Acta Biologica Hungarica 67(4), pp. 431–441 (2016)
DOI: 10.1556/018.67.2016.4.9

PEPTAIBOL PROFILES OF IRANIAN *TRICHODERMA* ISOLATES

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(Received: July 02, 2016; accepted: July 28, 2016)

Five Iranian *Trichoderma* isolates from species *T. viride*, *T. viridescens, T. asperellum*, *T. longibrachiatum* and *T. citrinoviride* – selected from the Fungal Collection of the Bu Ali Sina University, Hamedan, Iran – were investigated for their peptaibol production. All examined isolates showed remarkable antibacterial activities during the screening of their extracts for peptaibol content with a *Micrococcus luteus* test culture. HPLC-ESI-IT MS was used for identification and elucidation of the amino acid sequences of peptaibols. The detected peptaibol compounds contain 20 or 18 amino acid residues and belong to the trichobrachin and trichotoxin groups of peptaibols, respectively. *T. longibrachiatum* and *T. citrinoviride* produced trichobrachins, while trichotoxins could be detected in *T. viride*, *T. viridescens* and *T. asperellum.* Out of 37 sequences detetermined, 26 proved to be new, yet undescribed compounds, while others were identified as previously reported trichotoxins (trichotoxin A-50s and T5D2) and trichobrachins (longibrachins AI, AII, AIII, BII and BIII). Compounds within the two groups of detected peptaibols differed from each other only by a single or just a few amino acid changes.

Keywords: Trichoderma – peptaibol – antimicrobial activity – high performance liquid chromatography – electrospray ionization mass spectrometry

INTRODUCTION

The genus *Trichoderma* was described in 1794 by Persoon [37]. It is a worldwide distributed soil saprophytic fungal genus, the representatives of which have been used as biological control agents against various fungal pathogens. The first report about the biocontrol potential of *Trichoderma* was published by Weindling [47]. Presently, about 60% of all registered biofungicides worldwide are *Trichoderma*-based products [44]. These fungi can adapt to different ecological environments [17] and are mycoparasites [21], antibiotic producers [38], plant growth promoters, metabolizers of xenobiotics [18], plant disease control agents and commercial biofungicides [11]. Some species of this genus are clinically important (e.g. *T. longibrachiatum* and *T. citrinoviride* [13]) or harmful in the mushroom industry (e.g. *T. aggressivum* [16]), while others are used for the bioremediation of organic and inorganic wastes includ-

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ing heavy metals [10, 11, 15]. *Trichoderma* species can confer biotic and abiotic stress tolerance [21], and control fungi belonging to taxonomically diverse groups as well as oomycetes [31]. They also restrict pathogenic bacterial growth on foliage [12] and parasitize nematodes [42].

Trichoderma species are rich sources of different secondary metabolites (SMs), some of which have antimicrobial activities [45]. They produce a wide range of SMs that can affect phytopahogens directly or change plant metabolism to induce resistance or promote plant growth [46]. The production of SMs correlates with the different stages of morphological development [6]. Pyrones, koninginins, viridins, nitrogen heterocyclic compounds, azaphilones, butenolides and hydroxy-lactones, diketopiperazines, isocyano metabolites and peptaibols are secondary metabolites known to be toxic to many fungi [46].

Peptaibols form a class of antibiotics that act as microbe-associated molecular patterns and elicitors, and also can trigger the plant defense responses against pathogens. These short linear, α-helical peptides of 5–20 residues can be characterized with α-aminoisobutyric acid-rich content, an acetylated N-terminus and a C-terminal amino alcohol (e.g. phenylalaninol, valinol, leucinol, isoleucinol or tryptophanol). They contain high amount of non-proteinogenic amino acids (e.g. α-aminoisobutyric acid and isovaline) [43]. According to the sequence alignment and special amino acid composition, peptaibols have been classified into 9 subfamilies (SF1 to SF9) [7]. Members of SF1, 4, 5 and 9 are produced by *Trichoderma* species [30].

Alamethicin is the firstly characterized peptaibol, isolated from the culture broth of *Trichoderma viride* [26, 33], however, the producer strain was later re-identified as *T. arundinaceum* [20]. This peptide was shown to react with the cell membrane in target cells [29]. U-22324F was the first name of this compound but later its name was changed to alamethicin [20].

Peptaibols are synthesized by multifunctional non-ribosomal peptide synthetases (NRPs), e.g. *tex1, tex2* and *tex3* [30, 50], which have modular structures where each single module is adding a single residue to the final peptide [22]. A minimum unit of a NRPS contains adenylation (A), thiolation (T) and condensation (C) domains [40].

Peptaibols have antifungal, antibacterial, antiviral and anticancer activities [9]. The biological activities of alamethicin on oysters (*Crassostrea gigas*), as well as of tetrodotoxin, saxitoxin and longibrachin on Diptera larvae are also known from the literature [36].

Online protein databases such as PDB (www.rcsb.org/pdb/home/home.do) and UniProt (www.uniprot.org) do not contain peptaibol sequences because of their special characteristics such as unusual amino acids, short lengths and absence of genetically coded sequences [32, 49]. In 1997 the first online peptaibol database containing 9 subfamilies of peptaibols was released [39]. Whitmore et al. [48] gathered more than 300 peptaibols in a freely accessible database at http://peptaibol.cryst.bbk.ac.uk/home.shtml containing sequence, structure, biological source, crystallography data, groupings of sequences, specified sequence motifs and some other information [48] and in 2004 updated it [49]. In 2013, Stoppacher et al. [39] constructed "The comprehensive peptaibiotics database" containing information of 1062 Aib-containing non-

ribosomal fungal peptides. This database was originally a downloadable software tool based on Microsoft (MS) Access, but it was later developed to an online resource available at peptaibiotics-database.boku.ac.at [49]. Till 2015, 235 new peptaibol sequences were added to this database [32].

In this study we present the sequences of 18- and 20-residue peptaibols detected in *Trichoderma* strains isolated from Iranian soil samples.

MATERIALS AND METHODS

Strains, culture conditions and extraction procedures

Isolates from the species *T. viride, T. viridescens, T. asperellum, T. longibrachiatum* and *T. citrinoviride* deriving from the Bu Ali Sina University Fungal Collection (BASUFC), Hamedan, Iran were examined during this study (Table 1). The strains were previously isolated from soil samples collected in different regions of Iran and after purification by the hyphal tip method they were identified by morphological characteristics and the sequence analysis of their internal transcribed spacer (ITS) region using the online software *TrichO*key 2.0 (www.isth.info). Fungal strains were cultured on malt extract agar (MEA: 5 g l^{-1} malt extract, 2.5 g l^{-1} yeast extract, 10 g l^{-1} glucose and 20 g l^{-1} agar) before extraction.

Preparation of crude extracts from the cultures was performed according to Marik et al. [23]. Screening of extracts for peptaibol content was carried out with *Micrococcus luteus* test culture SZMC 0264 as described by Marik et al. [24]. The bioactive crude extracts were fractionated by solid phase extraction on Silica gel 60 (0.015–0.040 mm) with methanol/chloroform mixtures as mobile phase. Conditioning was performed by washing the cartridge with 3 ml methanol and 3 ml chloroform. The 3 ml chloroform was added to the stored dry extract and passed with vacuum using a 12 port Visiprep SPE manifold (Supelco, USA) through a conditioned cartridge. Stepwise elution was performed with 3 ml portions of methanol/chloroform successively from 0% to 100% methanol in 11 steps. The effluents were collected into clean tubes and evaporated to dryness using nitrogen. Then 200 µl methanol was added to each tube and kept at –20 °C for further analyzes. The efficiency of the SPE procedure was tested with the bioassay screening procedure on *Micrococcus luteus* as described above [24].

Table 1
Trichoderma strains involved in the study

Species	BASUFC culture collection number	Geographic location	Source of isolation
T. viride	06886H	Iran, Hamedan	Soil
T. viridescens	06768A	Iran, Hamedan	Rhizosphere of potato
T. asperellum	06256F	Iran, Kordistan	Rhizosphere of potato
T. citrinoviride	06758A	Iran, Guilan	Soil
T. longibrachiatum	06983B	Iran, Markazi	Soil

BASUFC: Bu Ali Sinai University Fungal Collection, Hamedan, Iran.

High performance liquid chromatography – ion trap mass spectrometry

Reversed phase high performance liquid chromatography (HPLC) – electrospray ionization (ESI) – mass spectrometry (MS) was carried out on an Agilent 1100 modular HPLC system (Palo Alto, USA) coupled to Varian 500 ion trap (IT) MS (Agilent, USA) as described by Marik et al. [25]. The sequence of y ions originating from the split of the labile Aib-Pro bond in MS full scan mode were predicted by calculations based on the sequences with identical masses found in the Comprehensive Peptaibiotics Database and b-ion series sequence homologies [39].

The newly described peptaibols were designated with individual identifiers. Names of the compounds were generated from 3 characteristic parts of the molecules with a "Pept" prefix followed by the molecular mass (first number), the type of the C-terminal part from the Aib-Pro residues (second alphabetic character) and the elution order in our separation system (third number). For the sequences the three letter amino acid codes were used and supplemented with "ol" ending in the case of amino alcohol residues. The C-terminal parts (major y ion sequence) predicted in this study were the following: -Pro-Lxx-Aib-Aib-Gln-Vxxol (a), -Pro-Lxx-Aib-Vxx-Gln-Vxxol (b), -Pro-Vxx-Aib-Vxx-Gln-Gln-Pheol (c) and -Pro-Vxx-Aib-Vxx-Glu-Gln-Pheol (d). The nomenclature for fragment ions observed on the MS^1 spectra followed the terminology published by Biemann [1] as well as by Roepstorff and Fohlman [35].

RESULTS

Antibacterial activity of crude extracts and SPE fractions of Iranian Trichoderma *isolates*

All examined isolates showed remarkable antibacterial activities. The largest inhibition zone (22 mm in diameter) was around the crude extract of *T. asperellum*, while the least effective inhibition with an inhibition zone of 9 mm in diameter occurred around *T. citrinoviride*. Alamethicin equivalent concentrations [24] of the crude extracts were 541.64, 635.59, 635.59, 150.65 and 79.45 µg/ml for *T. viride, T. viridescens, T. asperellum, T. longibrachiatum* and *T. citrinoviride,* respectively. Bioassays were also performed with the SPE fractions, then the active fractions were pooled for each isolate and subjected to HPLC-IT-MS measurements.

Mass spectrometric analysis of peptaibols detected from Iranian Trichoderma *isolates*

Singly and double charged sodium adducts of the peptaibols were formed in the ESI source and could be easily identified on the MS spectra, which determined the molecular masses of the molecules (Fig. 1). The fragmentation of peptides already occurred

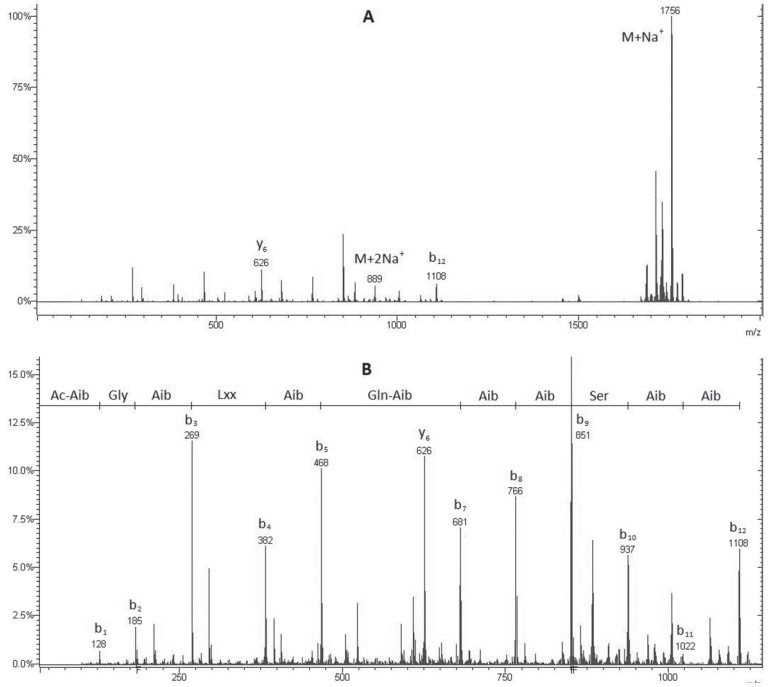

Fig. 1. Full scan mass spectrum of compound Pept-1733-b-2 investigated by HPLC-ESI-MS. A: Full range MS spectrum with marked characteristic ions, B: enlarged region of the b-series ions with the amino acid residues

between the labile Aib-Pro bonds forming the major y ions y_6 and y_7 for 18- and 20-residue peptaibols, respectively, as well as the series of b ions (Fig. 1A). The b series ions of the N-terminal peptide parts were clearly detected on the MS spectra (Fig. 1B). For both the 18- and 20-residue peptaibols, two types of y-ions containing 6 (*m/z* 612 and 626) and 7 (*m/z* 770 and 790) amino acids were predicted. The initial part of the sequences showed high similarities between the detected peptaibol molecules, which was followed by variable inner residues involving the Gln-Aib residue pairs at the 6-7 and 7-8 positions in the case of 18- and 20-residue peptaibols, respectively.

Full scan mass spectra of the crude extracts showed that *T. longibrachiatum* and *T. citrinoviride* produced 20-residue peptaibols, while 18-residue peptaibols could be detected in *T. viride*, *T. viridescens* and *T. asperellum* (Table 2). The results revealed the following numbers of compounds: *T. asperellum* 06256F: 26, *T. viridescens* 06768A: 26, *T. viride* 06886H: 27, *T. citrinoviride* 06758A: 10, and *T. longibrachiatum* 06983B: 10.

In the case of *T. viride*, *T. viridescens* and *T. asperellum*, 18-residue trichotoxins were detected, most of which were produced by all three isolates. However, compounds Pept-1705-a-3 and Pept-1733-b-1 were not detected from *T. viridescens* and

Table 2

Sequences of peptaibols detected in the examined *Trichoderma* strains

Name	Producing isolate	Mw	Amino acid sequence
Trichotoxin T5D2	06886H, 06768A, 06256F	1675	Ac Aib Gly Aib Lxx Aib Gln Aib Aib Ala Ala Aib Pro Lxx Aib Aib Gln Vxxol
Trichotoxin A-50 F (T5F)	06886H, 06768A, 06256F	1689	Ac Aib Gly Aib Lxx Aib Gln Aib Aib Ala Ala Aib Pro Lxx Aib Aib Gln Vxxol
Trichotoxin A-50 E (T5E)	06886H, 06768A, 06256F	1689	Ac Aib Gly Aib Lxx Aib Gln Aib Aib Aib Ala Ala Aib Pro Lxx Aib Iva Gln Vxxol
Pept-1689-a-1	06886H, 06768A, 06256F	1689	Ac Aib Gly Aib Lxx Aib Gln Aib Aib Ala Aib Ala Aib Pro Lxx Aib Aib Gln Vxxol
Pept-1689-a-2	06886H, 06768A, 06256F	1689	Ac Aib Gly Aib Lxx Aib Gln Aib Aib Ala Aib Ala Aib Pro Lxx Aib Aib Gln Vxxol
Pept-1691-a-1	06886H, 06768A, 06256F	1691	Ac Aib Gly Aib Lxx Aib Gln Aib Aib Ala Ser Ala Aib Pro Lxx Aib Aib Gln Vxxol
Pept-1703-a-1	06886H, 06768A, 06256F	1703	Ac Aib Gly Aib Lxx Aib Gln Aib Aib Aib Ala Aib Pro Lxx Aib Aib Gln Vxxol
Trichotoxin sequence 05	06886H, 06768A, 06256F	1703	Ac Aib Gly Aib Lxx Aib Gln Aib Aib Ala Aib Aib Pro Lxx Aib Aib Gln Vxxol
Pept-1703-b-1	06886H, 06768A, 06256F	1703	Ac Aib Gly Aib Lxx Aib Gln Aib Ala Aib Aib Pro Lxx Aib Vxx Gln Vxxol
Pept-1703-b-2	06886H, 06768A, 06256F	1703	Ac Aib Gly Aib Gln Aib Gln Aib Aib Gly Ala Aib Pro Lxx Aib Vxx Gln Vxxol
Pept-1703-b-3	06886H, 06768A, 06256F	1703	Ac Aib Gly Aib Lxx Aib Gln Aib Ala Aib Aib Pro Lxx Aib Vxx Gln Vxxol
Pept-1705-a-1	06886H, 06768A, 06256F	1705	Ac Aib Gly Aib Lxx Aib Gln Aib Aib Ala Ser Aib Aib Pro Lxx Aib Aib Gln Vxxol
Pept-1705-a-2	06886H, 06768A, 06256F	1705	Ac Aib Gly Aib Lxx Aib Gln Aib Aib Aib Ser Ala Aib Pro Lxx Aib Aib Gln Vxxol
Pept-1705-a-3	06886H, 06256F	1705	Ac Aib Gly Aib Lxx Aib Gln Aib Aib Aib Ser Ala Aib Pro Lxx Aib Aib Gln Vxxol
Trichotoxin A-50 I (T5I)	06886H, 06768A, 06256F	1717	Ac Aib Gly Aib Lxx Aib Gln Aib Aib Aib Ala Aib Aib Pro Lxx Aib Vxx Gln Vxxol
Pept-1719-a-1	06886H, 06768A, 06256F	1719	Ac Aib Gly Aib Lxx Aib Gln Aib Aib Aib Ser Aib Aib Pro Lxx Aib Aib Gln Vxxol
Pept-1719-b-1	06886H, 06768A, 06256F	1719	Ac Aib Gly Aib Lxx Aib Gln Aib Aib Aib Ser Ala Aib Pro Lxx Aib Vxx Gln Vxxol
Pept-1719-b-2	06886H, 06768A, 06256F	1719	Ac Aib Gly Aib Lxx Aib Gln Aib Aib Aib Ser Ala Aib Pro Lxx Aib Vxx Gln Vxxol
Trichotoxin A-50 J (T5J)	06886H, 06768A, 06256F	1731	Ac Aib Ala Aib Lxx Aib Gln Aib Aib Ala Aib Aib Pro Lxx Aib Vxx Gln Vxxol
Pept-1731-b-1	06886H, 06768A, 06256F	1731	Ac Aib Gly Aib Lxx Aib Gln Aib Aib Aib Aib Aib Pro Lxx Aib Vxx Gln Vxxol
Pept-1733-b-1	06886H, 06768A	1733	Ac Aib Gly Aib Lxx Aib Gln Aib Aib Aib Ser Aib Aib Pro Lxx Aib Vxx Gln Vxxol

Pept-1733-b-2	06886H, 06768A, 06256F	1733	Ac Aib Gly Aib Lxx Aib Gln Aib Aib Aib Ser Aib Aib Pro Lxx Aib Vxx Gln Vxxol
Pept-1733-b-3	06886H, 06768A, 06256F	1733	Ac Aib Gly Aib Lxx Aib Gln Aib Aib Aib Ser Aib Aib Pro Lxx Aib Vxx Gln Vxxol
Pept-1733-b-4	06886H, 06768A, 06256F	1733	Ac Aib Gly Aib Lxx Aib Gln Aib Aib Aib Ser Aib Aib Pro Lxx Aib Vxx Gln Vxxol
Pept-1733-b-5	06886H, 06768A, 06256F	1733	Ac Aib Gly Aib Lxx Aib Gln Aib Aib Aib Ser Aib Aib Pro Lxx Aib Vxx Gln Vxxol
Pept-1747-b-1	06886H, 06768A, 06256F	1747	Ac Aib Gly Aib Gln Aib Gln Aib Aib Aib Aib Pro Lxx Aib Vxx Gln Vxxol
Pept-1749-b-1	06886H, 06768A, 06256F	1749	Ac Aib Gly Aib Lxx Aib Gln Aib Aib Aib Ser Vxx Aib Pro Lxx Aib Vxx Gln Vxxol
Longibrachin A I Trilongin BI	06758A, 06983B	1937	Ac Aib Ala Aib Ala Aib Ala Gln Aib Vxx Aib Gly Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol
Longibrachin B II Trilongin CI	06758A, 06983B	1938	Ac Aib Ala Aib Ala Aib Ala Gln Aib Vxx Aib Gly Lxx Aib Pro Vxx Aib Aib Glu Gln Pheol
Longibrachin A III	06758A, 06983B	1951	Ac Aib Ala Aib Ala Aib Aib Gln Aib Vxx Aib Gly Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol
Pept-1951-c	06758A, 06983B	1951	Ac Aib Ala Aib Ala Aib Ala Gln Aib Vxx Aib Gly Lxx Aib Pro Vxx Aib Vxx Gln Gln Pheol
Pept-1952-d	06758A, 06983B	1952	Ac Aib Ala Aib Ala Aib Ala Gln Aib Vxx Aib Gly Lxx Aib Pro Vxx Aib Vxx Glu Gln Pheol
Longibrachin B III Trilongin CIII	06758A, 06983B	1952	Ac Aib Ala Aib Ala Aib Aib Gln Aib Vxx Aib Gly Lxx Aib Pro Vxx Aib Aib Glu Gln Pheol
Longibrachin A II Trilongin BII	06758A, 06983B	1952	Ac Aib Ala Aib Ala Aib Ala Gln Aib Vxx Aib Gly Lxx Aib Pro Vxx Aib Vxx Glu Gln Pheol
Pept-1965-c-1	06758A, 06983B	1965	Ac Aib Ala Aib Ala Aib Aib Gln Aib Lxx Ala Gly Lxx Aib Pro Vxx Aib Vxx Gln Gln Pheol
Pept-1965-c-2	06758A, 06983B	1965	Ac Aib Ala Aib Ala Aib Aib Gln Aib Lxx Ala Gly Lxx Aib Pro Vxx Aib Vxx Gln Gln Pheol
Pept-1966-d	06758A, 06983B	1966	Ac Aib Ala Aib Ala Aib Aib Gln Aib Lxx Ala Gly Lxx Aib Pro Vxx Aib Vxx Glu Gln Pheol

M: Molecular weight

T. asperellum, respectively. Twenty-residue, trichobrachin-like peptaibols were produced by the isolates belonging to the two clinically most relevant species of the genus, *T. longibraciatum* and *T. citrinoviride*, which could be characterized by completely identical peptaibol profiles.

DISCUSSION

The toxic effects of peptaibols are reliable properties during their detection. Results of the bioassay tests on *Bacillus subtilis, Escherichia coli, M. luteus* and *Serratia marcescens* showed that the largest inhibition zones of methanolic crude extracts deriving from *Trichoderma* species were detected in the case of *M. luteus* [24], therefore the optimized screening method based on this bacterium was applied during the present study. The bioassay revealed that all examined isolates of *T. viride, T. viridescens, T. asperellum, T. citrinoviride* and *T. longibrachiatum* have bioactive compounds in their crude extracts.

Peptaibols have the same chemical characteristics and molecular masses, therefore they cannot be completely separated by HPLC and are eluted together [28]. The complete separation of peptaibols is usually difficult but certain methods such as high energy collision, or collision-induced dissociation mass spectrometry were applicable to characterize peptaibols in crude extracts [34]. The ESI-IT-MS technology is a more simple method for the sequencing of peptaibols [28]. We used HPLC for separation and ESI-MS for sequencing and identification of peptaibol compounds. ESI-MS produced pseudomolecular ions such as $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, $[2M+H]^+$ and $[M+2Na]^+$ or $[M–H]^–$ in negative-mode. An advantage of this method is that only small samples are needed [9, 25].

Two groups of peptaibols were detected in the examined isolates: 18-residue peptaibols from *T. viride*, *T. viridescens* and *T. asperellum,* and 20-residue peptaibols from *T. citrinoviride* and *T. longibrachiatum*. The separated peptaibols are sequence analogues of 18-residue trichotoxins and 20-residue trichobrachins. Compounds within the two groups differed from each other only by single or few amino acid changes.

Trichotoxins were shown already in the early studies to induce voltage-dependent pores in bilayer lipid membranes [2]. Some of the compounds detected in this study were similar to peptaibol sequences available in the Comprehensive Peptaibiotics Database [39] as trichotoxin A-50 E (T5E), A-50 F (T5F), A-50 I (T5I) and A-50 J (T5J) [5] as well as trichotoxin T5D2 and trichotoxin sequence 05 [41]. We also found 21 yet undescribed trichotoxin sequences obtained as microheterogeneous mixtures produced by members of the *Viride* clade. Besides *T. viride* [4, 5, 14], the production of trichotoxins was also reported in the literature for *T. asperellum* (clade *Hamatum* [3, 8]) and *T. harzianum* (clade *Harzianum* [41]), suggesting that this group of peptaibols can be produced by various subtaxa within the genus *Trichoderma*. Our results are in agreement with these previous data by confirming the trichotoxin production ability of further *T. asperellum* and *T. viride* isolates, and in addition they

reveal the production of trichotoxins also in the case of *T. viridescens* from clade *Viride*.

Longibrachins [19] and trilongins [27] are belonging to the group of trichobrachins with high similarities to the *T. citrinoviride* and *T. longibrachiatum* sequences presented in Table 2. Some of the compounds detected in this study were similar to known sequences including Longibrachin AI (Trilongin BI), BII (Trilongin CI), AIII, BIII (Trilongin CIII) and AII (Trilongin BII) [39]. Five yet undescribed longibrachin-related sequences were also detected in both Iranian isolates. In a recent study, similar compounds were reported from forest-derived isolates of *T. aethiopicum*, *T. novae-zelandiae* and *T. pseudokoningii* from section *Longibrachiatum* of the genus [23].

The recent study is the first attempt to detect peptaibols from Iranian *Trichoderma* isolates. Members of *Trichoderma* section *Longibrachiatum* were producing entirely different peptaibols than members of sections *Viride* and *Hamatum*. Further studies are needed to clear the role of the individual compounds in the producer *Trichoderma* species.

ACKNOWLEDGEMENTS

This work was supported by grants NKFI K-105972 (National Research, Development and Innovation Office, Hungary) and GINOP-2.3.3-15-2016-00006 (Széchenyi 2020 Programme, Hungary).

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