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Trichothecene Production by *Trichoderma brevicompactum*

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Trichoderma brevicompactum, *T. viride*, *T. harzianum*, *T. atroviride*, *T. longibrachiatum*, *T. erinaceum*, *T. citrinoviride*, and *Hypocrea lutea* were screened for production of trichothecenes after growth on one or several solid and liquid media. Trichothecenes were detected by liquid chromatography combined with online UV/vis spectroscopy and electrospray high-resolution mass spectrometry. *T. brevicompactum* produced trichodermin and/or harzianum A on all media investigated, with liquid media yielding the largest amounts. Detection of octa-2*Z*,4*E*,6*E*-trienedioic acid in the harzianum-A-producing strains indicated that harzianum A was synthesized directly by esterification of trichodermol with octa-2*Z*,4*E*,6*E*-trienedioic acid. Both the *T. viride* strain from which trichodermin was originally isolated and the *T. harzianum* strain from which harzianum A was originally isolated were shown to belong to *T. brevicompactum* based on four independent criteria: metabolite profiles, micromorphology, macromorphology on yeast extract sucrose agar and potato dextrose agar, and DNA sequences of the ITS1/ITS2 regions of the nuclear ribosomal DNA.

KEYWORDS: *Fusarium*; *Stachybotrys*; *Myrothecium*; *Hypocrea*; alamethicin; LC−MS; dereplication; peptaibol; mycotoxin; biosynthesis; biocontrol

INTRODUCTION

The genus *Trichoderma* is widely used as biocontrol agent for fungal phytopathogens and for production of enzymes, and thus, the potential mycotoxin production of this genus is an important safety issue for the industry (*1*, *2*). Mycotoxins reported from *Trichoderma* include the cytotoxic and immunosuppressive trichothecenes (*3*−*5*), gliotoxin (*6*, *7*), and ribosome-inactivating proteins (*8*). Other bioactive metabolite groups are peptides and peptaibols, volatile pyrones and lactones, isonitriles, and various low-molecular weight compounds (*9*, *10*).

Recent taxonomic re-evaluations of the genus *Trichoderma* and its teleomorphic state *Hypocrea* published, e.g., by Chaverri and Samuels (*11*) and Druzhinina and Kubicek (*12*), are underpinned by the current knowledge of molecular phylogeny. As for many other fungal genera, in the past decade, the use of DNA sequencing has exposed new insights to the systematics of *Trichoderma*. This has revealed the morphological species concepts of Bisby (*13*), Rifai (*14*), and Bissett (*15*) to accommodate several paraphyletic taxa. *Trichoderma brevicompactum*, a new anamorphic species closely related to *Hypocrea lutea*, recently has been described from soil and tree bark in North, Central, and South America and southern Asia (*16*). This species is characterized by a *Pachybasium*-type morphology, resembling other small-spored species referable to *Trichoderma* section *Pachybasium* but with essentially subglobose conidia.

In the early 1960s, trichodermin and trichodermol (**Figure 1**) were isolated from a proposed *T. viride* and were the first trichothecenes fully elucidated by NMR and X-ray crystallography (*3*, *4*). This strain labeled ND8 was deposited as American Type Culture Collection (ATCC) 14910 (*17*) but is no longer available from ATCC. Since then, *Trichoderma* strains have been considered trichothecene producers, although very few reports actually have been published. The only recent strain was reported by Corley et al. (*5*), who isolated and structure-elucidated harzianum A (**Figure 1**) from a culture of *T. harzianum* ATCC 90237 and very recently from a *Hypocrea* sp. Centraalbureau voor Schimmelcultures (CBS) 113214 by Lee et al. (*18*). A few other studies have claimed production of trichothecenes (*19*−*22*), but in most of the studies, nonspecific analytical methods such as TLC and HPLC were used. In addition, the strains have not been deposited in a culture collection or are no longer available. To add even more confusion, a crude bioactive liquid fraction from a *Trichoderma* culture has been named trichodermin (*23*).

Trichodermin, trichodermol, or harzianum A were not detected in any of 44 *Trichoderma* strains (*T. atroviride*, *T.*

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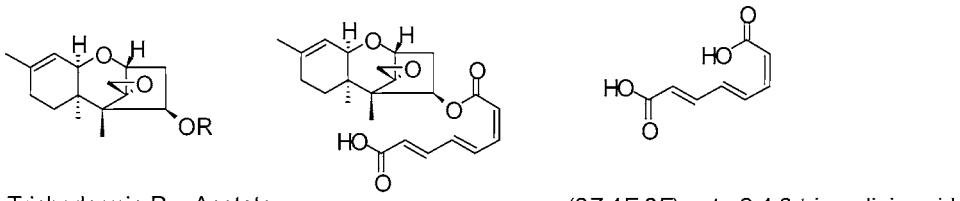

Figure 1. Structures of the trichothecenes and octa-2*Z*,4*E*,6*E*-trienedioic acid detected.

citrinoviride, *T. hamatum*, *T. harzianum*, *T. longibrachiatum*, and *T. viride*), which were analyzed by HPLC−DAD for species identification by direct image analysis of chromatograms (*24*). The ND8 strain, ATCC 90237, or strains of the newly described *T. brevicompactum* were not included in this study. In a previous screening study (*25*) on 150 *Trichoderma* strains, trichothecenes were detected only in the strain of Corley et al. (*5*). Because this study was performed by GC−MS/MS after hydrolysis, trichodermin, trichodermol, and harzianum A were all detected as trichodermol.

Godtfredsen and Vangedal (*3, 4*) reported their *Trichoderma* strain to be a *T. viride*, and Corley et al. (*5*) reported their strain to be a *T. harzianum.* Except for the *Hypocrea* sp. of Lee et al. (*18*), these two strains are the only sufficiently documented to produce trichothecenes. It is unclear if the lack of more trichothecene-producing strains has been due to improper culture conditions used so far or that the capability to produce trichothecenes is rare in *Trichoderma*. In the present study, we show that common culture conditions are sufficient and that both strains belong to the same species *T. brevicompactum*.

MATERIALS AND METHODS

Solvents were HPLC-grade, and all other chemicals were analytical-grade unless otherwise stated. Yeast extract sucrose agar (YES), oat meal agar (OA), potato dextrose agar (PDA), and synthetic low-nutrient agar (SNA) have been described elsewhere (*26*). Difco yeast extract was used in YES. To all media, trace metals were added (0.001% $ZnSO_4 \cdot 7H_2O$ and 0.0005% $CuSO_4 \cdot 5H_2O$) and 2% agar was used in the semisolid media. Liquid cultivations were performed in 25-mL Blue cap bottles containing 4 mL of liquid medium of YES (YESliq), potato dextrose (PDliq), or Raulin−Thom (RTliq) (*27*). Water was purified from a Milli-Q system (Millipore, Bedford, MA).

Source of reference standards: Trichodermin was a gift from LEO Pharma A/S, and other reference standards were available from previous studies in our laboratory (*28*). Harzianum A was extracted by acidic ethyl acetate (EtOAc) from the producer strain, ATCC 90237, and purified on an Oasis MAX SPE mixed reversed-phase anion-exchange cartridge (Waters, Milford, MA), first washed with acetonitrile (CH_3CN) and then fractioned by acidic water−CH_3CN starting at 15% CH_3CN and increasing in steps of 10% CH_3CN. The 55% CH_3CN fraction contained harzianum A (>90% pure by LC−UV), which was identified by UV and LC−high-resolution negative and positive electrospray MS data as described below. Octa-2*Z*,4*E*,6*E*-trienedioic acid was produced by hydrolyzing harzianum A in 0.1 M NaOH in methanol for 6−24 h.

Cultivation. The origin of strains, their accession numbers in culture collections, and GenBank accession numbers are shown in **Table 1**. For morphological identification, cultures were grown on OA, PDA, and SNA media for 7−14 days at room temperature (~20 °C) under ambient daylight. Microscopic observations and measurements were made from slides mounted in water. For metabolite production, the strains were inoculated onto PDA media and incubated for 10 days at 25 °C in the dark. Digital images of the cultures were acquired using a Nikon D100 camera fitted with a macro lens. Selected strains (**Table 2**) were also cultivated on YES, OA, and in liquid media placed in a rotary shaker at 120 rpm (3 cm throw) for 10 days at 25 °C in the dark. After cultivation, the bottles were stored at −20 °C until extraction.

Extraction for Chemical Analysis. Agar cultures were extracted by scraping off the fungal biomass from the agar surface, transferred to a 4-mL vial and frozen for 3−4 days. The biomass was then extracted overnight on a rotary shaker at 200 rpm with 3 mL of EtOAc, which were transferred to a new 4-mL vial and evaporated in vacuo. Samples were redissolved in 500 μL of CH_3CN and filtered through a 4 mm 0.45 μm PFTE syringe filter (Chromacol, Herts, U.K.).

Liquid cultures were extracted overnight on a rotary shaker at 200 rpm with 15 mL of EtOAc. The upper EtOAc was filtered through a Whatman PS1 (Brentford, U.K.) phase separation filter and evaporated in vacuo. Samples were then redissolved in 500 μL of CH_3CN and filtered through a 4 mm 0.45 μm PFTE syringe filter.

Chemical Analysis. High-resolution LC−DAD−MS was performed on an Agilent 1100 system equipped with a photodiode array detector (DAD) and a 50 × 2 mm i.d., 3 μm, Luna C_{18} II column (Phenomenex, Torrance, CA). The LC system was coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, U.K.), with a Z-spray electrospray ionization (ESI) source, and a LockSpray probe (*28*) and controlled by the MassLynx 4.0 software.

Samples were analyzed in positive ESI (ESI^+) using a water−CH_3CN gradient system starting from 15% CH_3CN, which was increased linearly to 100% in 20 min holding this for 5 min or starting from 5% CH_3CN keeping this for 2 min and increasing to 100% in 18 min keeping this for 5 min. The water was buffered with 10 mM ammonium formiate and 20 mM formic acid (HCOOH) and the CH_3CN with 20 mM HCOOH as previous described (*28, 29*). The only changes were that two scan functions (1 s each) were used: the first with a potential difference of 10 V between the skimmers and using a scan range of *m*/*z* 100−900 and the second with 30 V between the skimmers and using a scan range of *m*/*z* 100−2000. In ESI^+, the instrument was tuned to a resolution >7000 (at half-peak height).

Samples were also analyzed in negative ESI (ESI^-) mode using the same instrument and gradient systems, except that only the water was buffered (100 μL/L of HCOOH). ESI^- was performed at a resolution >5000 (at half-peak height), and two scan functions (1 s each) were used: the first with a potential difference of 10 V between the skimmers and the second with a potential difference of 30 V between the skimmers. The desolvation temperature was 450 °C; source block temperature was 120 °C; and the desolvation flow (nitrogen 99.9%) was ca. 0.53 m^3/h.

The capillary was held at −1800 V, and data were collected as centroid data from *m*/*z* 100−900. A solution of 3,4-dihydroxybenzoic acid in water−methanol (1:1, v/v) was infused (10 μL/min) into the lock spray source (second ESI^- spray) using a syringe pump. The $[M-H]^-$ ion of 3,4-dihydroxybenzoic acid was subsequently used for online mass correction every 3 s.

Analysis of Chemical Data. The presence of trichodermin was detected in ESI^+ from the first scan function of the reconstructed ion chromatograms of *m*/*z* 293.18 $[M + H]^+$ and confirmed by the fragments *m*/*z* 233.15 and 215.15, and the presence of trichodermol was detected in ESI^+ from the same scan function as *m*/*z* 251.16 $[M + H]^+$ and confirmed by the fragment *m*/*z* 233.15. Harzianum A was detected in ESI^- from the second scan function of the reconstructed ion chromatograms of *m*/*z* 399.18 $[M - H]^-$ and confirmed from the UV absorption at 306 ± 4 nm (as well as the full UV spectrum). For all extracted ion chromatograms, a window of *m*/*z* ±0.02 was used. Other peaks, excluding those found in negative controls, were matched against an internal reference standard database (~630 compounds) (*28*).

DNA Extraction, Sequencing, and Analysis. For total genomic DNA extraction, strains were grown on liquid malt-yeast-peptone

Table 1. *Trichoderma* and *Hypocrea* Strains Included in This Study[a]

strain	other collections	species	habitat	geographic origin	trichothecene production on PDA	GenBank accession number ITS1/ITS2
CBS 109720[b]	MA 3296, DAOM 231232, IBT 40866	*T. brevicompactum*	soil in sunflower field	Geneva, NY	trichodermin	AY324173/ AY324183
CBS 112443	MA 761, IBT 40867	*T. brevicompactum*	Ex rhizosphere of *Glycosmis sapindoides*	Kuriva forest, Papua New Guinea	trichodermin	AY324174/ AY324183
CBS 112444	MA 1374, IBT 40861	*T. brevicompactum*	soil	Union Island, Saint Vincent and the Grenadines	trichodermin	AY324173/ AY324183
CBS 112445	MA 4105, IBT 40868	*T. brevicompactum*	soil, maize field	Costa Rica	harzianum A	AY324173/ AY324183
CBS 112446	MA 4106, IBT 40862	*T. brevicompactum*	soil in backyard	Trivandrum, India	trichodermin	AY324173/ AY324183
IBT 40836	D.Z. 02, lr. 500	*T. brevicompactum*	soil	Khoramabad, Iran	harzianum A[c]	AY154921
IBT 40837	D.Z. 47, lr. 503	*T. brevicompactum*	soil	Alshter Road, Khoramabad, Iran	harzianum A[c]	AY154943
IBT 40838		*T. brevicompactum*	soil	Iran	trichodermin[c]	AY154920
IBT 40839[d]	D.Z. 01, lr. 29	*T. brevicompactum*	soil	Qazvin, Iran	trichodermin[c]	AY154920
IBT 40840[d]		*T. brevicompactum*	soil	Iran	trichodermin[c]	AY154920
IBT 40841[d]		*T. brevicompactum*	soil	Iran	trichodermin[c]	AY154920
IBT 40842[d]		*T. brevicompactum*	soil	Hamadan, Iran	harzianum A[c]	AY154921
IBT 40863		*T. brevicompactum*	soil	Shahr-e Kord, Chahar Mahall va Bakhtiari, Iran	harzianum A	AY154921
IBT 40864		*T. brevicompactum*	soil	Hamadan, Iran	harzianum A	AY154921
LEO ND8		*T. brevicompactum* (originally identified as *T. viride*)	soil	Papua New Guinea	trichodermin[c]	AY154920
ATCC 90237[d]	IBT 9471	*T. brevicompactum* (originally identified as *T. harzianum*)	micaceous clay from stream bed	Windhoek, Namibia	harzianum A[c]	DQ080074
CBS 238.81		*H. lutea*		Brazil	ND	
CBS 678.74		*H. lutea*	Decayed trunk of *Fraxinus angustifolia*	Southern Moravia, junction of the rivers Morava and Dyje near Lanzhot and Breclav, Czech Republic	ND	
CBS 228.48	ATCC 10097, NRRL 1086	*H. lutea*	soil	Washington	ND	
CBS 597.95		*H. lutea*	hapalopilus rutilans	Tartu, Estonia	ND	
CBS 109832		*H. lutea*	estuarine sediment	São Paulo State, Cabatão, Santos Estuary, Brazil	ND	
CBS 355.97		*H. lutea*	log	Luquillo Mts., El Verde Research Area, Puerto Rico	ND	
CBS 102037		*H. lutea*	pyrenomycete and decorticated wood	Estatoe South, North Carolina	ND	
IBT 8850		*T. harzianum*	air sample	recycling plant, Denmark	ND[c]	
IBT 9153	BBA 70224	*T. harzianum*	aluminia-paper vapor barierer	public building, Denmark	ND[c]	AJ230664
IBT 9134		*T. harzianum*	building material	Denmark	ND[c]	
IBT 8965	BBA 70227	*T. atroviride*	building material	Denmark	ND[c]	AJ230659
IBT 8964		*T. atroviride*	indoor wood construction	Denmark	ND[c]	
DAOM 230019[b]	CBS 117088	*T. erinaceum*	soil	Coral Island (Koh Lann), Thailand	ND	
IBT 8866	BBA 70471	*T. viride*	building material	Denmark	ND[c]	AJ230682
IBT 8212	BBA 70470	*T. viride*	gypsum board	private home, Denmark	ND[c]	AJ230682
IBT 9155	BBA 70226	*T. longibrachiatum*	top side of bitumen layer	public building, Denmark	ND[c]	AJ230665
IBT 9128	BBA 70225	*T. longibrachiatum*	building material	Denmark	ND[c]	AJ230665
IBT 9135	BBA 70222	*T. citrinoviride*	building material	public building, Denmark	ND[c]	AJ230663
IBT 9149	BBA 70223	*T. citrinoviride*	building material	public building, Denmark	ND[c]	AJ230663

[a] Abbreviations: ATCC, American Type Culture Collection, Manassas, VA; BBA, Biologische Bundesanstalt für Land- und Forstwirtschaft, Berlin, Germany; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; DAOM, Eastern Cereal and Oilseed Research Centre, Ottawa, Canada; D.Z., collection of D. Zafari, Hamadan, Iran; IBT, BioCentrum-DTU, Kgs. Lyngby, Denmark; MA, Austrian Center of Applied Mycology and Bioresources, Vienna, Austria; NRRL, ARS Culture Collection, Northern Regional Research Laboratory, National Center for Agricultural Utilization Research, Peoria, IL; ND, not detected; PDA, potato dextrose agar. [b] Ex-type strains. [c] Strains grown on additional media (**Table 2**). [d] Images from 10 to 12 day cultures on OA, PDA, and YES media are available on the journal web site.

medium (MYP) (*30*) without agar. The mycelium was obtained by vacuum filtration, and cells were disrupted either by pulverization in a precooled mortar using liquid nitrogen or using a glass bead beater method [mycelium, 750 μL of 1% SDS/10 mM EDTA, and 250 μL of 0.5 mm diam glass beads in 1.5 mL screw-cap reaction tubes; agitated in a mixer mill (Retsch GmbH and Co. KG, Haan, Germany) at the highest speed for 30 min]. DNA was further isolated and purified according to the method described by Hering (*31*).

The ITS1 and ITS2 regions of the ribosomal DNA were amplified by using the primers ITS5 and ITS4 (*32*). The length of the fragments was determined on 1% agarose gels. PCR conditions were optimized until all strains yielded a single PCR product, which was purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), according to the instructions of the manufacturer. The purified double-stranded PCR products were directly sequenced using Amersham Thermo-Sequenase reactions (3 μL scale) and IRD-labeled primers (ITS1 and ITS4) (*32*). Primary nucleotide sequences were determined on a LI-COR 4000L automated DNA sequencer. The entire product was sequenced bidirectionally. Sequences were assembled and corrected using Sequencher 4.0.5 (Gene Codes Corporation, Ann Arbor, MI). DNA sequences were aligned with published sequence data (*16*) visually and improved manually with the BioEdit Sequence Alignment Editor 7.0.1 (*33*). The complete ITS1/ITS2 sequences (including 5.8S) obtained in this study have been submitted to GenBank, acession numbers AY154920, AY154921, AY154943, and DQ080074.

RESULTS

Analysis, Extraction, and Sample Preparation. Type A trichothecenes are very labile; therefore, a very low potential difference is needed between the skimmers to avoid extensive fragmentation resulting in a lack of detection. To get good sensitivity of high mass ions, a much higher potential difference is needed between the cones, otherwise the ions will not be introduced into the analyzer; thus, a second scan function is needed. This has another advantage because small molecules are being fragmented as a result of the higher velocity in the skimmer region, and thus, pseudo-MS/MS data can be obtained by comparing the MS spectra from the two scan functions.

Table 2. Trichothecene Production from Selected *Trichoderma* Strains on Semisolid and Shaken Media, Incubated for 10 Days in Darkness

medium		YES[a]		PDA		OA		PDliq		YESliq		RTliq	
strain number	species	tric	harz A	tric	harz A	tric	harz A	tric	harz A	tric	harz A	tric	harz A
IBT 40841	*T. brevicompactum*	++[b]	ND	+++	ND	++	ND	++++	ND	+++	ND	+++	ND
IBT 40840	*T. brevicompactum*	++	ND	+++	ND	++	ND	++++	ND	+++	ND	+++	ND
IBT 40839	*T. brevicompactum*	++	ND	+++	ND	++	ND	++++	ND	+++	ND	+++	ND
IBT 40838	*T. brevicompactum*	+	ND	+++	ND	++	ND	++++	ND	+++	ND	+++	ND
LEO ND8	*T. brevicompactum*	++	ND	+++	ND	++	ND	not performed		not performed		not performed	
IBT 40837	*T. brevicompactum*	+	+++	+	+++	ND	+++	ND	++++	ND	++++	ND	+++
IBT 40836	*T. brevicompactum*	ND	++++	ND	++++	+	+++	ND	++++	ND	++++	ND	+++
IBT 40842	*T. brevicompactum*	ND	+++	+	++++	ND	+++	ND	++++	ND	++++	ND	+++
ATCC 90237	*T. brevicompactum*	+	+++	+	+++	+	+++	ND	++++	ND	++++	ND	+++
IBT8866	*T. viride*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IBT8212	*T. viride*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IBT8850	*T. harzianum*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IBT9153	*T. harzianum*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IBT9134	*T. harzianum*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IBT8965	*T. atroviride*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IBT8964	*T. atroviride*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IBT9155	*T. longibrachiatum*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IBT9128	*T. longibrachiatum*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IBT9135	*T. citrinoviride*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IBT9149	*T. citrinoviride*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] ND, not detected; YES, yeast extract sucrose agar; OA, oat meal agar; PDA, potato dextrose agar; YESliq, shaken yeast extract sucrose medium; PDliq, shaken potato dextrose medium; RTliq, shaken Raulin–Thom medium. [b] +, peak area of 5–100 counts; ++, peak area of 100–500 counts; +++, peak area of 500–3000 counts; ++++, peak area of 3000–30 000. The trichodermin peak area was determined from the *m*/*z* 293.18 (ESI^+) and harzianum A from *m*/*z* 399.18 (ESI^-).

As with many organic acids, harzianum A had very poor response factors in ESI^+, whereas it was ionized strongly in ESI^-. Therefore, it was necessary to analyze all samples in both ionization polarities, because trichodermin and trichodermol, as with most type A trichothecenes, are not detected in ESI^- at all. A number of the extracts contained some isocoumarin derivatives (on the basis of their UV spectra), which eluted close to trichodermin and which had the same nominal masses (results not shown); therefore, data should be evaluated carefully when analyzing for trichodermin even by LC−MS and using LC−MS/MS or LC−HRMS.

Harzianum A has a very distinct UV spectrum (**Figure 2**), and this should provide sufficient specificity by LC−DAD analysis as long as the full UV spectrum is being used, including the shoulders at 292 and 318 nm, otherwise it could be confused with, e.g., 6-pentyl-α-pyrone (*34*) (not produced by *T. brevicompactum*), which has a UV max at 302 nm and eluted ca. 1 min before harzianum A in the solvent systems used. Interestingly, all of the samples containing harzianum A also contained a peak (almost unretained if starting at 15% CH_3CN and retention time ca. 5.8 min if starting a 5% CH_3CN) with a UV spectrum that could be superimposed on the harzianum A UV spectrum (**Figure 2E**) and had a $[M-H]^-$ ion of *m*/*z* 167.0357. This was only 1.2 mDa from the calculated mass of 167.0345 Da of octa-2*Z*,4*E*,6*E*-trienedioic acid (the ester moiety of harzianum A). Because the acid was not commercially available, it was produced by hydrolysis of harzianum A in methanolic NaOH, yielding a peak with the same retention time as the proposed octa-2*Z*,4*E*,6*E*-trienedioic acid in the crude extract, thus validating the identity of this peak (results not shown). It should be noted that the main product of the non-trichothecene moiety from the hydrolysis of harzianum A in methanolic NaOH was the monomethylated octa-2*Z*,4*E*,6*E*-trienedioic acid (3 times the amount of the free diacid). The methylation was also seen upon hydrolysis of the macrocyclic trichothecene roridin A, where its non-trichothecene moiety was only detected as the mono- and dimethylated products in the ratio of 3:1 (results not shown).

Metabolite Profiling Results. In **Tables 1** and **2**, it can be seen that two *T. brevicompactum* chemotypes were found, one producing trichodermin and one producing harzianum A. All strains consistently produced trichothecenes on the media tested (**Table 2**), and it was demonstrated that potato dextrose medium was a good substrate for trichothecene production. Shake conditions compared with semisolid agar also gave 5−10 times higher yields of trichothecenes. The esterification of trichodermol to trichodermin and harzianum A was very effective because only traces of trichodermol were detected in a few extracts (results not shown). In **Table 1**, it can also be seen that the ability to produce trichothecenes was restricted to *T. brevicompactum*, because no trichothecenes were detected in the closely related species (*16*).

Peptaibols are the most common class of metabolites from *Trichoderma*, *Hypocrea*, and *Gliocladium* numbering 220 of the 436 compounds described from these genera in Antibase 2003 (Wiley and Sons, Hoboken, NJ). However, the only peptaibols that could be unambiguously identified in this study were the alamethicins (*35*), which matched a reference standard and were detected as their $[M + Na]^+$ and $[M + K]^+$ adducts. These peptaibols were only found in the two *T. brevicompactum* types. It appeared that the peptaibols were mainly found in the mycelium, whereas the trichothecenes were excreted into the medium (results not shown). Thus, an incubation time of 10 days was chosen to obtain good yields of trichodermin and/or harzianum A.

The overall chemical profile of all the 10−40 peaks detected in the various species clearly showed that each species had a specific profile (results not shown). In all the nine trichodermin-producing *T. brevicompactum* strains, the ca. 30 peaks detected in UV and ESI^+ were consistently produced (results not shown), further validating that the ND8 strain is indeed a *T. brevicompactum*. The seven harzianum-A-producing strains had, except for the difference in octa-2*Z*,4*E*,6*E*-trienedioic acid production and harzianum A, the same chemical profile as the trichodermin-producing type (results not shown).

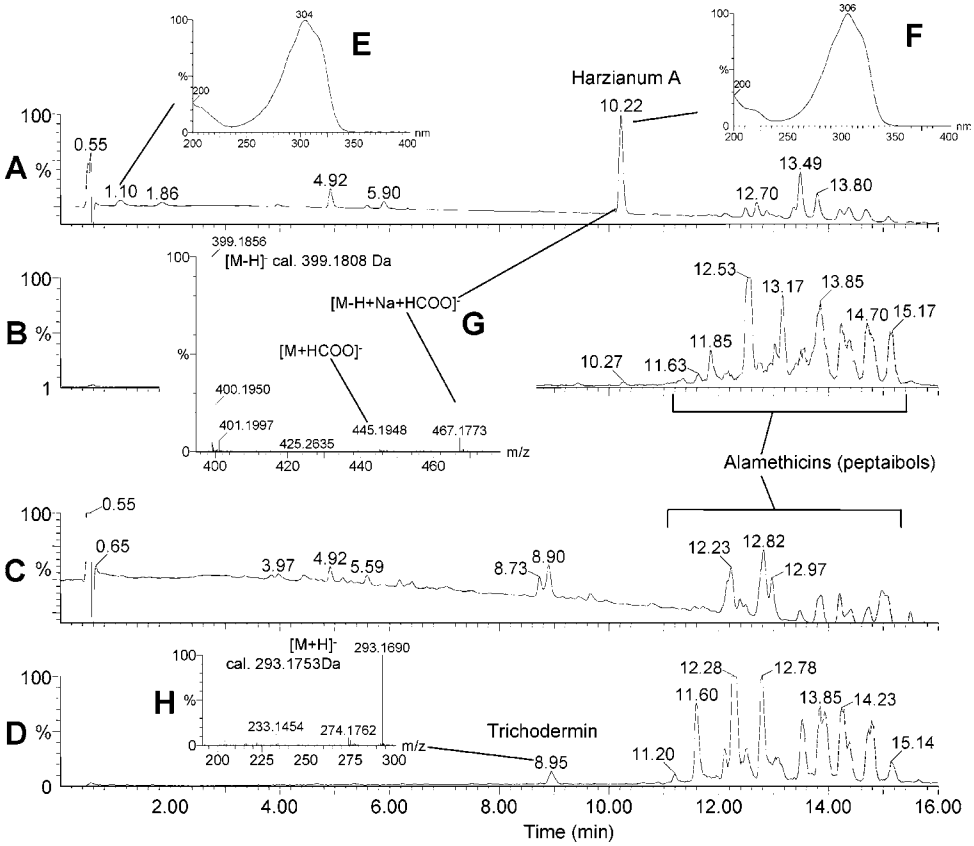

Figure 2. LC–UV–ESI$^+$–MS chromatograms (starting at 15% CH_3CN) of extracts of *Trichoderma brevicompactum* grown on potato dextrose agar. (A) UV trace (200–700 nm) from IBT 40837. (B) ESI$^+$ chromatogram of IBT 40837. (C) UV trace (200–700 nm) from LEO ND8. (D) ESI$^+$ chromatogram of LEO ND8. (E) UV spectrum of assumed (2*Z*,4*E*,6*E*)-octa-2,4,6-trienedioic acid. (F) UV spectrum of harzianum A. (G) ESI$^-$ spectrum of harzianum A from LC–ESI$^-$ run. (H) ESI$^+$ spectrum of trichodermin in the LEO ND8 strain. The time delay between UV and MS signals is 0.05 min.

Morphology Results. Strains investigated (**Table 1**) showed a *Pachybasium*-type micromorphology, including the characters and measurements of *T. brevicompactum* described by Kraus et al. (*16*). Microscopically, the cultures observed had mainly subglobose conidia that distinguished them well from other known species of the section *Pachybasium*, except for *T. harzianum*. However, cultures of *T. brevicompactum* grown on SNA tended to form pustules that were absent in *T. harzianum*.

The *T. brevicompactum* strains could easily be differentiated from the other species by its marcomorphology on YES, where it produced white fluffy mycelia without sporulation, which was very different from its normal greenish appearance on PDA and OA. This is illustrated for ATCC 90237, IBT 40839, IBT 40840, IBT 40841, and IBT 40842 in the pictures of 10−12 day cultures on YES, OA, and PDA available as Supporting Information on the journal website.

DNA Sequencing. The result from sequencing of the nuclear ribosomal DNA revealed identical sequences for the ITS1/ITS2 regions of all strains of *T. brevicompactum* investigated. In the alignment of published ITS sequence, they were also identical to the ITS1 and ITS2 sequences (see **Table 1**) (*16*) of other *T. brevicompactum* strains, viz. CBS 109720, CBS 112443, CBS 112444, CBS 112445, and CBS 112446.

DISCUSSION

To understand the confusion about trichothecene production by members of the genus *Trichoderma*, first of all, one has to consider the taxonomic knowledge available at the times of original publication. Godtfredsen and Vangedal's identification (*3*, *4*) of the "*T. viride*" strain ND8 from Papua New Guinea (*17*) probably was owed to Bisby's "single species concept" (*13*) and the lack of an identification key to the genus *Trichoderma* at that time. Moreover, this strain has never been reinvestigated by other scientists, and the species name published became undisputed. The identification of ATCC 90237 by Corley et al. (*5*) was probably due to the fact that the morphological species description of *T. harzianum* agreed best on the characters and measurements of the harzianum A strain. As pointed out by Kraus et al. (*16*), *T. harzianum* resembles many characters of *T. brevicompactum*. The *Hypocrea* species of Lee et al. (*18*) seems also to be a member of *T. brevicompactum*. It produces harzianum A (*18*) and shows 100% sequence homology to the ITS1/ITS2 regions of the strains IBT 40836 and IBT 40837. The scanning electron microscopy illustrations given by Lee et al. (*18*) show somewhat longer and narrower conidia that may be an artifact because of the fixation method used. However, our own morphological observations on cultures of CBS 113214 confirmed the identification as *T. brevicompactum*.

The consistent production of trichothecenes on all media (**Table 2**) showed that in our previous studies we likely would have detected any possible trichothecene production in other *Trichoderma* strains. Trichothecene production is presumable not widely spread within the genus *Trichoderma*, and the separation of the two *T. brevicompactum* chemotypes, the trichodermin producers and the harzianum A producers, into distinct taxa is currently envisaged by the authors. The identification of the free octa-2*Z*,4*E*,6*E*-trienedioic acid in the extracts from the harzianum-A-producing *T. brevicompactum* strains strongly indicates that harzianum A is synthesized in

one step from trichodermol and the octa-2*Z*,4*E*,6*E*-trienedioic acid. This proposed biosynthetic route may be the same in the other fungal genera such as *Stachybotrys* and *Myrothecium*, which are producing the highly cytotoxic macrocyclic diesters of verrucarol (15-hydroxy-trichodermol) (*36*−*41*).

In conclusion, we have shown that *T. brevicompactum* consistently produced trichodermin and/or harzianum A. These metabolites were unambiguously detected using LC−UV−HRMS. We also showed that the three trichothecene-producing *Trichoderma* reference strains known to science belong to this species and not to *T. viride* or *T. harzianum* as given in the original publications (*5*, *17*). The results have important safety implications for agricultural industries because *T. harzianum*, which is widely used for biocontrol purposes (*2*), does not produce trichothecenes in this study. The assignment of *T. brevicompactum* being the trichothecene-producing species in *Trichoderma* and re-identifications of known strains into this taxon were based on four independent methods all showing the same metabolite profiling, macro- and micromorphology, and DNA sequencing.

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Supporting Information Available: Pictures of 10−12 day cultures on YES, OA, and PDA. This material is available free of charge via the Internet at http://pubs.acs.org.

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