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Rapid bioactivity-based pre-screening method for the detection of peptaibiotic-producing *Trichoderma* strains

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ABSTRACT Peptaibiotics are bioactive secondary metabolites belonging to a constantly growing family of fungal peptide antibiotics. They are linear, amphipathic oligopeptides consisting of 5-20 amino acids, usually containing nonproteinogenic amino acid residues as characteristic building blocks of the structure. The majority of the peptaibiotics described so far are produced by members of the filamentous fungal genus *Trichoderma*. Species of this genus are known as important sources of antibiotics and enzymes, and they are also known as promoters of plant growth, decomposers of xenobiotics and as commercial biofungicides. In this work, a rapid, bacterium-based screening method was developed and optimized for the detection of peptaibiotic production in liquid cultures of *Trichoderma* strains. The method was validated with the commercially available alamethicin as a peptaibiotic reference compound.

Acta Biol Szeged 57(1):1-7 (2013)**KEY WORDS**peptaibiotics
Trichoderma
bioactivity
pre-screening

Species of the imperfect filamentous fungal genus *Trichoderma* with teleomorphs belonging to the *Hypocreales* order of Ascomycota are of great economic importance as sources of antibiotics, enzymes, as plant growth promoters, decomposers of xenobiotics, and as commercial biofungicides (Chet and Inbar 1994; Harman et al. 2004). However, *Trichoderma* strains have also been reported in an increasing number of cases as etiologic agents in human infections (Kredics et al. 2011) and as causal agents of green mould epidemics in the commercial production of mushrooms (Hatvani et al. 2008; Kredics et al. 2010).

Peptaibiotics are secondary metabolites, which constitute a family of fungal peptide antibiotics growing constantly since alamethicin was isolated from cultures of strain NRRL 3199 of *Trichoderma arundinaceum* (initially identified as *T. viride*) (Reusser 1967). The structure, microheterogeneity and biological effects of this molecule were investigated over 45 years as reviewed in details by Leitgeb et al. (2007) and Kredics et al. (2013). The structure and properties of a considerable part of more than 1000 peptaibiotics are presented in the Comprehensive Peptaibiotics Database, which is freely available from the internet at <http://peptaibiotics-database.boku.ac.at> (Stoppacher et al. 2013). Most of the peptaibiotic producers are fungal species from genera belonging to the *Hypocreales* order of the Ascomycota division, with members of the genus *Trichoderma* being the most important, responsible for the production of about two third of these molecules (Szekeres et al. 2005).

Peptaibiotics are linear, amphipathic oligopeptides composed of 5-20 amino acids. Their sequences contain high amounts of non-proteinogenic amino acid residues like isovaline (Iva), and α -aminoisobutyric acid (Aib), which are α,α -dialkylated amino acids occurring in high proportions and representing characteristic building blocks of the structure. The N-terminal residues of these peptides are usually acetylated or rarely acylated, and an amino alcohol, mostly phenylalaninol or in certain cases valinol, leucinol, isoleucinol or tryptophanol is linked by a peptide bond at the C-terminal end (Szekeres et al. 2005).

Because of the growing number of peptaibiotics discovered, screening methods are required which provide sufficient and reliable diagnostic information on peptaibiotic production potential. Such assays may be based on analytical or biological methodologies. The characteristic amino acid residues and amino alcohols could be used to monitor the peptaibiotic production of biocontrol *Trichoderma* strains based on the gas chromatographic separation of their crude mycelial extracts after acidic hydrolysis (Solfrizzo et al. 1994). Krause et al. (2006) also presented a chromatographic screening method enabling the sensitive detection of fungal peptides with Aib content. Their method comprises solid-phase extraction and preconcentration procedure followed by on-line reversed-phase high performance liquid chromatography (HPLC) coupled to electrospray ionization tandem mass spectrometry, where the presence of the marker amino acid Aib was detected by characteristic mass differences of fragment ions of 85.1 Da (Aib-H₂O) on the mass spectra (Krause et al. 2006).

Peptaibiotics exert a broad range of biological activities depending on their chain length and particular structural

Accepted October 21, 2013

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Table 1. *Trichoderma* strains involved in the experiments.

Isolate code	Location of isolation	Species	Pre-screening test result	Inhibition zone in mm (alamethicin equivalent in µg/ml)
SZMC 1012	wheat rhizosphere, Deszk	<i>T. longibrachiatum</i>	-	-
SZMC 1628	wheat rhizosphere, Rúzsza	<i>T. brevicompactum</i>	-	-
SZMC 1631	wheat rhizosphere, Rúzsza	<i>T. rossicum</i>	-	-
SZMC 1649	wheat rhizosphere, Rúzsza	<i>T. spirale</i>	-	-
SZMC 1656	wheat rhizosphere, Rúzsza	<i>T. gamsii</i>	-	-
SZMC 1762	<i>Agaricus</i> compost, Hungary	<i>T. ghanense</i>	-	-
SZMC 1813 (CBS 100527)	<i>Agaricus</i> compost, North-America	<i>T. aggressivum</i> f. <i>ag-gressivum</i>	-	-
SZMC 1811 (CBS 433.95)	<i>Agaricus</i> compost, Great-Britain	<i>T. aggressivum</i> f. <i>euro-paeum</i>	-	-
SZMC 1746	<i>Agaricus</i> compost, Hungary	<i>T. aggressivum</i> f. <i>euro-paeum</i>	-	-
SZMC 12498	wild growing <i>Agaricus</i> sp., fruiting body	<i>T. atroviride</i>	+	8 (67.71)
SZMC 1886	wild growing <i>Agaricus</i> sp., fruiting body	<i>T. tomentosum</i>	+	12.5 (139.07)
SZMC 12617	wild growing <i>Agaricus</i> sp., fruiting body	<i>T. citrinoviride</i>	+	8 (67.71)
SZMC 1873	wild growing <i>Agaricus</i> sp., fruiting body	<i>T. hamatum</i>	-	-
SZMC 1881			-	-
SZMC 1885			-	-
SZMC 12492	wild growing <i>Agaricus</i> sp., fruiting body	<i>T. harzianum</i>	+	12.5 (139.07)
SZMC 1880			-	-
SZMC 12487			-	-
SZMC 1874)			+	15 (207.45)
SZMC 1884	wild growing <i>Agaricus</i> sp., fruiting body	<i>T. koningii</i>	-	-
SZMC 12500	wild growing <i>Agaricus</i> sp., fruiting body	<i>T. koningiopsis</i>	-	-
SZMC 12727	wild growing <i>Agaricus</i> sp., fruiting body	<i>T. pleurotica</i>	+	11.5 (118.51)
SZMC 12454	<i>Pleurotus</i> compost	<i>T. pleurotum</i>	+	50 (OCR)
SZMC 1718	-	<i>T. velutinum</i>	-	-
SZMC 12515	wild growing <i>Agaricus</i> sp., fruiting body	<i>T. virens</i>	+	38 (OCR)
SZMC 12553 (CNM-CM-2171)	clinical	<i>T. longibrachiatum</i>	-	-
SZMC 12554 (CNM-CM-2277)	clinical	<i>T. longibrachiatum</i>	-	-
SZMC 1775 (IMI 291914)	Antarctica	<i>T. longibrachiatum</i>	-	-
SZMC 1776 (CECT 20105)	Biocontrol strain, Spain	<i>T. longibrachiatum</i>	-	-
SZMC 1773 (CECT 2412)	<i>Agaricus</i> compost, Wales	<i>T. longibrachiatum</i>	+	14 (176.78)

OCR: out of calibration range.

features (Szekeres et al. 2005), which could also be utilized for their detection. They have been described to exhibit antibacterial activity against Gram-positive bacteria and wall-less bacteria (Dornberger et al. 1995; Leclerc et al. 2001), and display antifungal (Dornberger et al. 1995; Berg et al. 1996) as well as antiviral activities (Kim et al. 2000; Yun et al. 2000). The bioactivity of peptaibiotics also includes toxicity towards mammalian cells (Peltola et al. 2004) and insecticidal action on larvae (Matha et al. 1992; Bandani et al. 2001; Landreau et al. 2002).

Based on the literature, the best choice for the detection of fungal peptaibiotic production is the sequential combination of a sensitive and low-cost biological pre-screening assay followed by a sophisticated analytical technique. In accordance with this strategy, the present work describes the development and optimization of a rapid, bacterium-based method for the pre-screening of fungal ferment broth extracts for peptaibiotic production.

Materials and Methods

Strains and culture conditions

The examined 30 *Trichoderma* strains were derived from the the Microbiological Collection of the University of Szeged (SZMC; Table 1) and were maintained on yeast extract medium (YE: 2 g l⁻¹ yeast extract, 5 g l⁻¹ KH₂PO₄, 20 g l⁻¹ agar in tap water) at 25°C. The isolates were identified previously based on their internal transcribed spacer (ITS) sequences using the *TrichOkey* 2.0 online barcoding program (www.isth.info, Druzhinina et al. 2005).

The *Bacillus subtilis* (SZMC 0209), *Escherichia coli* (SZMC 0582), *Micrococcus luteus* (SZMC 0264) and *Serratia marcescens* (SZMC 0567) strains also derived from the SZMC culture collection and were maintained on LB (Luria-Bertani) agar medium (10 g l⁻¹ tryptone, 5 g l⁻¹ yeast extract, 10 g l⁻¹ NaCl and 20 g l⁻¹ agar-agar) at 37 C° with weekly subculturing.

Testing of inhibitory effects of crude extracts on bacteria

Four different species of bacteria (*B. subtilis*, *E. coli*, *M. luteus* and *S. marcescens*) were involved in the initial test of antibacterial effects in the case of the crude ferment broths and mycelial extracts of 15 tested *Trichoderma* isolates (*T. aggressivum* f. *aggressivum* SZMC 1813, *T. atroviride* SZMC 12498, *T. brevicompactum* SZMC 1628, *T. citrinoviride* SZMC 12617, *T. cerinum* SZMC 1886, *T. gamsii* SZMC 1656, *T. ghanense* SZMC 1762, *T. hamatum* SZMC 1873, *T. harzianum* SZMC 1874, SZMC 12487 and SZMC 12492, *T. koningiopsis* SZMC 12500, *T. rossicum* SZMC 1631, *T. spirale* SZMC 1649 and *T. virens* SZMC 12515). *Trichoderma* strains were grown in shaken (rotary shaker, 200 rpm) or standing cultures in liquid yeast extract-glucose medium (YEG: YE + 10 g l⁻¹ glucose) for 5 days. Mycelia and the lyophilized ferment broths were separately dissolved in 5 and 2 ml methanol, respectively.

Agar plates were prepared containing 20 ml media corresponding to the test organisms: beef extract agar (BEA) medium (4 g l⁻¹ beef extract, 4 g l⁻¹ peptone, 10 g l⁻¹ glucose, 1 g l⁻¹ yeast extract and 20 g l⁻¹ agar) for *B. subtilis* and *S. marcescens*, and LB agar medium for *E. coli* and *M. luteus*. Bacteria were mixed into 5 ml covering agar (BEA or LB with 10 g l⁻¹ agar) at 40°C and poured onto the surface of the base medium.

Fifty µl amounts of the methanolic extracts of the *Trichoderma* ferment broths were filled into holes (5 mm in diameter) bored into the surface of the agar plates. The diameter of the inhibition zones were observed and recorded after 2 days of incubation at 37°C. After the selection of the test bacterium and the optimization of bacterial pre-cultivation time and inoculated cell density (see below), the above procedure was applied under optimal conditions for the entire set of *Trichoderma* isolates.

Optimization of bacterial pre-cultivation time and inoculated cell density in the pre-screening system

The ferment broths deriving from shaken cultures of two *Trichoderma* strains showing antibacterial effects in the initial test, *T. citrinoviride* SZMC 12617 and *T. virens* SZMC 12515 were tested. The *Trichoderma* strains were grown in shaken cultures in liquid YEG medium for 5 days. After culturing, a 2 ml chloroform extract was made from 2 ml of ferment broth, evaporated and finally dissolved in 100 µl methanol, and 50 µl of that extract was used in the optimization of the assay with *M. luteus*.

The examined *M. luteus* strain was precultured on LB agar medium at 37°C for different periods of time (1, 2, 3, 4, 5 and 6 days). For the purposes of pour plating, a suspension was prepared from *M. luteus* in physiological salt solution. The

cell concentration of the suspension was determined with the aid of a calibration curve after the measurement of the optical density (OD) of the suspensions at 620 nm. The calculated amounts (10⁵, 2×10⁵, 3×10⁵, 4×10⁵, 5×10⁵, 10⁶, 1.5×10⁶, 2×10⁶ and 3×10⁶ cells) of the *M. luteus* suspension were mixed at 40°C into 5 ml molten LB agar medium containing 10 g l⁻¹ agar, which was subsequently poured onto the surface of the base LB agar medium. Finally, holes of 5 mm in diameter were bored into the surface of the LB agar plates and 50 µl amounts of the extracts were filled into them. The diameters of the inhibition zones were observed and recorded in mm along two perpendicular axes after 1 and 2 days of incubation at 37°C.

Calibration of inhibitory effects

An accurately weighed amount (5 mg) of alamethicin (Sigma, Hungary) was placed separately in a 5 ml volumetric flask and dissolved in methanol (VWR, Hungary) to produce a 1000 µg ml⁻¹ standard stock solution. This stock solution was diluted serially with methanol, revealing a solution series with the concentrations of 500.00, 250.00, 125.00, 62.50, 31.25, 15.63, 7.81 and 3.91 µg ml⁻¹. Fifty µl amounts of the solutions were filled into holes (5 mm in diameter) bored into the surface of LB agar plates inoculated with 10⁵ *M. luteus* cells. The diameters of the inhibition zones were observed and recorded as described above. Three replicates were performed and the mean of the recorded values was used for the calibration. The resulting calibration curve was used to express the inhibitory effect of the examined *Trichoderma* ferment broths as 'alamethicin equivalent'.

Results

Selection of the bacterial strain

Initially, four different bacterial strains (*B. subtilis* SZMC 0209, *E. coli* SZMC 0582, *M. luteus* SZMC 0264 and *S. marcescens* SZMC 0567) were tested with extracts of ferment broths and mycelia of standing and shaken cultures of 15 *Trichoderma* strain. Inhibition zones were detected in the case of *B. subtilis* for 15 samples (standing culture, mycelium: SZMC 1649, 1656, 1631, standing culture, ferment broth: SZMC 12500, shaken culture, mycelium: SZMC 1649, 1873, 12492, 1631, 1874, shaken culture, ferment broth: SZMC 1631, 12500, 1813, 12492, 1873, 1874), in the case of *E. coli* for 6 samples (standing culture, ferment broth: SZMC 1873, 12515, 1656, 1886, 12498, 12500), in the case of *M. luteus* for 12 samples (standing culture, mycelium: SZMC 12617, standing culture, ferment broth: SZMC 12515, 1762, 1649, shaken culture, mycelium: SZMC 1873, 12492, 1631, 1874, shaken culture, ferment broth: SZMC 12515, 12617, 1873, 12498), and in the case of *S. marcescens* for 9 samples (standing culture, mycelium: SZMC 1762, 1649, standing culture, ferment broth: SZMC 12500, 1886, 12515, 12617,

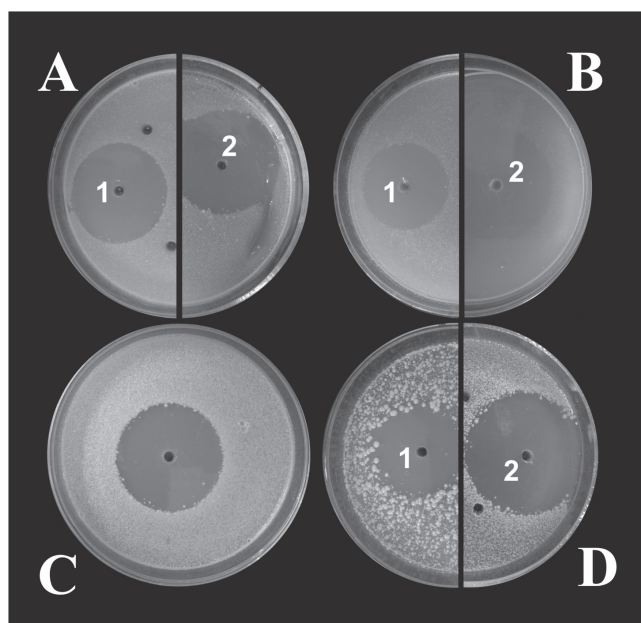


Figure 1. Inhibition zones of extracts of *Trichoderma virens* strain SZMC 12515 on solid medium inoculated with 2×10^5 cells of *Micrococcus luteus*. The bacteria were pre-cultured for 1 (A1), 2 (A2), 3 (B1), 4 (B2, C), 5 (D1) and 6 (D2) days and the results were recorded after one (A1, A2, B1, B2) and two (C, D1, D2) days of incubation at 37°C.

1813, 1631, 1656). Altogether, the largest inhibition zones were detected in the case of ferment broth extracts of shaken cultures on *M. luteus* therefore these conditions were selected for the optimization of the biological pre-screening test.

Effect of the pre-cultivation period and bacterial cell density on the inhibitory effects

Based on the initial test, two antibacterial strains of *Trichoderma* species, *T. citrinoviride* SZMC 12617 and *T. virens* SZMC 12515 were selected for the optimization of the culture age of the test bacteria and the applied concentration of the cells. One, 2, 3, 4, 5 or 6 days old *M. luteus* cultures were applied in different amounts (10^5 , 2×10^5 , 3×10^5 , 4×10^5 , 5×10^5 , 10^6 , 1.5×10^6 , 2×10^6 and 3×10^6 cells, Table 2, Figure 1). Bacteria pre-cultured for 1, 2 and 3 days formed a merged bacterial lawn in contrast to the bacteria pre-cultured for 4, 5 and 6 days, where it was not possible to unambiguously determine the size of the inhibition zone after 1 or 2 days of incubation (Figure 1). Consequently, the pre-culturing time should be less than 3 days. However, there are significant differences between the size of the inhibition zones after different pre-culturing times. In the case of *T. citrinoviride* SZMC 12617, the largest zones could be detected for most of the bacterial concentrations, when the bacteria were pre-cultured for 2 days (Table 2). Altogether, the increasing concentration of test bacteria caused decreasing diameters of the inhibition zone. The test was not working under the cell concentration

Table 2. Effect of pre-cultivation time and applied cell amount of *Micrococcus luteus* on the diameter of the inhibition zones (mm) caused by the ferment broth extracts of strains *Trichoderma virens* SZMC 12515 and *Trichoderma citrinoviride* SZMC 12617.

<i>T. virens</i> SZMC 12515		Age of <i>Micrococcus luteus</i> (days)			
Cell number ($\times 10^6$)	1	2	3	4	
0.1	33	38	36	42	
0.2	34	37	38	40	
0.3	33	35	35	45	
0.4	32	33	35	45	
0.5	31	33	35	44	
1	27	33	35	36	
1.5	31	32	30	36	
2	27	28	29	37	
3	29	32	23	35	
<i>T. citrinoviride</i> SZMC 12617		Age of <i>Micrococcus luteus</i> (days)			
Cell number ($\times 10^6$)	1	2	3	5	6
0.1	38	50	36	33	52
0.2	37	43	32	34	47
0.3	36	38	32	31	43
0.4	38	33	33	31	43
0.5	33	35	32	31	43
1	28	35	35	27	40
1.5	31	34	32	28	37
2	28	34	27	27	32
3	27	31	28	30	32

ND: not determined

of 10^5 *M. luteus* cells ml^{-1} as the inhibition zones were not unambiguous due to the low cell number. In conclusion, pre-culturing of *M. luteus* for 2 days and applying 10^5 cells per plate proved to be the optimal conditions.

Inhibitory properties of alamethicin in the pre-screening system

The effect of alamethicin as a peptaibiotic reference compound was tested on *M. luteus* at different concentrations (Fig. 2). The 500.0, 250.0, 125.0 and 62.5 $\mu\text{g ml}^{-1}$ levels of the alamethicin dilution resulted in mean inhibition zones of 19.8, 16.2, 10.6 and 7.3 mm (limit of quantitation, LOQ), respectively, while the further dilution steps with lower concentrations showed no measurable inhibitory effect on *M. luteus* (Figure 3). The relative standard deviations (RSD) of the repeated tests ($n=3$) were below 10%, and the accuracy of the regression line was below $\pm 5\%$, while the limit of detection was at the level of 31.25 $\mu\text{g ml}^{-1}$ with low visibility.

Screening the ferment broths of the *Trichoderma* isolates under the optimized conditions

The optimized test was applied to test 30 strains of *Tricho-*

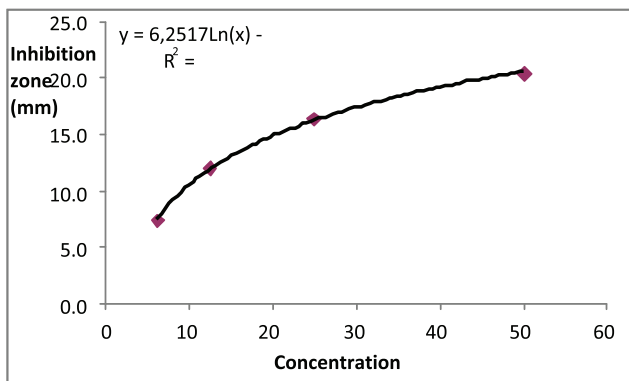


Figure 2. Calibration curve prepared with alamethicin as peptaibiotic reference compound.

derma listed in Table 1. The test proved to be positive for 9 strains: *T. atroviride* SZMC 12498, *T. tomentosum* SZMC 1886, *T. citrinoviride* SZMC 12617, *T. harzianum* SZMC 12492, SZMC 1874, *T. pleuroticola* SZMC 12727, *T. pleurotum* SZMC 12454, *T. virens* SZMC 12515 and *T. longibrachiatum* SZMC 1773 (CECT 2412) with inhibition zones of 10, 19, 8, 12, 15, 14, 8, 38 and 16 mm in diameter, respectively (Table 1). No antibacterial effects could be detected for the other 21 strains under the applied test conditions.

Discussion

In this study we report the development and optimization of a rapid bioactivity-based pre-screening method for the detection of peptaibiotic production in *Trichoderma* strains. Bioactivity-based assays for the study of peptaibiotics have been applied in previous studies. The toxic effects of peptaibiotics were already applied in their detection and correlation was found between the peptaibiotic production and the biological activity in the case of brine shrimp (*Artemia salina*) (Solfrizzo et al. 1994) and blowfly larvae (*Phormia terrae novae*) (Landreau et al. 2002). Poirier et al. (2007) performed embryotoxicity bioassays with oysters (*Crassostrea gigas*) in order to assess the acute toxicity of alamethicin and different groups of peptaibiotics produced by a *T. longibrachiatum* strain isolated from marine environment. Alamethicin and a mixture of long and short-sequence peptaibiotics were shown to disturb the embryogenesis of *C. gigas* in the nanomolar concentration range. Although statistically significant differences were not observed among the applied three groups of peptaibiotics because of the remarkable variations between replicates, toxic effects based on the estimation of EC_{50} appeared appreciably different. However, compared to the short-sequence peptaibiotic mixture, long-sequence peptaibiotics were associated with a higher toxicity and the abnormality effects of alamethicin were between them. The initial peptaibiotic concentrations in all of the three groups of the tested

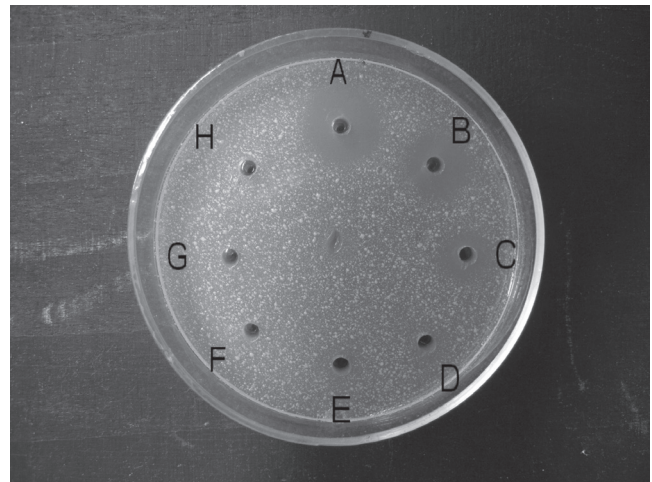


Figure 3. Effect of serial dilution steps of alamethicin on *Micrococcus luteus*: (A) 500.0 $\mu\text{g ml}^{-1}$, (B) 250.0 $\mu\text{g ml}^{-1}$, (C) 125.0 $\mu\text{g ml}^{-1}$, (D) 62.5 $\mu\text{g ml}^{-1}$, (E) 31.25 $\mu\text{g ml}^{-1}$, (F) 15.63 $\mu\text{g ml}^{-1}$, (G) 7.81 $\mu\text{g ml}^{-1}$ and (H) 3.91 $\mu\text{g ml}^{-1}$.

peptaibiotics were significantly correlating with the abnormality of embryogenesis by a non-linear regression (Poirier et al. 2007). Boar spermatozoa have also been successfully used as tools in the search for peptaibiotics from *T. harzianum* (Peltola et al. 2004), *T. longibrachiatum* (Mikkola et al. 2012) and *Acremonium exuviarum* (Andersson et al. 2008).

Trichoderma species, the tested isolates of which proved to be positive in the bacterium-based test optimized during this study were *T. atroviride*, *T. citrinoviride*, *T. harzianum* (2 strains), *T. longibrachiatum*, *T. pleuroticola*, *T. pleurotum*, *T. tomentosum* and *T. virens*. Among them, *T. atroviride* is known from the literature to produce atroviridins (Oh et al. 2000), neoatroviridins (Oh et al. 2005) and trichoatrokointins (Stoppacher et al. 2008), *T. citrinoviride* to produce paracelsins (Pócsfalvi et al. 1997) and other 20-residue peptides from the paracelsin family (Maddau et al. 2009), *T. longibrachiatum* to produce trichobrachsins (Mohamed-Benkada et al. 2006; Ruiz et al. 2007), trichorovin (Mohamed-Benkada et al. 2006) and trilongins (Mikkola et al. 2012), while *T. virens* to produce trichorzins (Wiest et al. 2002) as well as 11- and 14-residue peptaibiotics (Mukherjee et al. 2011). A series of peptaibiotic compounds could be isolated previously also from *T. harzianum* strains, including harzianins (Rebuffat et al. 1995), trichokindins (Iida et al. 1994), trichorozins (Iida et al. 1995), trichorzianins (El Hajji et al. 1987; Rebuffat et al. 1989; Pócsfalvi et al. 1998), trichorzins (Goulard et al. 1995; Duval et al. 1997) and trichotoxins (Suwan et al. 2000).

The isolates of the other tested species, *T. aggressivum* f. *aggressivum*, *T. aggressivum* f. *europaeum*, *T. brevicompactum*, *T. gamsii*, *T. ghanense*, *T. rossicum* and *T. spirale* were negative under the test conditions. Among these species, *T. brevicompactum* is well-known for its ability to produce

a series of peptaibiotics (trichobrevins, trichocompactins, trichocryptins, trichoferins) (Degenkolb et al. 2006), and *T. aggressivum* f. *europaeum* was also shown to produce short peptaibiotics (Krause et al. 2006). However, it can be supposed that not all isolates of a particular species are capable of peptaibiotic production, furthermore, the production may also depend from the culture conditions.

To confirm our results on the suspected presence or absence of peptaibiotic production, detailed analytical examinations will be carried out based on characteristic amino acid residues of known peptaibiotics such as α -aminoisobutyric acid and isovaline. Although it is possible that the pre-screening method described here detects not just peptaibiotics but also other antibacterial compounds of non-peptaibiotic nature, the known effects of peptaibiotics on Gram-positive bacteria (Dornberger et al. 1995; Leclerc et al. 2001; Szekeres et al. 2005) suggest that this method may prove to be a powerful as well as time- and cost-consuming tool for the pre-screening of large numbers of fungal isolates for peptaibiotic production.

Acknowledgments

The research of András Szekeres was supported by the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP 4.2.4.A/2-11-1-2012-0001 'National Excellence Program'. For Cs. V.: this research was realized in the frames of TÁMOP 4.2.4. A/2-11-1-2012-0001 „National Excellence Program – Elaborating and operating an inland student and researcher personal support system”. The project was subsidized by the European Union and co-financed by the European Social Fund. The relating research group was also supported by the grant OTKA K-105972 from the Hungarian Scientific Research Fund providing infrastructure and research equipment.

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