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Peptaibols from *Trichoderma asperellum* TR356 strain isolated from Brazilian soil

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Abstract

The *Trichoderma* genus consists of a group of free-living filamentous fungi, including species able to act as biological control agents (BCAs) against pathogenic fungi. It is believed that this ability is due to synergy between several mechanisms, including the production of a wide variety of secondary metabolites by these organisms. Among these, we highlight the production of peptaibols, an antibiotic peptide group characterized by the presence of non-proteinogenic amino acids such as α -aminoisobutyrate (Aib), as well as by N-terminal modifications and amino alcohols in the C-terminal region. This study aimed to outline a profile of peptaibol production and to identify secreted peptaibols from the *Trichoderma asperellum* TR356 strain, described as an efficient BCA against *S. sclerotiorum*. The fungus was grown on TLE 0.3% glucose medium for 5 days, with agitation at 120 rpm in the dark. Liquid medium filtrate was used as the metabolite source. These extracts were subjected to high performance liquid chromatography (HPLC) and subsequent analysis by matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF). The results indicate the production of two classes of peptaibols for this *T. asperellum* strain. Primary structures of two asperelines (A and E) and five trichotoxins (T5D2, T5E, T5F, T5G and 1717A) have been elucidated. Most of these peptaibols had been previously described in *T. viride* and *T. asperellum* marine strains. This is the first report of some of these compounds being produced by a *T. asperellum* strain from soil. Future analyses will be necessary to elucidate the three-dimensional structures and their activities against pathogens.

Keywords: *Trichoderma asperellum*; Peptaibols; Mass spectrometry; Asperelines; Trichotoxins

Introduction

The *Trichoderma* genus belongs to the Ascomycota phylum, Hypocrales order, Hypocreaceae family and corresponds to filamentous fungi that are free-living and widely distributed in nature. One of the main characteristics of this genus is its ability to act as a biological control agent (BCA) against phytopathogenic microorganisms such as *Sclerotinia sclerotiorum*, *Rhizoctonia solani* and *Fusarium* spp. (Harman, 2006; Schuster & Schmoll, 2010).

The biological control mechanisms can be divided into direct and indirect effects. Direct effects include nutrient and space competition, volatile and non-volatile antibiotic production, hydrolytic enzyme production and parasitism. Indirect effects include all aspects that promote morphological and biochemical changes in the host plant, such as nutrient availability, stress responses

and induction of resistance to diseases caused by plant pathogens (Harman *et al.*, 2004; Vinale *et al.*, 2008; Vinale *et al.*, 2012). It is believed that *Trichoderma's* extensive mycoparasitism ability is also related to the wide variety of secondary metabolites produced by these organisms (O'Brian & Wright, 2011; Mukherjee *et al.*, 2012).

Among secondary metabolites produced by *Trichoderma* spp., there are reports and descriptions of such compounds as pyrones, terpenoids, steroids, gliotoxins, gliovirins (Reino *et al.*, 2008), and some antibiotic peptides, known as peptaibols (Daniel, 2007). The discovery of peptide antibiotics produced by fungi has attracted attention because they represent a possible weapon against pathogens. Peptaibols are included in a class of compounds called peptaibiotics. They are defined as peptides derived from fungal secondary metabolism, consisting of 4 to 21 amino acid residues approximately. One of their main features is the presence of non-proteinogenic amino acids such as α -aminoisobutyrate acid (Aib), isovaline (Iva), ethylnorvaline (EtNor) and hydroxyproline (Hyp). (Stoppacher *et al.*,

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2013). At the N-terminus there are modifications such as acyl or acetyl groups, and at the C-terminus there is the presence of an amino alcohol such phenylalaninol (Phe-OH), prolinol (Pro-OH) or valinol (Val-OH) (Daniel, 2007). The name “peptaibol” is derived from *peptide*, *Aib* and amino alcohol, referring to these main features.

Several peptaibols from *Trichoderma* have already been described. Recently, “The Comprehensive Peptaibiotics Database” was created, including information such as amino acid sequences, molecular formulae, monoisotopic masses, and groupings for 1043 peptaibiotics (Stoppacher et al., 2013; <http://peptaibiotics-database.boku.ac.at>). Because some of these compounds present antimicrobial activity, there is a growing interest in their identification and structure elucidation. Peptaibols from *Trichoderma* have been reported as showing activity against mycoplasma (Beven et al., 1998), *Staphylococcus aureus* and *Sclerotium rolfsii* (Rebuffat et al. 1995). Some peptaibols, called trichorzianins, acted in synergy with *T. harzianum* chitinases and β -1,3-glucanases against *Botrytis cinerea*, and it was reported that these same peptaibols inhibited the activity of *B. cinerea* β -1,3-glucan synthases, preventing cell wall reconstruction (Schirmböck et al., 1994; Lorito et al., 1996).

There are also reports of *T. harzianum* peptaibols causing biological changes in human lung epithelial carcinoma cells (Peltola et al. 2004), and of *T. koningii* peptaibols inhibiting hepatocellular carcinoma cell growth by causing an increase in calcium influx and consequent activation of the apoptosis pathway (Wang et al., 2010). Some studies suggest that their biological activity is related to their amphipathic nature, allowing these compounds to form ion channels in lipid membranes (Mikkola et al., 2012; Rahaman & Lazaridis, 2014). Some studies have focused

on peptaibols produced by *Trichoderma* in genus classification and taxonomy (Degenkolb et al., 2008).

Peptaibols fit into a group of metabolites called non-ribosomal peptides. This is because these compounds do not result from gene transcription and subsequent translation, but are formed from multi-enzyme complex called Non-Ribosomal Peptide Synthetases (NRPSs). In summary, these multienzyme complexes are formed by a set of modules, where each module has catalytic domains responsible for the synthesis steps (Schwarzer et al., 2003). In general, there are three main steps, starting in domain A (adenylation), where the biosynthetic process begins with amino acid entry, which is activated by adenylation. The activated amino acid is attached to a PCP protein cofactor (HS-4'PP) that acts as a carrier between the catalytic centers, and finally, in domain C (condensation), peptide bond formation occurs (Mootz et al., 2002; Schwarzer et al., 2003).

In this context, this study aimed to draw a production profile and identify peptaibols secreted by the *Trichoderma asperellum* TR356 strain, based on a recent study conducted by Geraldine and colleagues (2013), showing that this strain had high efficiency in the control of white mold, a disease caused by the pathogen *Sclerotinia sclerotiorum*. In that study the authors attributed the biological control efficiency to the high production of hydrolytic enzymes such as NAGase and β -1,3-glucanase, involved in host cell wall degradation. However, the involvement of other secondary metabolites such as peptaibols, was not analyzed. In the literature there are several reports of peptaibols identified from strains of *T. asperellum*, but the production mechanisms of these compounds between strains are not identical (Chutrakul et al., 2008; Ren et al., 2009; Ren et al., 2013). Based on this, we determined the need to investigate

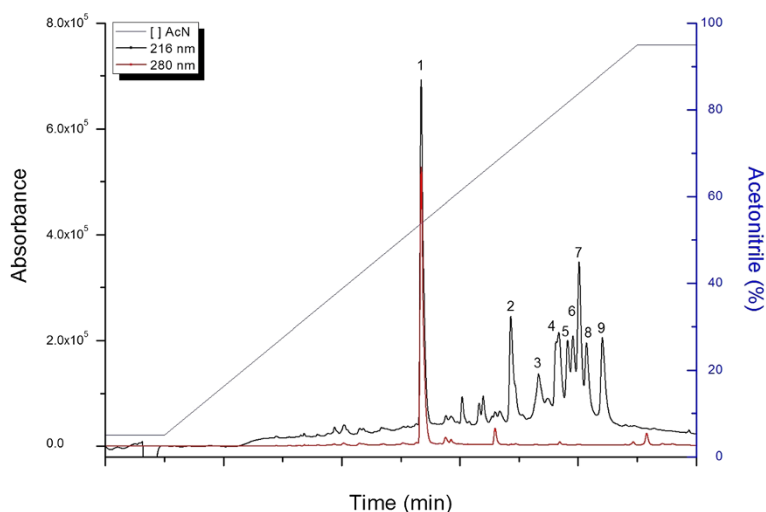


Figure 1 *T. asperellum* TR356 extract chromatography (HPLC). Detection at 216 nm (black) and 280 nm (red). The gradient of acetonitrile + 0.1% TFA is shown in blue.

The Asperelin group was previously described only in *Trichoderma asperellum* marine strains (Ren *et al.*, 2009; Ren *et al.*, 2013). We believe that this is the first description of these compounds being produced by a soil strain of *Trichoderma asperellum*.

In the fraction 4 analysis (Figure 3a), interpretation of just two ions were possible. The ion with m/z 1698.1 $[M + Na^+]$ (Figure 3c) showed most of b-series and part of the γ -series. In the N-terminal portion of the b-series there is a difference of 270 Da, and between isovaline and proline, there is a sodium ion, also observed in the γ -series. In the C-terminal portion of the γ -series it is possible to find an ion of m/z 612. In Jaworski & Brückner (1999) *T. viride* trichotoxins study, it was noted that the bond between Aib and proline on these peptaibols is extremely labile, thus generating two ions resulting from this break.

This feature would explain the binding of sodium ions in this exact position in both series, and the presence of the m/z 612 ion, which can also be observed in the spectrum of fraction 4 (Figure 3a). After fragmentation,

this ion seems to confirm what had been observed in the b-series (Figure 3b). The difference of 211 Da suggested the presence of a proline and leucine/isoleucine. After comparing the sequence to those in the databases, it was possible to identify a peptaibol named trichotoxin T5D2 with molecular weight of 1675 Da (Suwan *et al.*, 2000). From this reference then we can assume that the difference of 270 Da corresponds to an Aib + acetyl group, a glycine, and a second Aib. The trichotoxin T5D2 identified in this spectrum has not yet been described in *T. asperellum*.

In fraction 5 (Figure 4a) only an ion of m/z 1728.0 $[M + K^+]$ was interpretable (Figure 4b). Interpretation of the ion with m/z 1712.1 was not possible because there seemed to be more than one peptide being fragmented together. Again, it is possible to see part of γ -series, most of the b-series, and a difference of 270 Da at the beginning of the b-series. We also noted the presence of a potassium ion between Aib and proline due to the fragility of that bond, as discussed previously. In this fraction, the ion with m/z 612 was also seen (data not shown), and when fragmented

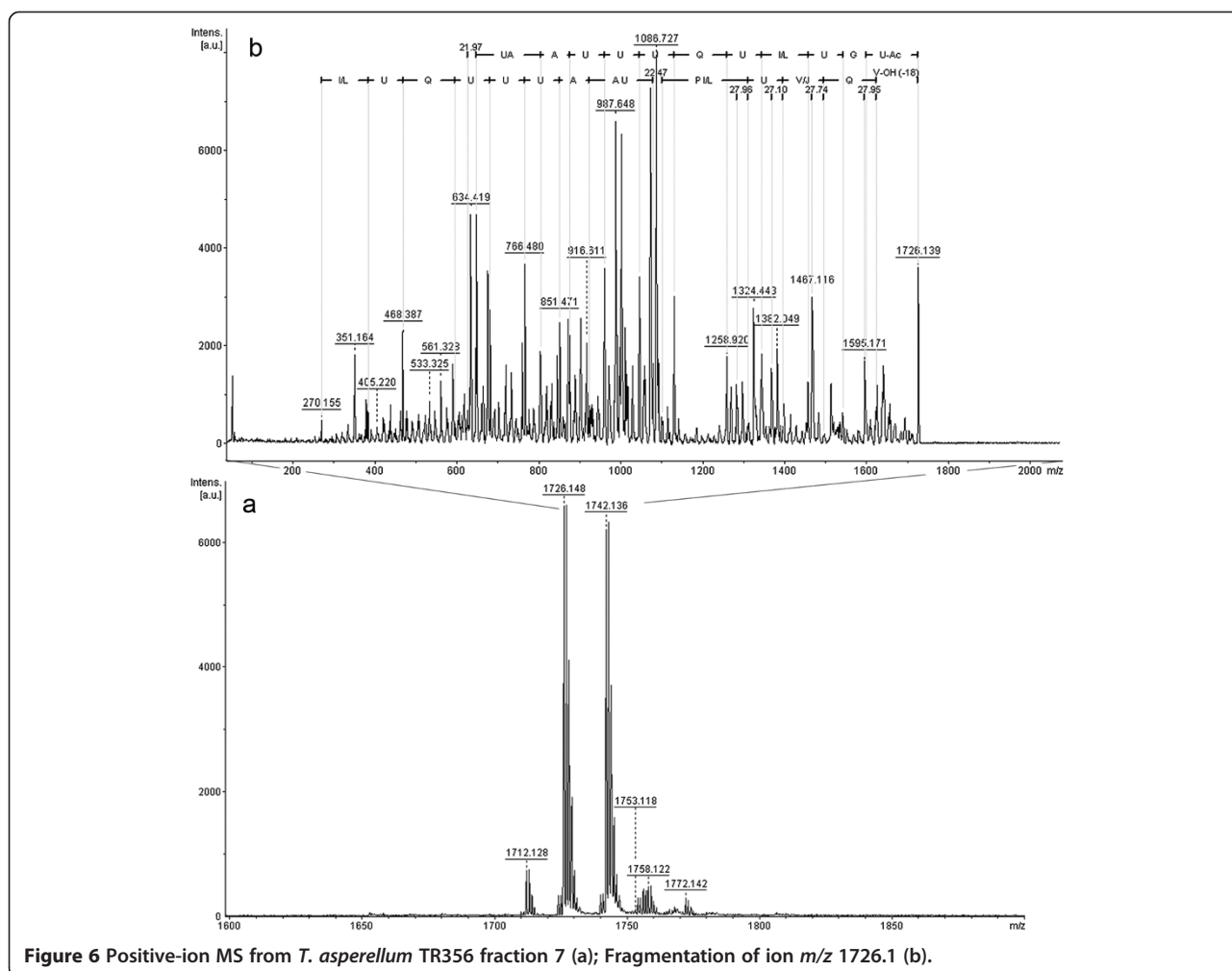


Figure 6 Positive-ion MS from *T. asperellum* TR356 fraction 7 (a); Fragmentation of ion m/z 1726.1 (b).

this has the same sequence as that described above. Sequence analysis suggests that this peptaibol is a trichotoxin A-50 E (T5E), first identified by Bruckner & Przybylski (1984) and isolated from *T. viride*. From this information, it is assumed that the difference of 270 Da corresponds to Aib + acetyl group, glycine, and a second Aib. This peptaibol has not previously been described in isolates of *T. asperellum*.

In the analysis of fraction 6, the two most prominent features are ions of m/z 1712.1 $[M + Na^+]$ and 1728.0 $[M + Na^+]$ (Figure 5a). In the m/z 1712.1 $[M + Na^+]$ ion fragmentation we observed part of the γ -series and most of the b -series. Again, as reported previously, there is a difference of 270 Da in the beginning of the b -series, and the presence of sodium ions, which in this analysis are in different positions in each series (Figure 5b). The Y -series appears to finish at m/z 626.4, probably due to the labile bond between proline and Aib as discussed above. Thus, when we look at the fraction 6 mass spectrum in this region, it is possible to detect and

fragment this ion, confirming part of the series that was not observed in the m/z 1712.1 $[M + Na^+]$ fragmentation. Based on these data, the analysis of this sequence was similar to the trichotoxin A-50 F (T5F) also described by Bruckner & Przybylski (1984) in *T. viride*.

Fragmentation of the m/z 1728.0 ion elucidated only a small part of γ -series, and in the b -series there is the presence of a sodium ion between Aib and alanine residues (Figure 5c). This sequence, when compared to the databases is similar to trichotoxin A-50 G (T5G), also identified by Bruckner & Przybylski (1984). Neither of the peptaibols found in this fraction (T5F and T5G) have been previously described as being produced by *T. asperellum* strains.

In fraction 7, two prominent ions were observed (Figure 6a), but only the m/z 1726.1 $[M + Na^+]$ profile was elucidated. On this fragmentation mass spectrum (Figure 6b), we can see part of both series ($-\gamma$ and $-b$). Again we see the presence of a sodium ion between Aib and proline residues, and in this mass spectrum it

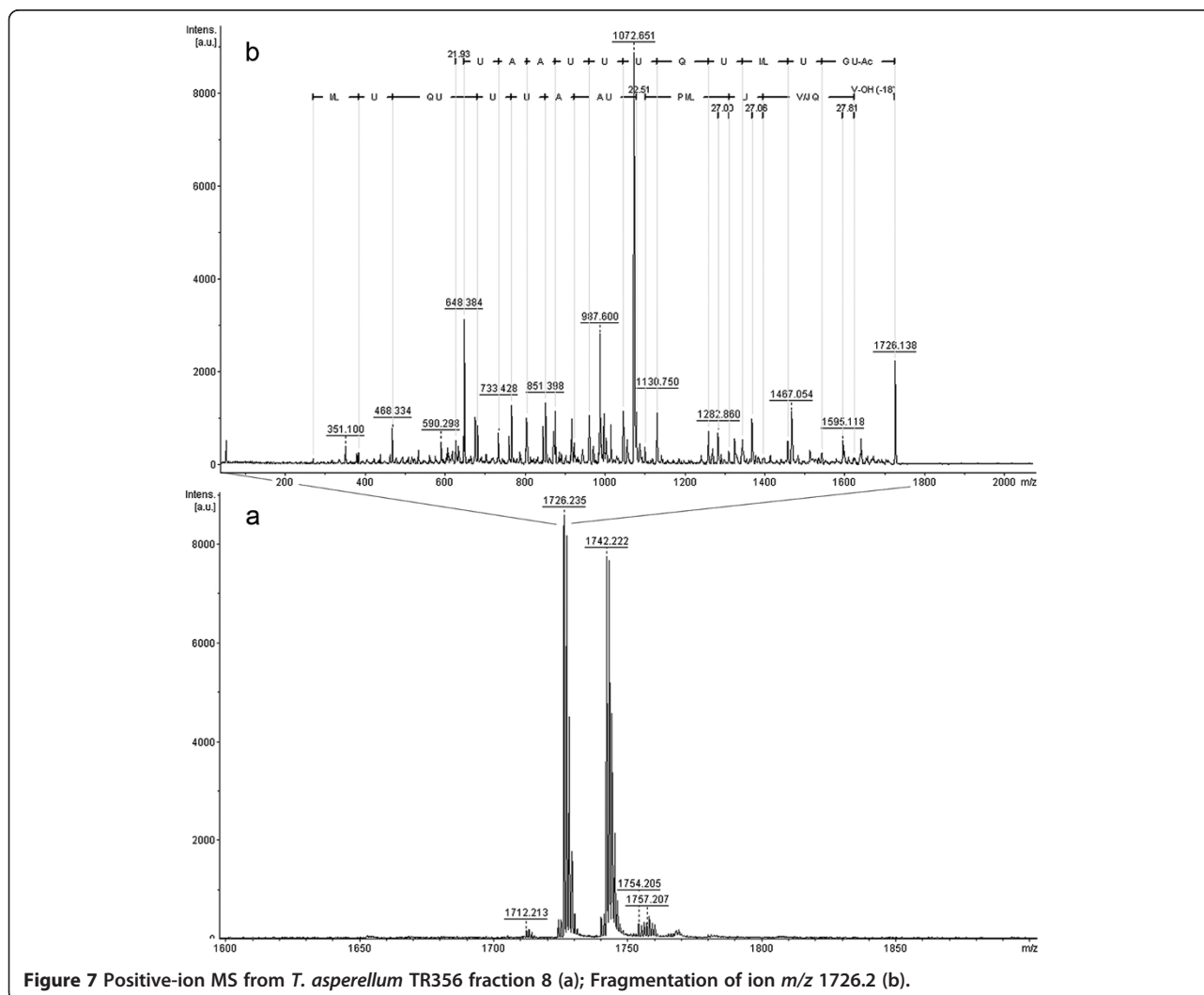
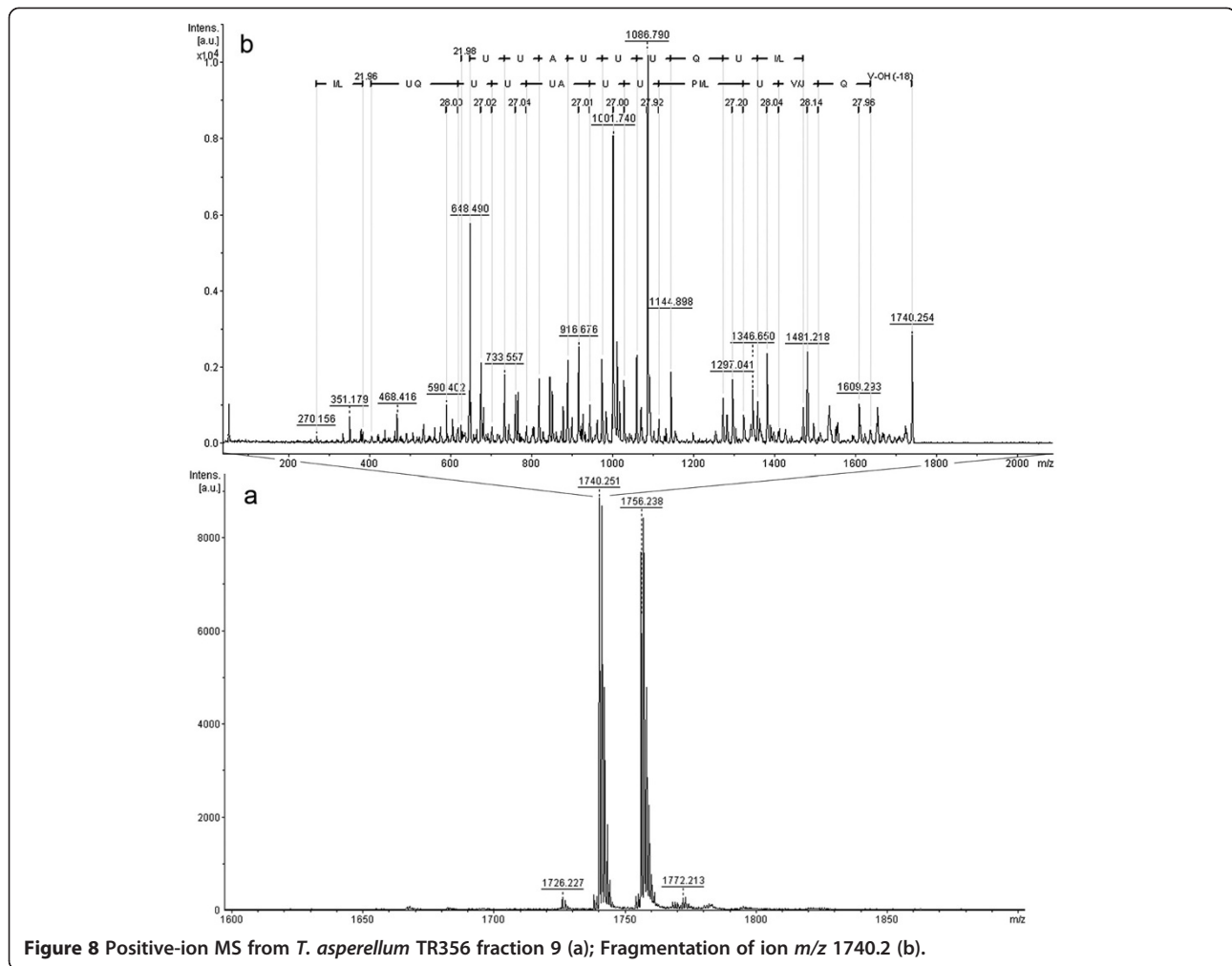


Figure 7 Positive-ion MS from *T. asperellum* TR356 fraction 8 (a); Fragmentation of ion m/z 1726.2 (b).



was possible to identify the amino acid residues present in the γ -series N-terminus, confirming what we find in literature. The analysis of fraction 8 (Figure 7a) was identical to fraction 7, and again only the ion of m/z 1726.2 [$M + Na^+$] was interpreted (Figure 7b). By comparing these sequences with the databases, in both fractions there are peptaibols similar to the trichotoxin A-50 G (T5G), also found in the previous fraction.

In the last fraction collected from the *T. asperellum* TR356 extract (Figure 8a), two ions were observed, and only the ion with m/z 1740.2 [$M + Na^+$] was interpreted (Figure 8b). In this MS we observed the presence of sodium ions between Aib and proline in the γ -series, and between leucine/isoleucine and Aib in the β -series. This sequence is similar to trichotoxin 1717A (Chutrakul et al. 2008), isolated from *T. asperellum* strains.

Table 1 Peptaibol sequences from *T. asperellum* TR356 strain extract; Mod.: N-terminal modification; Lxx: leucine or isoleucine; Vxx: valine or isovaline

Peptaibol	Mod.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Asperelin E	Ac	Aib	Aib	Val	Aib	Lxx	Aib	Aib	Ser	Aib	Prool								
Asperelin A	Ac	Aib	Aib	Val	Aib	Lxx	Aib	Aib	Ala	Aib	Prool								
Trichotoxin T5D2	Ac	Aib	Gly	Aib	Lxx	Aib	Gln	Aib	Aib	Ala	Ala	Ala	Aib	Pro	Lxx	Aib	Aib	Gln	Valol
Trichotoxin A-50 E (T5E)	Ac	Aib	Gly	Aib	Lxx	Aib	Gln	Aib	Aib	Ala	Ala	Ala	Aib	Pro	Lxx	Aib	Aib	Gln	Valol
Trichotoxin A-50 F (T5F)	Ac	Aib	Gly	Aib	Lxx	Aib	Gln	Aib	Aib	Ala	Ala	Ala	Aib	Pro	Lxx	Aib	Vxx	Gln	Valol
Trichotoxin A-50 G (T5G)	Ac	Aib	Gly	Aib	Lxx	Aib	Gln	Aib	Aib	Ala	Ala	Ala	Aib	Pro	Lxx	Aib	Vxx	Gln	Valol
Trichotoxin 1717A	Ac	Aib	Gly	Aib	Lxx	Aib	Gln	Aib	Aib	Ala	Aib	Aib	Pro	Lxx	Aib	Vxx	Gln	Valol	

Table 2 Further information regarding *T. asperellum* TR356 strain peptaibols; RT: chromatography retention time

Peptaibol	m/z	Fraction	RT (min)
Asperelin E	953,6 [M + H ⁺]	2	34,3
Asperelin A	937,6 [M + H ⁺]	3	36,6
Trichotoxin T5D2	1698,0 [M + Na ⁺]	4	38,4
Trichotoxin A-50 E (T5E)	1728,0 [M + K ⁺]	5	39,1
Trichotoxin A-50 F (T5F)	1712,0 [M + Na ⁺]	6	39,5
Trichotoxin A-50 G (T5G)	1726,1 [M + Na ⁺]	6 7 8	39,5 40,1 40,7
Trichotoxin 1717A	1740,2 [M + Na ⁺]	9	42

The trichotoxins are a group of approximately 27 peptaibols. They were initially identified by Brückner & Przybylski (1984) from isolates of *T. viride*, and subsequently further characterized by Jaworski & Brückner (1999). Later, new trichotoxins were identified from *T. harzianum* (Suwan et al., 2000) and *T. asperellum* strains (Chutrakul et al., 2008), who demonstrated for the first time their antimicrobial activity against *B. stearothersophilus*.

Tables 1 and 2 summarize the information obtained from each *T. asperellum* TR356 fraction analysis.

Conclusion

The genus *Trichoderma* is known to act as an ACB through several mechanisms including the production of peptaibols. Among various species, the *T. asperellum* TR356 strain was recently described as an efficient ACB against *S. sclerotiorum* in the field, but the production of secondary metabolites had not been previously reported. Using data obtained in this study, it was concluded that *T. asperellum* TR356 strain is able to produce different peptaibols, such as asperelins A and E, and trichotoxins T5D2, 1717A and A-50 E, F and G. Many of these peptaibols have already been described as being produced by *T. asperellum* strains and other *Trichoderma* species; however, this is the first report of these compounds being produced by a *T. asperellum* strain isolated in Brazil. It was not possible to measure the antimicrobial activity of these peptaibols here, since production levels were insufficient for such tests. Further analysis will be needed to elucidate the involvement of these compounds in the *Trichoderma* biological control process against plant pathogens, and to elucidate the three-dimensional structures of these molecules.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JB, MM and MR carried out the biochemistry studies, participated in the identification of the peptaibol and drafted the manuscript. LS participated in the design of the study and performed the statistical analysis. CU conceived

of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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