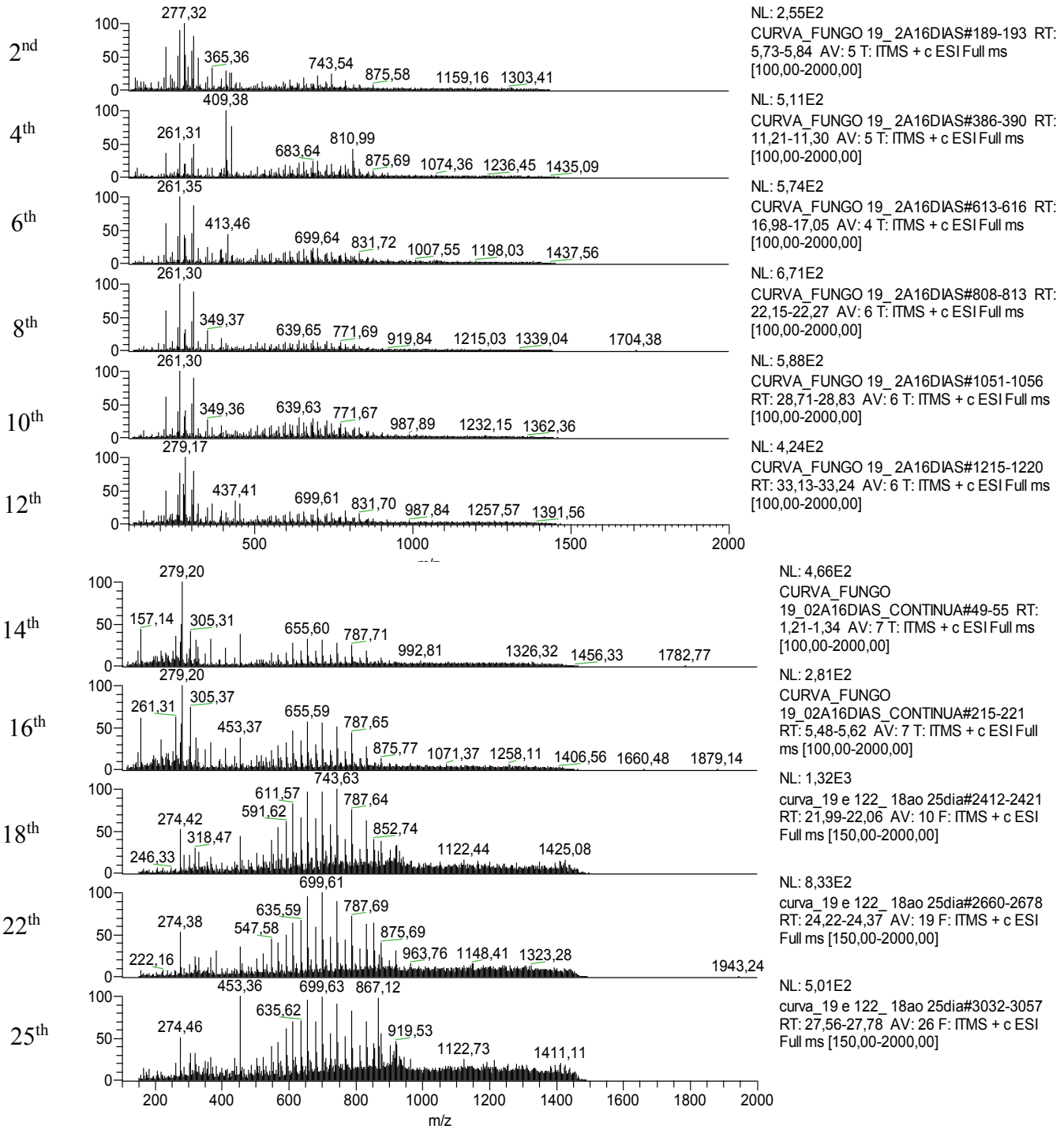

Figure S2. Chemical profile of extracts of mycelium in the shaking method by *T. lentiforme* (T.19) in 2nd, 4th, 6th, 8th, 10th, 12th, 14th, 16th, 18th, 22th, 25th days respectively.

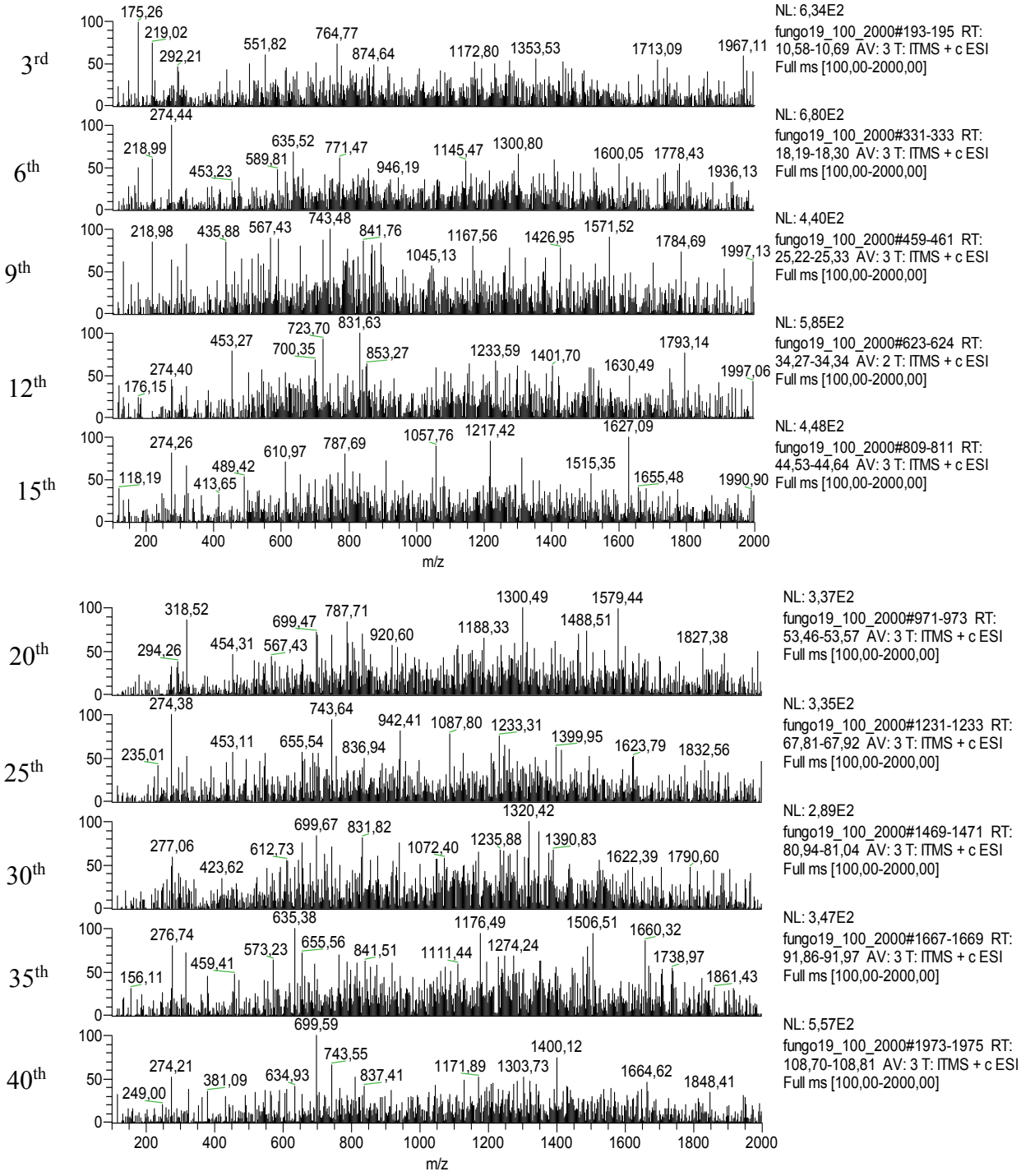

Figure S3. Chemical profile of extracts of the cultivated medium in the static method by *T. lentiforme* (T. 19) in 3rd, 6th, 9th, 12th, 15th, 20th, 25th, 30th, 35th, 40th days respectively.

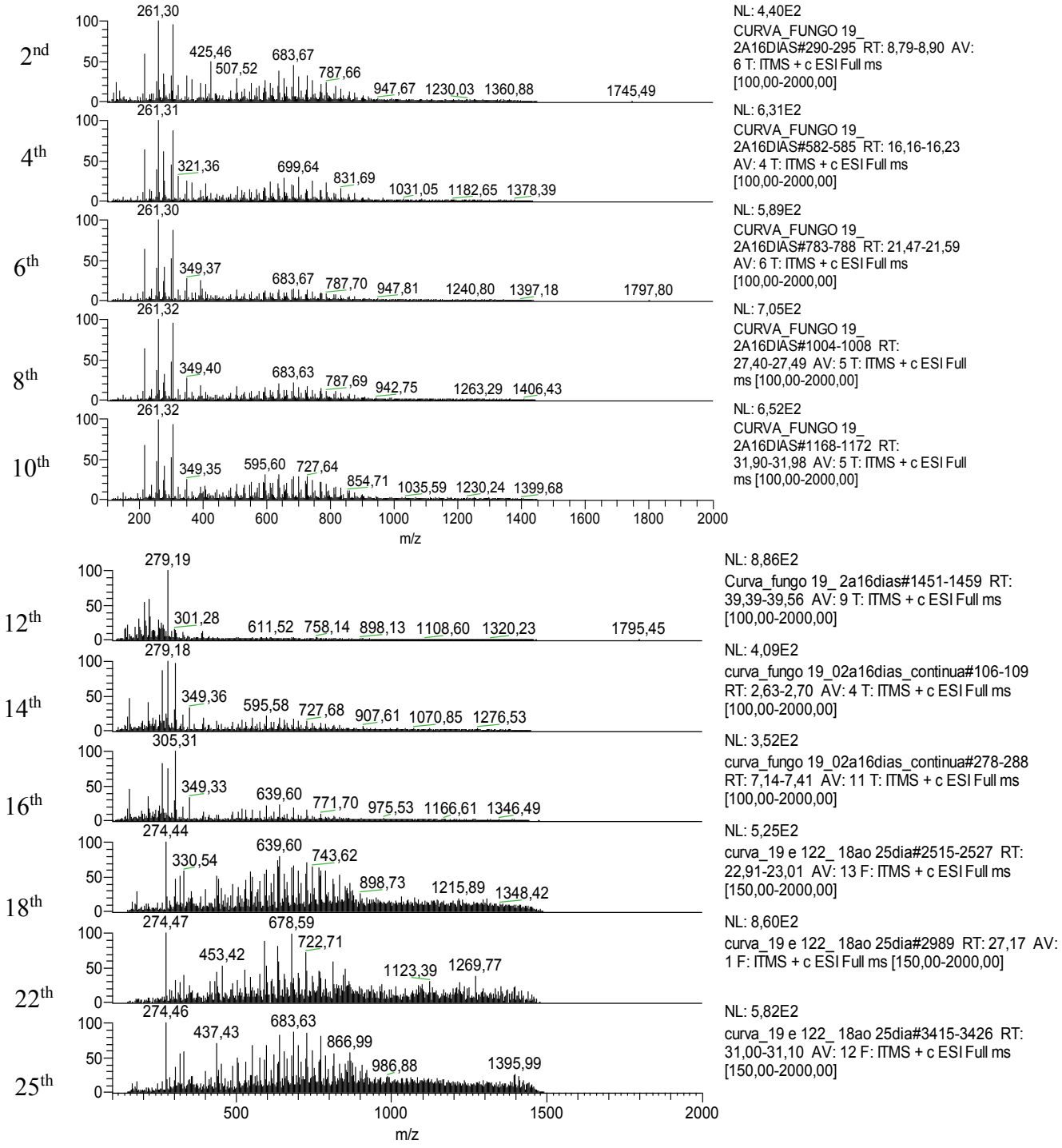

Figure S4. Chemical profile of extracts of the cultivated medium in the shaking method by *T. lentiforme* (T.19) in 2nd, 4th, 6th, 8th, 10th, 12th, 14th, 16th, 18th, 2th, 25th days respectively.

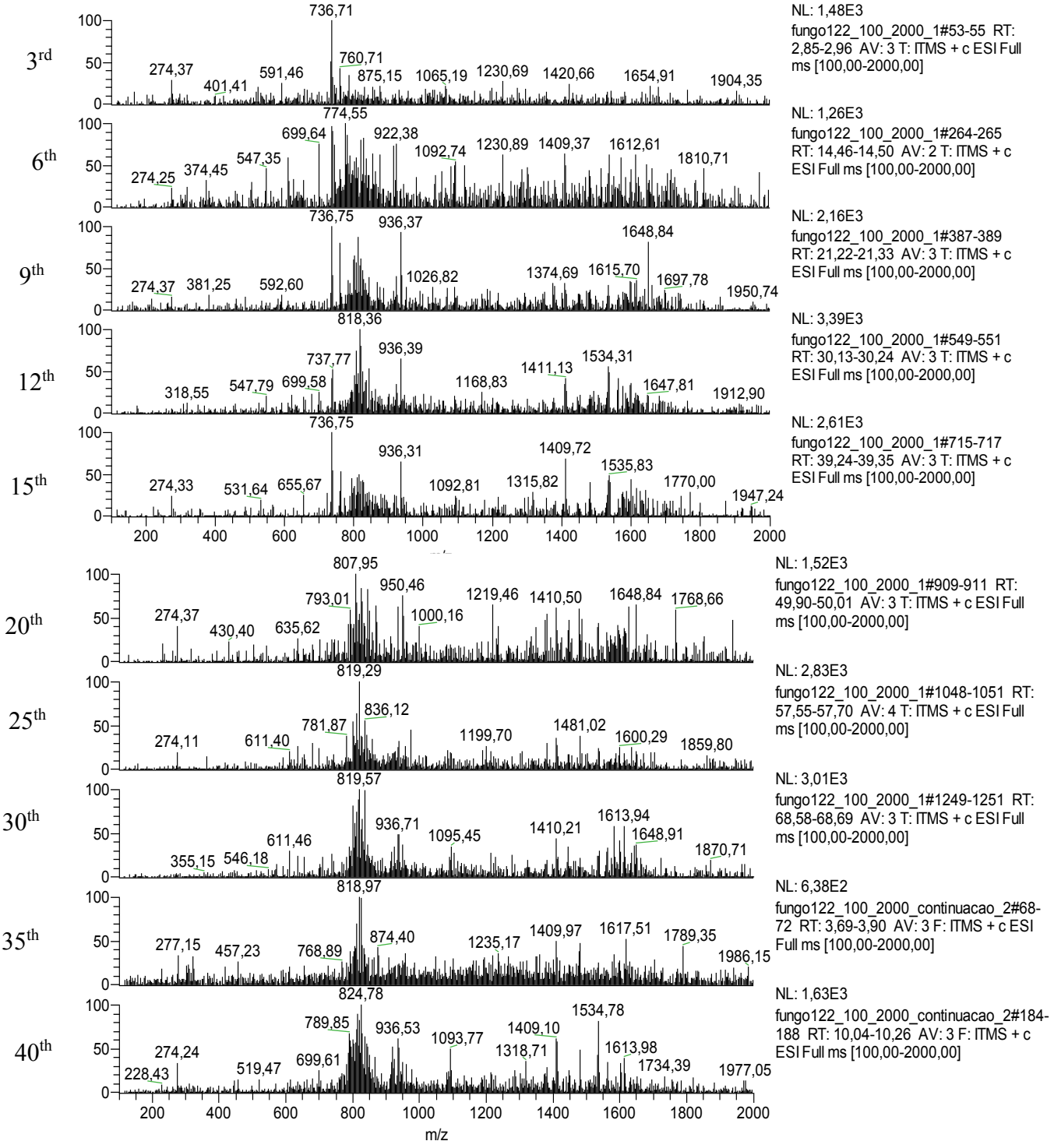

Figure S5. Chemical profile of extracts of mycelium in the static method by *T. harzianum* (T. 122) in 3rd, 6 th, 9th, 12th, 15th, 20th, 25th, 30th, 35th, 40th days respectively.

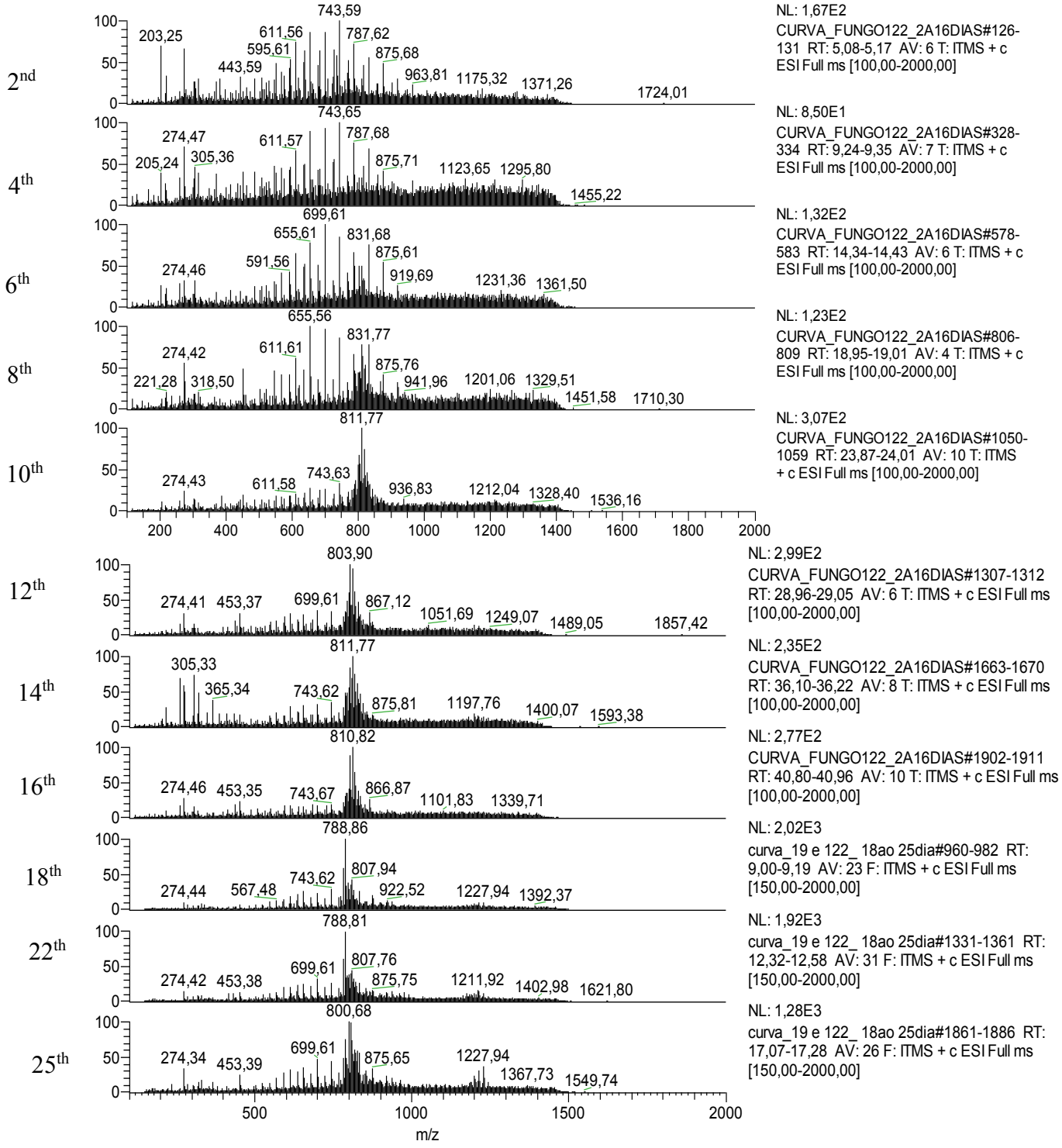

Figure S6. Chemical profile of extracts of mycelium of the shaking method in by *T. harzianum* (T. 122) in 2nd, 4 th, 6th, 8th, 10th, 12th, 14th, 16th, 18th, 22th, 25th days respectively.

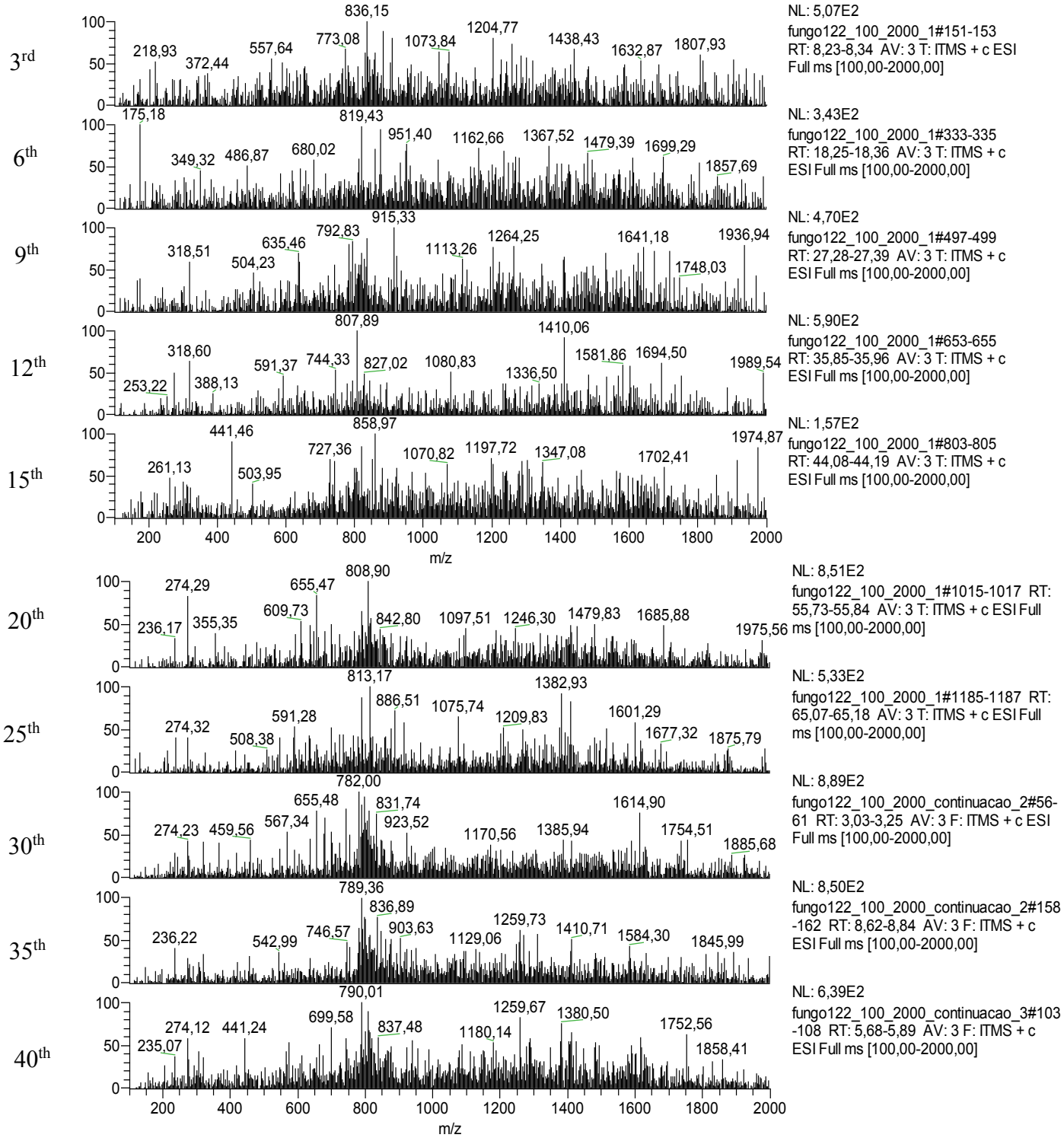

Figure S7. Chemical profile of extracts of the cultivated medium in the static method by *T. harzianum* (T. 122) in 3rd, 6th, 9th, 12th, 15th, 20th, 25th, 30th, 35th, 40th days respectively.

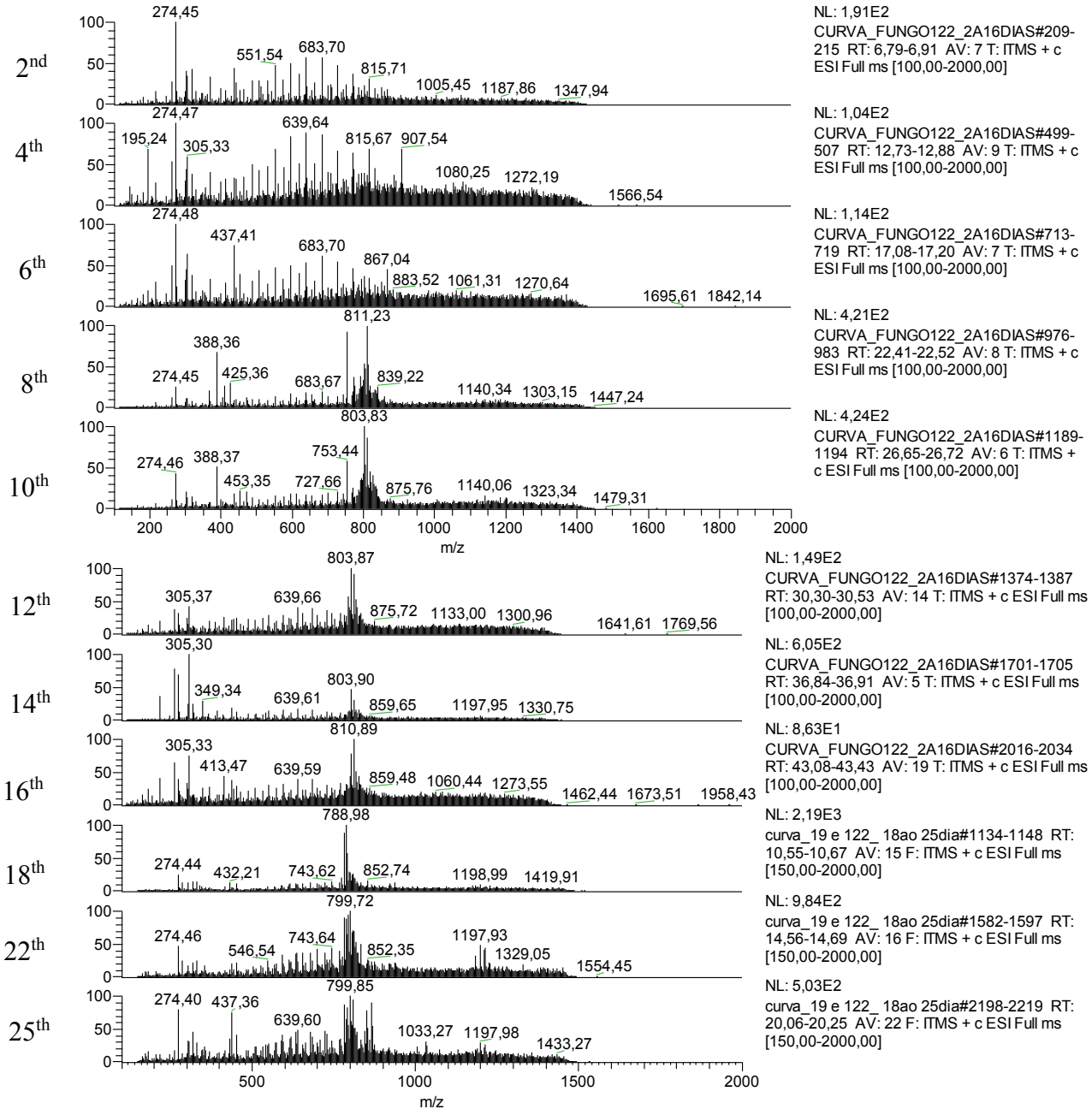

Figure S8. Chemical profile of extracts of the cultivated medium in the shaking method by *T. harzianum* (T.122) in 2nd, 4th, 6th, 8th, 10th, 12th, 14th, 16th, 18th, 22th, 25th days respectively.

CAPÍTULO 4

Avaliação do potencial antimicrobiano de *Trichoderma* spp.

O presente capítulo refere-se ao quinto objetivo da tese, o mesmo foi elaborado com as informações obtidas neste trabalho que não foram contemplados nos capítulos anteriores, organizadas em materiais e métodos e resultados e discussão. Deverá futuramente compor outro manuscrito, com adição de novas análises ou resultados do grupo de pesquisa.

1 Materiais e métodos

1.1 Obtenção dos extratos de *Trichoderma* spp.

Foram selecionadas 17 linhagens representando cada grupo morfológico de *Trichoderma* (Tabela 1). Para o cultivo das linhagens, 20 µL das suspensões de conídios (McFarland No. 6) foram inoculados em triplicata em frascos de 300 mL contendo 125 mL do meio BDL (Batata, Dextrose e 0,2 % de extrato de levedura). O material foi incubado por 20 dias a 28 °C no modo estático. Após cultivo, o meio líquido foi separado do micélio por filtração a vácuo. A extração de metabólitos do meio cultivado foi realizada três vezes por partição com AcOEt/2-PrOH (9:1 v/v) em funil de separação. O micélio foi macerado com EtOAc/MeOH (1:1 v/v) por 48 h e então filtrado. Todos os extratos foram submetidos a evaporador rotativo de baixa pressão (Fisotam), secos, pesados e armazenados a 4 °C para testes biológicos (Souza et al., 2004).

1.2 Cepas bacterianas e fúngicas

Patógenos humanos bacterianos e fúngicos, como *Staphylococcus aureus* (S 007), *Escherichia coli* (E 004), *Pseudomonas aeruginosa* (P 004), *Enterococcus faecalis* (E 002), *Candida albicans* (CC 001), *C. tropicalis* (CC 002), foram obtidos em culturas microbianas mantidas no laboratório Cefar Diagnóstica, São Paulo-SP, para realização de ensaios antimicrobianos.

1.3 Ensaio antimicrobiano - screening e concentrações inibitórias mínimas (CIM)

Os ensaios foram realizados em placas de 96 poços com 100 µL de caldo Mueller Hinton (MHB) ou Sabouraud (SB), 100 µL de soluções de extrato teste e 10 µL de suspensões bacterianas/fúngicas a 1,0 x 10^8 UFC mL^{-1}. Os extratos fúngicos testados foram dissolvidos em dimetilsulfóxido (DMSO) a 10% (v/v) na concentração inicial de

2.000 µg.mL^{-1}, e a incubação foi realizada a 37° C por 24 h. A cor rosa nos poços indicou viabilidade celular após o uso de 2% (v/v) de TTC (cloreto de 2,3,5-trifeniltetrazólio). Fluconazol, tetraciclina (Gram-negativos) e ampicilina (Gram-positivos) foram utilizados durante os testes como controles positivos na concentração de 2000 µg.mL^{-1} e DMSO como controle negativo. As CIMs foram determinadas a partir da diluição seriada da solução estoque de 2.000 µg.mL^{-1}, para a obtenção de faixas de concentração (1000 a 7,81 µg.mL^{-1}). Os resultados foram registrados de acordo com a metodologia descrita por Koolen et al., (2012).

2 Resultados e discussão

Dos trinta e quatro extratos, metade de micélio e metade dos meios cultivados pelas linhagens em estudo, sete (cinco linhagens) apresentaram atividade antimicrobiana contra os patógenos selecionados. Os extratos T.19 CM (meio cultivado), T.261 CM, T.269 MY (micélio) e T.221 MY mostraram atividade contra *C. albicans* a 1.000 µg.mL^{-1}. T.261 CM e T.221 CM mostraram atividade bacteriostática para *S. aureus* na mesma concentração. Os melhores resultados obtidos foram dos extratos T.145 CM e T.221 MY contra *E. faecalis*, ambos usando CIM no valor de 500 µg.mL^{-1} (Tabela 2). O extrato de T.145 CM também mostrou resultados fungicidas para *C. albicans* no teste inicial (screening) e no ensaio de CIM. Nenhum dos extratos foi eficiente contra *E. coli*, *P. aeruginosa*, e *C. tropicalis*.

Os resultados de atividade antimicrobiana destacam a redução de dezessete linhagens para cinco, dentre essas cinco, todas são endofíticas, três foram identificadas como *T. harzianum* (T.261, T.269, T.221), uma linhagem identificada como *T. lentiforme* (T.19) e uma última como *T. asperelloides* (T. 145) (Tabela 1). Pôde-se também verificar que estes cinco extratos são de vias metabólicas e extrações diferentes, ou seja, intra e extracelulares (CM e MY), o que demonstra a versatilidade do gênero na produção de moléculas bioativas utilizando várias rotas biossintéticas.

Das cinco linhagens em destaque, quatro delas (T.19, T.261, T.269 e T.221) apresentaram CIM de 1000 µg.mL^{-1} frente a *C. albicans* e duas linhagens (T.261 e T.221) contra *S. aureus*, valor considerado alto se comparado com trabalhos anteriores, porém vale ressaltar que esses resultados são referentes a extratos brutos, ou seja, material sem qualquer tipo de tratamento cromatográfico, sendo assim, um trabalho mais

aprofundado com suas frações poderia nos indicar uma concentração em potencial, estimulando novas pesquisas.

Tais afirmações baseiam-se em trabalhos anteriores com algumas dessas linhagens, como é o caso de *T. lentiforme* (T.19), do qual Souza (2005) descreve os resultados de atividade antimicrobiana frente a diversos patógenos, incluindo atividade de koningininas isoladas de T.19, tais como A, D, E e F, esta última destacando-se frente a *Aspergillus flavus* com CIM de 31µg.mL^{-1}.

As linhagens *T. asperelloides* (T.145) e *T. harzianum* (T.221) apresentaram a menor concentração de inibição (500 µg.mL^{-1}) contra *E. faecalis*, se comparados aos outros valores de CIM (Tabela 2). Este resultado estimulou o estudo químico das frações de T.145, gerando um trabalho (Fernandes et al., 2020), pioneiro na identificação de substâncias produzidas por *T. asperelloides*, onde uma fração metanólica contendo asperelinas apresentou atividade antimicrobiana contra *S. aureus*, *S. mutans* e *L. monocytogenes*. Neste trabalho foi sugerido que tal atividade seja ocasionada pela presença dos peptaibois e que a espécie em estudo pode ser promissora para o desenvolvimento de novos antibióticos contra bactérias.

Ao longo dos anos, diversos trabalhos reportaram muitas espécies do gênero *Trichoderma* como produtoras de moléculas antimicrobianas (Souza et al., 2004; Vizcaíno et al., 2015; Maha et al., 2018). No trabalho Santos et al. (2018), os extratos etanólicos foram usados nos bioensaios contra *S. aureus*, causando erradicação e desnaturação completa da matriz com redução da biomassa bacteriana em concentrações tão baixas quanto 2,3 µg.mL^{-1}. Nesse mesmo trabalho, foi possível observar, por meio de técnicas de microscopia de varredura, o ataque à parede celular, o distúrbio de membrana e morte de células bacterianas, este trabalho sugeriu que moléculas de alto peso molecular sejam as principais responsáveis por tal atividade.

Em 2013, o trabalho de Moreira revelou que os extratos etanólicos de uma linhagem endofítica de *Trichoderma*, isolada das folhas de *Araucária angustifólia*, apresentaram atividade contra *S. aureus* e *E. coli.* Ishi e colaboradores relataram em 2013 o isolamento e elucidação estrutural de uma molécula anitimicrobiana: citosporona S, produzida por uma linhagem de *Trichoderma*. Esta molécula apresentou atividade tanto para bactérias Gram-positivas quanto para Gram-negativas e alguns fungos.

Em 2015, o trabalho de Pires e colaboradores, relataram linhagens endofíticas de *Trichoderma* com potencial antimicrobiano contra bactérias patogênicas, em destaque *T. longibrachiatum* considerada a mais promissora com inibição de sete dos 10 patógenos testados, incluindo *S. aureus.*

A partir dos resultados deste trabalho e dos trabalhos citados, fica evidente o potencial do gênero *Trichoderma* para a produção de moléculas bioativas para fins medicinais, farmacêuticos e agrícolas. Entretanto é necessário conduzir estudos mais aprofundados com diferentes tipos de substratos de cultivo. Também se devem incluir novas espécies de *Trichoderma* a fim de ampliar a investigação do potencial antimicrobiano de suas espécies. Neste estudo, identificamos T.145 (*T. asperelloides*) e T.221 (*T. harzianum*) como fortes candidatos a estudos químicos para a identificação das moléculas responsáveis pelas atividades antimicrobianas.

Tabela 1. Identificação e resultados de produção dos extratos de *Trichoderma*.

N° da coleção	**Grupo morfológico**	**Origem do isolado**	**Ocorrência**	**Id. Molecular (espécie)**	**N° de acesso *GenBank* ITS**	**N° de acesso *GenBank TEF1*-α**	**Massa do extrato MY (mg)**	**Massa do extrato CM (mg)**
T. 219	1	NI	Endofítico	*T. atroviride*	MN262492	*	404,0	285,0
T.145	2	*Victoria amazonica*	Endofítico	*T. asperelloides*	MN262514	MT281578	802,0	80,7
T. 531	3	NI	Endofítico	*T. afroharzianum*	MN262485	MT681949	234,0	116,3
T.122	4	*Scleronema micranthum*	Decompositor de madeira	*T. harzianum*	MN262505	MT681970	660,7	31,0
T.263	5	*S. micranthum*	Decompositor de madeira	*T. spirale*	MN283156	MT681974	184,0	96,0
T.137	6	*S. micranthum*	Decompositor de madeira	*T. lentiforme*	MN262496	MT681961	572,0	76,0
T.264	7	NI	Endofítico	*T. lentiforme*	MN262483	MT681953	850,0	76,0
T. 269	8	NI	Endofítico	*T. harzianum*	MN262484	MT681968	585,0	121,0
T.139	9	*S. micranthum*	Decompositor de madeira	*T. harzianum*	MN262497	MT681969	959,0	227,2
T.261	10	NI	Endofítico	*T. harzianum*	MN262500	MT681967	753,7	47,8
T. 60	11	*Rollinia* sp.	Endofítico	*T. lentiforme*	MN262481	MT681959	266,6	136,0
T. 28	12	*Rollinia* sp.	Endofítico	*T. lentiforme*	MN262487	MT681963	410	36,0
T. 19	13	*Strychnos cogens*	Endofítico	*T. lentiforme*	MN262491	MT681962	3.000,0	198,0
T. 37	14	*Murraya paniculata*	Endofítico	*T. koningiopsis*	MN262513	MT681980	227,6	132,3
T. 267	15	NI	Endofítico	*T. lentiforme*	MN382157	MT681955	273,0	104,4
T.221	16	NI	Endofítico	*T. harzianum*	MN262490	MT681971	480, 0	31,1
T. 869	17	River	Aquatic	*C. rosea*	MN262501	MT681984	697,0	72,4

NI: Não identificado; *: Não depositado; MY: extrato do micélio; CM: extrato do meio cultivado.

Tabela 2. Atividade antimicrobiana dos extratos de *Trichoderma* spp.

Patógenos	Concentração Mínima Inibitória (CIM)										
	T.19 CM	T.261MY	T.261CM	T.269 MY	T.145 CM	T.221MY		T.221 CM	C+ Fluoconazol	C+ Ampicilina	C- DMSO
	+	+	+	+	++	+	++	+	+/++	+/++	SA
C. albicans	1000	1000	SA	1000	SA	1000	SA	SA	7,81	SA	SA
E. feacalis	SA	SA	SA	SA	500	SA	500	SA	SA	7,81	SA
S. aureus	SA	SA	1000	SA	SA	SA	SA	1000	SA	7,81	SA

Nota: valores em µg. mL^{-1}

(+) Bacteriostático/Fungistático; (++) Bactericida/Fungicida (SA) Sem atividade; MY: extrato do micélio; CM: extrato do meio cultivado.

4 CONSIDERAÇÕES FINAIS

Neste trabalho avançamos no conhecimento de espécies do gênero *Trichoderma* que ocorrem na Amazônia, utilizando diferentes abordagens para analisar sua diversidade e amostrando-a através de trinta e sete linhagens, selecionadas de uma coleção de trabalho (LABMICRA-UFAM).

A macromorfologia foi útil para diferenciação em nível de gênero, e para prévia diferenciação de espécies de *Trichoderma*. Além disso, os resultados revelaram que é necessário definir níveis de relevância e associar como forma de complemento, dados de outras técnicas, juntamente aos dados morfológicos avaliados.

A identificação molecular revelou nove espécies de *Trichoderma* e uma linhagem do gênero *Clonostachys rosea*. Entre as espécies de *Trichoderma,* foi possível agrupar com eficiência as espécies mais numerosas como *T. harzianum* e *T. lentiforme*.

A análise de perfis químicos por espectrometria de massas mostrou eficiência para fins taxonômicos, na identificação de grupos mais definidos em nível de espécie, como observado, por exemplo, entre *T. asperellum* e *T. asperelloides*, *T. atroviride* e *T. reesei.* Foi possível verificar a riqueza de metabólitos secundários produzidos por diversas espécies, revelando suas aproximações, agrupamentos por similaridades metabólicas e associações com suas origens e aspectos moleculares e metabólicos.

A curva de produção metabólica possibilitou a compreensão do comportamento do gênero. De acordo com as variáveis: tempo e agitação, foi possível definir as fases de produção das linhagens selecionadas e avaliar o período adequado para análise de perfil químico e produção de substâncias.

Os testes biológicos demonstraram que quatro linhagens possuem atividade antimicrobiana frente a *C. albicans* na concentração de 1000 $\mu g.mL^{-1}$, duas foram bacteriostáticas para *S. aureus*, na mesma concentração e duas apresentaram atividade bactericida com CIM de 500 $\mu g.mL^{-1}$ para *E. faecalis*, confirmando o potencial de *Trichoderma* já reportado em diversos trabalhos.

A linhagem T.145 (*T. asperellum*) revelou-se como uma excelente produtora de peptaibois, confirmada através da elucidação estrutural de quinze sequências de asperelinas, nove inéditas e seis já relatadas na literatura. Essa linhagem também apresentou frações com potencial antimicrobiano contra bactérias Gram-negativas e baixa

toxicidade frente a células do tipo Vero. Acreditamos que tais resultados podem estar associados a presença das asperelinas nas amostras testadas, contudo, vale ressaltar que são necessários estudos mais aprofundados com estas moléculas, de forma isolada, para confirmar tais atividades.

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6 ANEXOS

Anexo 1. Protocolo de extração de DNA

Protocol

For optimal performance, add beta-mercaptoethanol (user supplied) to the Fungal/Bacterial DNA Binding Buffer to a final dilution of 0.5%(v/v) i.e., 500 µl per 100 ml.

1. Add 10-20 mg (wet weight) fungal or bacterial cells[1] that have been resuspended in up to 200 µl of water or isotonic buffer (e.g., PBS) or up to 40 mg of tissue to a ZR BashingBead™ Lysis Tube (0.1 & 0.5 mm). Add 750 µl Lysis Solution to the tube[2].

2. Secure in a bead beater fitted with a 2 ml tube holder assembly and process at maximum speed for ≥ 5 minutes.

   Note: Required processing time will vary depending on the device and application and therefore should be evaluated on a case by case basis.

   For example, processing times may be as little as 3 minutes when using high-speed cell disrupters (e.g., the portable TerraLyzer™ Sample Processor, FastPrep® -24, or similar) or as long as 20 minutes when using lower speeds (e.g., Disruptor Genie™, or standard benchtop vortexes). See manufacturer's literature for operating information.

3. Centrifuge the ZR BashingBead™ Lysis Tube in a microcentrifuge at 10,000 x g for 1 minute.

4. Transfer up to 400 µl supernatant to a Zymo-Spin™ IV Spin Filter (Orange Top) in a Collection Tube and centrifuge at 7,000 x g for 1 minute.

   Note: Snap off the base of the Zymo-Spin™ IV Spin Filter (Orange Top) prior to use.

5. Add 1,200 µl of Fungal/Bacterial DNA Binding Buffer to the filtrate in the Collection Tube from Step 4.

6. Transfer 800 µl of the mixture from Step 5 to a Zymo-Spin™ IC Column[3] in a Collection Tube and centrifuge at 10,000 x g for 1 minute.

7. Discard the flow through from the Collection Tube and repeat Step 6.

8. Add 200 µl DNA Pre-Wash Buffer to the Zymo-Spin™ IC Column in a new Collection Tube and centrifuge at 10,000 x g for 1 minute.

9. Add 500 µl Fungal/Bacterial DNA Wash Buffer to the Zymo-Spin™ IC Column and centrifuge at 10,000 x g for 1 minute.

10. Transfer the Zymo-Spin™ IC Column to a clean 1.5 ml microcentrifuge tube and add 20 µl (10 µl minimum) DNA Elution Buffer directly to the column matrix. Wait 2-3 minutes and then centrifuge at 10,000 x g for 30 seconds to elute the DNA.

Ultra-pure DNA is now ready for use in your experiments.

[1] This equates to approximately 2x10[8] bacterial cells, 2x10[7] yeast cells or 2x10[6] mammalian cells.

[2] Cap tube tightly to prevent leakage.

[3] The Zymo-Spin™ IC Column has a maximum capacity of 800 µl.

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Anexo 2. Protocolo para PCR - *Polymerase Chain Reaction*

6.3. PCR with Ready-To-Go PCR Beads

For general information concerning primer design and cycling parameters, refer to Appendixes 1–4.

When performing PCR amplifications, exercise extreme care to prevent DNA contamination as described above. Each PCR bead is designed for use in a 25 µl reaction volume (one PCR bead/tube).

When resuspended in a final volume of 25 µl, each reaction will contain 1.5 mM $MgCl_2$. Please refer to Appendix 3 if a higher concentration of $MgCl_2$ is desired.

1. For each reaction, add the following to a tube containing a PCR bead:

   Note: Do not mix the tube contents until all the components (below) have been added to the tube containing the bead.

5' (forward) primer (5–25 pmol)	X µl
3' (reverse) primer (5–25 pmol)	Y µl
Template DNA*	Z µl
Sterile high-quality water	to a final volume of 25 µl

   *Start with 50 pg for a simple template such as plasmid DNA, or 50 ng for a complex template such as genomic DNA. Avoid template amounts > 1 µg.

2. Snap the caps (provided) onto the tubes, pushing down firmly to ensure a tight fit. Mix the tube contents by gently flicking the tube with a finger. Vortex gently and then centrifuge the tube for a few seconds to bring the components to the bottom of the tube. The reaction is fully dissolved and mixed when it appears clear.

3. Place the reaction mixtures on ice or in a cold block until ready for cycling. Minimize the time on ice prior to cycling to prevent formation of background reaction products.

6.4. Thermal Cycling

The optimal cycling profile for a given PCR system and thermal cycler will vary and must be determined empirically. Cycle number can range from 20 to 40 depending on the desired yield of product. Thermal cycling results and product yield can vary with cycle conditions and thermal cycler used. Read the instructions provided with your thermal cycler and optimize reaction conditions accordingly.

Anexo 3. Protocolo Exosap – GE Healthcare.

Rapid, Simple Protocol

Setting up clean-up reactions with illustra™ ExoStar™ 1-Step requires only a single pipetting step, thanks to the pre-mixed enzyme formulation.

Recommended Protocol

1. Remove the illustra ExoStar 1-Step from the freezer and keep on ice whilst preparing the reaction.
2. Take a 5µl Aliquot of the completed PCR reaction mix.
3. Add 2µl of illustra ExoStar 1-Step to the reaction mix.
4. Incubate at 37°C for 15 minutes.
5. Incubate at 80°C for 15 minutes to inactivate the enzymes.

The PCR product is now ready for use in downstream reactions and processes. If a larger volume of PCR product is required, simply increase the volume of illustra ExoStar 1-Step added in proportion with the volume of PCR product.

Optimised for efficient primer digestion

The new illustra Alkaline Phosphatase and Exonuclease 1 enzymes have been optimised for highly efficient primer digestion, helping to improve the quality of downstream analysis. In analysis of primer digestion illustra ExoStar 1-Step was more efficient in digesting primers than the traditional USB® ExoSAP-IT® product when used under the manufacturer's standard operating protocol.

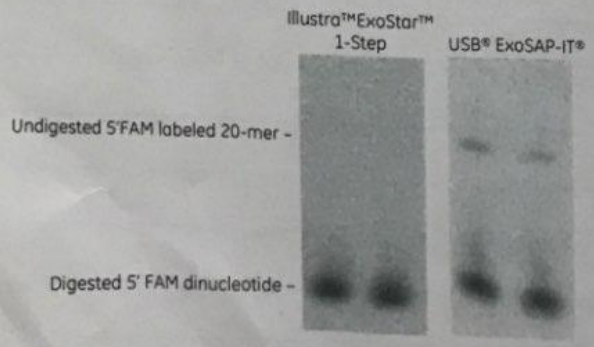


Fig. 1*: Electrophoretic analysis of the digestion of a 5'FAM labelled 20mer primer. Reactions were conducted according to manufacturer's instructions for illustra ExoStar 1-Step and USB® ExoSAP-IT® respectively, using 10 pmol of primer per reaction. No detectable primer remained in the samples digested using illustra ExoStar 1-Step but undigested primer remained in samples treated with USB® ExoSAP-IT®.

* Data presented in Fig. 1 was obtained by scientists at GE Healthcare, using experimental conditions as set out in the manufacturer's operating instructions for USB® ExoSAP-IT®.

No loss of PCR product

The use of an enzymatic digestion approach to clean up amplification reactions reduces losses of PCR product. The process has no intermediate transfer steps, spin columns or binding matrix to retain your PCR product and double stranded DNA is left intact by the Exonuclease 1 and Alkaline Phosphatase enzymes. The size of the PCR fragment does not affect the clean-up efficiency of the reaction.

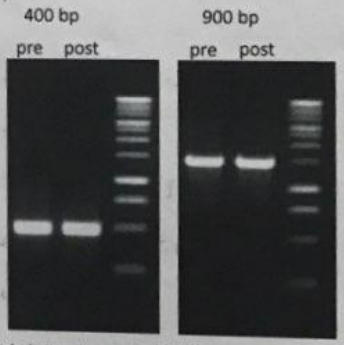


Fig. 2: Agarose gel electrophoresis of different size PCR products pre and post digestion with illustra ExoStar 1-Step. Samples were digested for 15 minutes at 37° followed by denaturation of the illustra ExoStar 1-Step enzymes at 80°C for 15 minutes as per the recommended operating protocol. No loss of PCR product was detected in any of the samples.

High quality sequencing results

Removal of unincorporated primers and nucleotides is essential to high quality DNA sequencing. Failure to fully remove these components leads to high background signals and miscalling of bases. With illustra ExoStar 1-Step, Phred20 quality scores were routinely achieved at read lengths >800 bp, equivalent to or better than other approaches to sample preparation.

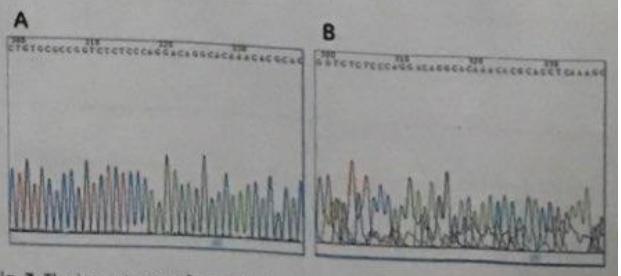


Fig. 3: The importance of sample clean up before DNA sequencing is illustrated in the comparison between panel A, showing PCR sequence quality following treatment with illustra ExoStar 1-Step and panel B showing sequence quality without this treatment. Read length, base calling and sequence quality are significantly improved by the use of illustra ExoStar 1-Step.

Anexo 4. Protocolo de reação de sequenciamento e purificação.

3ª ETAPA → Reação de Sequenciamento

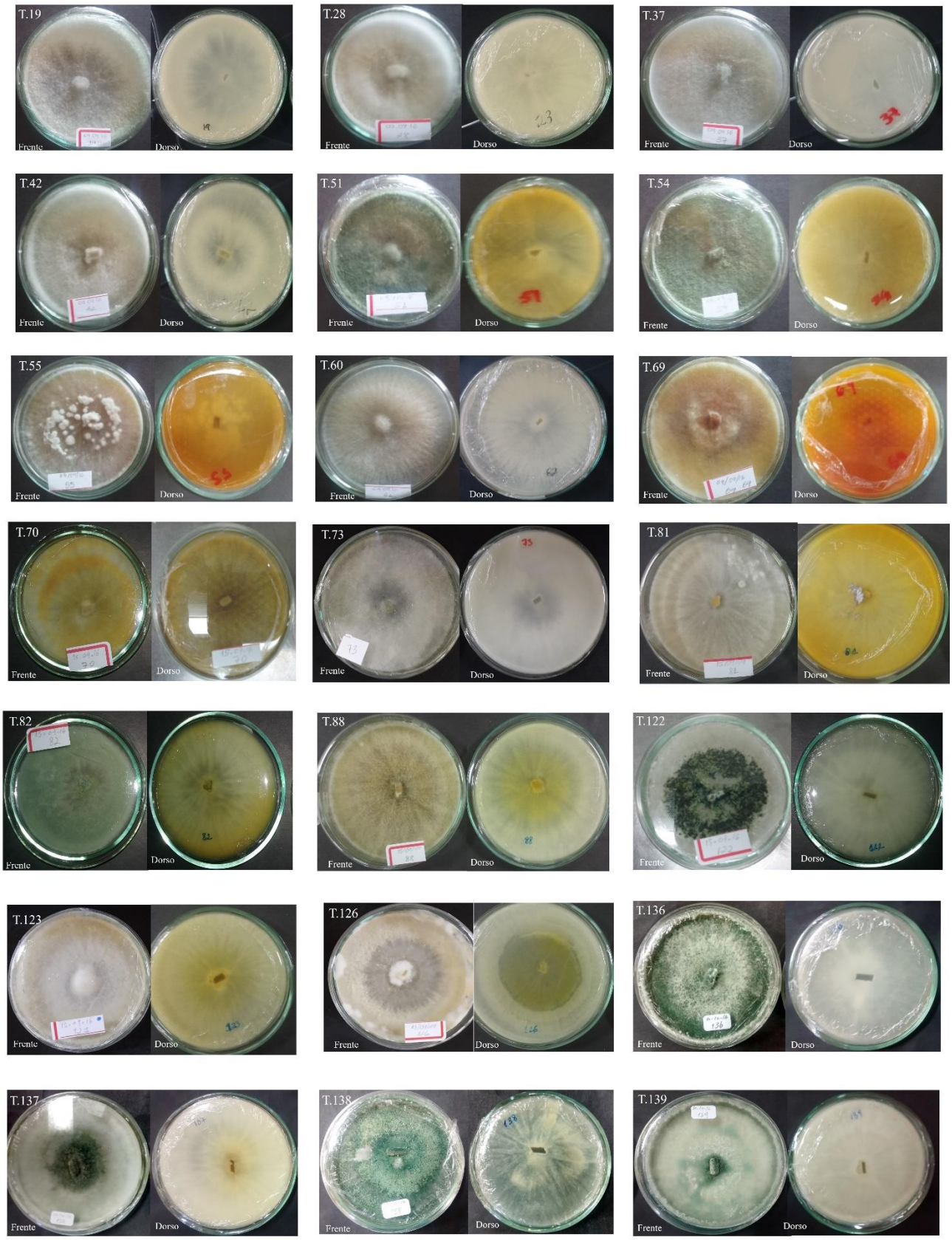
Reagentes	1 reação	12 reações
H_2O UP	2µL	24 µL
Tampão Big Dye	1,5 µL	18 µL
Big Dye Terminator (v3.1)	0,5 µL	6 µL
Primer F ou R (3,2pmol)	1 µL	12 µL
Produto de PCR	5 µL	60 µL
Volume Final	10 µL	120 µL

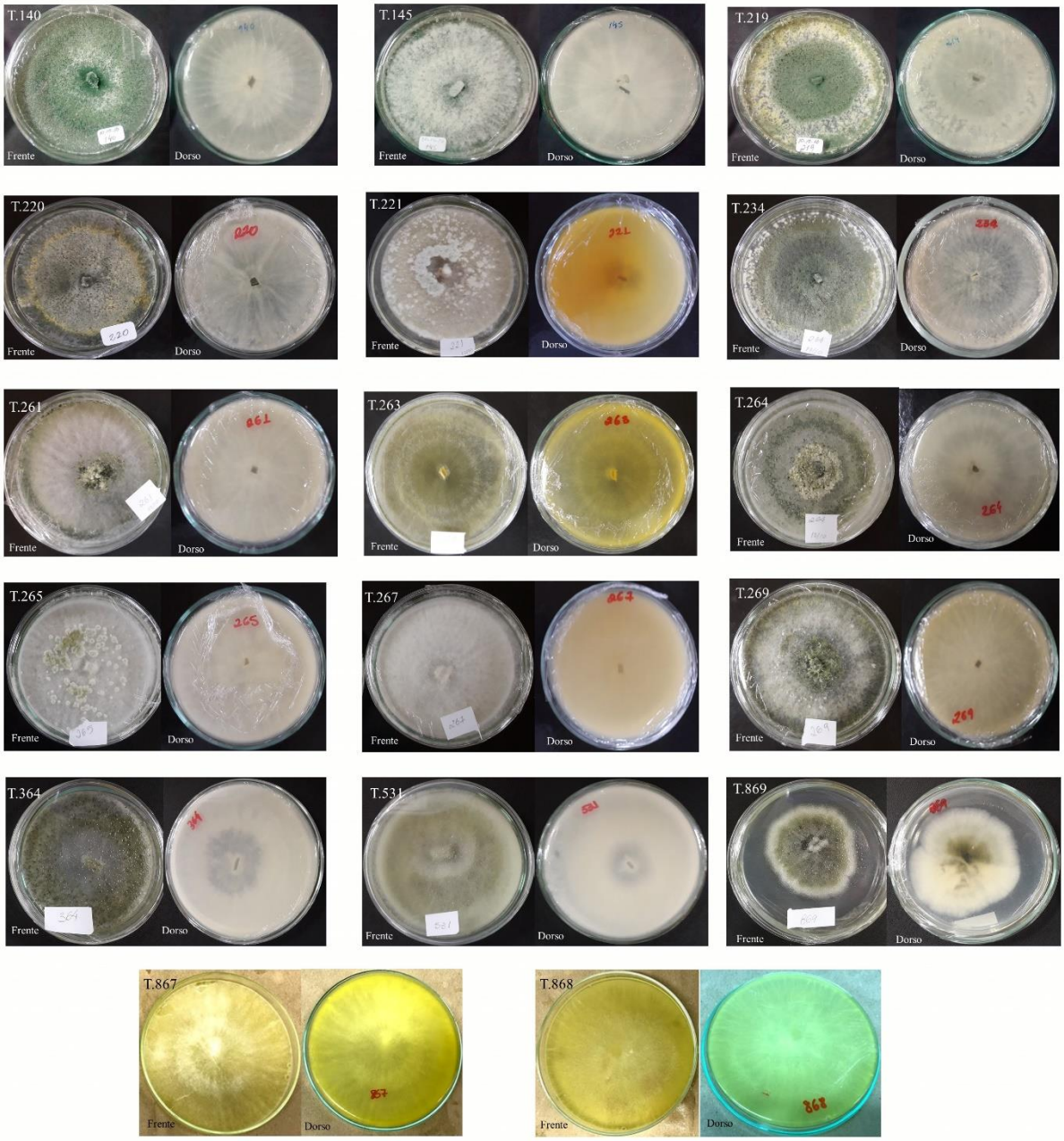
Incubar no termociclador (programa gravado)
96°C por 1min – 1x
96°C por 15seg
50°C por 15seg } 35x
60°C por 4min
10°C ∞

4ª ETAPA → Purificação para precipitação das reações de sequenciamento (placa c/96poços) 10 µL de reação

4.1 Remova a placa de reação de 96 poços do termociclador e centrifugue rapidamente (spin);
4.2 Adicione 5 µL de EDTA 125mM em cada poço;
4.3 Adicione 60 µL de Etanol 100% em cada poço;
4.4 Sele a placa, cubra com papel alumínio e misture com vortex;
4.5 Incube a temperatura ambiente por 15min;
4.6 Centrifugue à 4°C, 3000g x 30min;
4.7 Inverta a placa na centrífuga e centrifugue a 185g x 20seg, em seguida retire da centrífuga;
4.8 Adicione 60 µL de Etanol 70% em cada poço;
4.9 Centrifugue à 4°C, 1650g x 15min;
4.10 Inverta a placa na centrífuga e centrifugue a 185g x 20seg, em seguida retire da centrífuga (Nota: comece a contar o tempo (1') quando o rotor começar a se mover)
4.11 Evaporar o álcool em termociclador a 95°C por 3min;
4.12 Ressuspenda as amostras em 10 µL de tampão de injeção (HI-DI formamida);
4.13 Misture com vortex (3s) e centrifugue 2000g x 30seg;
4.14 Incubar em termociclador a 95°C por 3min e colocar imediatamente no gelo e levar para sequenciamento.

Anexo 5. Prancha com trinta e sete linhagens de *Trichoderma* e uma linhagem de *Clonostachys rosea.*

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