Occurrence and characterization of peptaibols from *Trichoderma citrinoviride*, an endophytic fungus of cork oak, using electrospray ionization quadrupole time-of-flight mass spectrometry

Lucia Maddau,¹ Annalisa Cabras,¹ Antonio Franceschini,¹
Benedetto T. Linaldeddu,¹ Salvatore Crobu,² Tonina Roggio²
and Daniela Pagnozzi²

Correspondence

Lucia Maddau
lmaddau@uniss.it

¹Dipartimento di Protezione delle Piante, Sezione di Patologia Vegetale, Università di Sassari,
Via E. De Nicola 9, 07100 Sassari, Italy

²Porto Conte Ricerche Srl, Tramariglio, Alghero, Italy

A cork oak endophytic strain of *Trichoderma citrinoviride*, previously selected for its antagonistic potential against various fungal pathogens involved in oak decline, was screened for the production of bioactive secondary metabolites. From liquid culture a mixture of polypeptide antibiotics (peptaibols) belonging to the paracelsin family was isolated and characterized. This peptide mixture was purified by column chromatography and preparative TLC on silica gel, and separated by analytical HPLC. It was analysed by MALDI-TOF MS and nano-ESI-QTOF MS. Tandem mass experiments were performed to determine the amino acid sequences based on the fragmentation pattern of selected parent ions. The mixture comprised 20-residue peptides with C-terminal phenylalaninol and N-terminal acetylation. Twenty-eight amino acid sequences were identified, and amino acid exchanges were located in positions 6, 9, 12 and 17. Among them, seven sequences are new as compared to those reported in the database specifically for peptaibols and in the literature. In addition, we obtained experimental evidence suggesting the existence of non-covalent dimeric forms (homo- and hetero-) of the various peptaibol species. The peptide mixture showed strong antifungal activity toward seven important forest tree pathogens, and it was highly toxic in an *Artemia salina* (brine shrimp) bioassay. These results emphasize the cryptic role of endophytic fungi as a source of novel bioactive natural products and biocontrol agents.

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INTRODUCTION

Cork oak (*Quercus suber* L.) is one of the most common evergreen forest tree species in Mediterranean countries and is the primary source of industrial cork. Since the beginning of the 1980s, decline phenomena have been observed in several cork oak ecosystems (Luque & Girbal, 1989; Bakry & Abourouh, 1995; Santos, 1995; Sanchez *et al.*, 2003; Linaldeddu *et al.*, 2007a). To date, this syndrome has represented the main phytopathological problem of oak forests. This is due to its complex aetiology, in which different adverse abiotic and biotic factors are involved. A primary role is played by various fungal pathogens, which are able to cause severe damage and also to persist in latency inside the living tissue of their hosts (Luque *et al.*,

2000; Franceschini *et al.*, 2005). In particular, the most widespread and virulent species are *Apiognomonia quercina* (Kleb.) Höhn., *Biscogniauxia mediterranea* (De Not.) O. Kuntze and *Botryosphaeria corticola* Phillips, Alves & Luque (Linaldeddu *et al.*, 2009).

At present, there are no effective means of controlling the pathogens associated with cork oak decline, and the need to define suitable measures in order to prevent this increasing phenomenon has become a priority (Luque *et al.*, 2008). Recently, promising results for the control of oak pathogens have been obtained using mutualistic endophytic fungi (Campanile *et al.*, 2007).

Endophytic fungal communities make up an important component of forest ecosystems (Stone *et al.*, 1996; Schulz & Boyle, 2005; Saikkonen, 2007; Sieber, 2007). Plants infected with endophytic fungi often exhibit a lower susceptibility to herbivorous insects and pathogens, partly due to the production *in planta* of secondary bioactive

Abbreviations: Ac, acetyl; Aib, α -aminoisobutyric acid; ESI-QTOF, electrospray ionization quadrupole time-of-flight mass spectrometry; Lx or Lxx, leucine or isoleucine; TFA, trifluoroacetic acid; Vx or Vxx, valine or isovaline; Fol or Pheol, phenylalaninol.

metabolites that act as deterrents (Bailey *et al.*, 2008; Sikora *et al.*, 2008; Vega *et al.*, 2008). These fungi can also enhance the resistance of their hosts to unfavourable conditions (drought, heavy metal presence, low pH and high salinity); they can influence the plant physiology or they can alter the mineral nutrient uptake. On the other hand, endophytic micro-organisms represent an important source of bioactive and chemically novel secondary metabolites that may also have potential in medicine, agriculture and industry and could be involved in the host–endophyte relationship (Schulz *et al.*, 2002). The capacity of endophytic fungi to synthesize compounds displaying a wide range of biological activities, from antimicrobial to anticancer, is well documented in several recent reviews (Gusman & Vanhalen, 2000; Tan & Zou, 2001; Strobel *et al.*, 2004; Gunatilaka, 2006; Zhang *et al.*, 2006). However, several ecological aspects and biochemical mechanisms involved in the interactions among endophytes, pathogens and hosts still have to be elucidated. Knowledge of these aspects has important implications for developing effective control strategies in forestry.

Studies on the cork oak endophytic community are recent, but practical guidelines for developing new biological control agents have already been evaluated (Linaldeddu *et al.*, 2005). Our previous findings showed that various cork oak endophytic *Trichoderma* species exhibited strong antagonistic activity *in vitro* and *in vivo* toward several oak pathogens (Maddau *et al.*, 2005; Linaldeddu *et al.*, 2007b). In particular, the isolate (S25) of *Trichoderma citrinoviride* Bissett showed the greatest ability to parasitize all tested pathogens, to colonize and protect host tissues and to produce strong inhibitory compounds. This experimental evidence suggested that the use of endophytes or their natural products may potentially represent an excellent eco-sustainable alternative to chemical pesticides to control plant pathogens. To our knowledge, no data are available in the literature concerning the production of antifungal metabolites from *T. citrinoviride*.

Thus, the objective of our research was to isolate and characterize the bioactive secondary metabolites produced by the selected endophytic strain of *T. citrinoviride*. This work reports the isolation, characterization and bioactivities of a peptide mixture from *T. citrinoviride* representative of the peptaibol family.

METHODS

Fungal strains, culture medium and growth conditions. The strain of *T. citrinoviride* was originally isolated from asymptomatic tissues of a cork oak tree growing in a natural stand in Sardinia (Italy). The fungus was identified on the basis of morphological characters and internal transcribed spacer (ITS) rDNA sequence. Pure cultures were maintained on potato-dextrose-agar (PDA, Fluka, Sigma-Aldrich Chemie) and stored at 4 °C in the collection of the Dipartimento di Protezione delle Piante, University of Sassari, Italy, as strain S25. For the production of bioactive metabolites, the fungus was grown in still liquid culture on Czapek medium. Roux bottles containing 170 ml autoclaved medium were inoculated with a

conidial suspension (10^6 spores ml⁻¹) of the fungus. Cultures were incubated at 25 °C in the dark for 21 days and then filtered through filter paper (Whatman no. 1).

Chemicals. α -Cyano-4-hydroxycinnamic acid was used as matrix for MALDI-TOF experiments. All solvents used in this study were of HPLC gradient grade, and were purchased from Merck-VWR.

Chromatography. For TLC, analytical and preparative plates pre-coated with silica gel (Kieselgel 60, F₂₅₄, 0.25 and 0.5 mm respectively; Merck-VWR) were used. The mobile phase was AcOEt/MeOH/H₂O (7:2:1). Spots were visualized by exposure to UV radiation (254 or 366 nm) and to I₂ vapour. Column chromatography was performed on silica gel (Kieselgel 60, 0.063–0.20 mm; Merck-VWR). For HPLC, an HP 1200 (Agilent Technologies) instrument equipped with a binary pump, connected to a UV/VIS detector of the same series was used. The peptide mixture was analysed on a Hypersil BDS C18 column (Alltech), 250 × 4.6 mm i.d., 5 µm particle size, using as eluent a linear gradient of eluent B [95% MeCN, 0.07% trifluoroacetic acid (TFA)] in A (0.1% TFA) from 30 to 100% in 70 min; the flow rate was 1 ml min⁻¹, the temperature 35 °C and the detector wavelength 220 nm.

Extraction and isolation. The culture broth (14 l) was extracted exhaustively with AcOEt. The organic extracts were combined, dried with Na₂SO₄ and evaporated under reduced pressure at 40 °C to give a red-brown oily residue (1.8 g). The residue was then subjected to bioassay-guided fractionation by silica gel column chromatography eluted with a gradient of petroleum ether/AcOEt (from 20:1 to 0:100, v/v) and AcOEt/MeOH (from 10:1 to 0:100, v/v). The collected fractions (15 ml each) were combined into 13 groups, T₁–T₁₃, on the basis of their silica gel TLC profiles. The purification process was monitored by testing the fractions on brine shrimp larvae (*Artemia salina*) and the fungal pathogen *Biscogniauxia mediterranea*. Fraction T₁₂ was found to be the most active against both test organisms. This fraction (1 g) was further purified by column chromatography eluted with a gradient of CHCl₃/MeOH (from 4:1 to 0:1, v/v), affording 800 mg of a peptide mixture. An 80 mg aliquot of this latter sample was purified by preparative TLC with system solvent AcOEt/MeOH/H₂O (7:2:1, by vol.). The silica gel was scraped off and the peptides dissolved in CH₂Cl₂/MeOH (1:1, v/v).

Mass spectrometry analysis. The purified peptide mixture was analysed using a MALDI micro MX (Waters) equipped with a reflectron analyser and used in delayed extraction mode. The peptide sample was mixed with an equal volume of matrix [10 mg ml⁻¹ in acetonitrile/0.2% TFA (70:30 v/v)], applied to the metallic sample plate and air-dried. Mass calibration was performed using the PEG-NaI mixture provided by the manufacturer as standard.

Peptide sequences were analysed on a Q-TOF hybrid mass spectrometer equipped with a nano Z-spray source (Waters). Sample solution (40 ng ml⁻¹ in 50% CH₃OH/0.1% HCOOH) was injected into the ion source at a flow rate of 1 µl min⁻¹. The mass spectrometer operated at a capillary voltage of 2000 V and a cone voltage ranging from 100 to 200 V. Mass spectra were acquired at 1 scan s⁻¹ in the mass range from 50 to 2000 *m/z* and the collision energy ranged between 2.0 and 50 V. Argon was used as the collision gas. Mass calibration was performed by using GluFibrinopeptide B standard provided by the manufacturer.

Antifungal assay. Organic crude extracts and the chromatographic fractions were tested for antifungal activity against *Biscogniauxia mediterranea*. The purified peptide mixture was also tested against *Apiognomonium quercina*, *Botryosphaeria corticola*, *Botryosphaeria parva* Pennycook & Samuels, *Botryosphaeria obtusa* (Schwein.) Shoemaker, *Diplodia pinea* (Desm.) J. Kickx f. and *Diplodia scrobiculata* J. de Wet,

Slippers & M.J. Wingf. All fungal pathogens tested are available in the collection of the Dipartimento di Protezione delle Piante. The antifungal activity was determined on PDA as inhibition of the radial growth at different concentrations. The crude extracts and residues of column chromatography fractions were assayed at concentrations of 500, 250 and 125 $\mu\text{g ml}^{-1}$. The purified peptide mixture was tested at concentrations of 10, 50, 100 and 200 $\mu\text{g ml}^{-1}$. Briefly, the test was carried out by growing each fungal species in Petri dishes (90 mm diameter) containing 10 ml 3% PDA supplemented with the appropriate amounts of toxic sample to obtain the concentrations reported above. PDA containing only the culture medium with the addition or not of methanol were used as controls for each fungus studied. The plates (three per fungal species) were seeded with an agar plug (6 mm diameter) cut from the edge of actively growing cultures and incubated at 25 °C. The radial mycelial growth (mm) of fungi in both treated and control Petri dishes was measured diametrically from three replicates every 24 h for 3–8 days depending on fungal species. The experiment was repeated twice and contained three replicates each time.

Artemia salina bioassay. Crude extract, chromatographic fractions and peptide mixture were assayed on brine shrimp larvae (*Artemia salina* L.). The assay was performed in cell culture plates with 24 cells (Corning) as described by Favilla *et al.* (2006). The samples were dissolved in methanol and serially diluted twofold to obtain the required experimental concentrations. The crude extract and the chromatographic fractions were tested at a concentration range of 20–200 $\mu\text{g ml}^{-1}$. The peptide mixture was tested at a concentration range of 0.1–20 $\mu\text{g ml}^{-1}$. Tests were performed in quadruplicate. The percentage of larval mortality was determined after incubation for 24 and 36 h at 27 °C in the dark. Percentage mortalities were corrected for the natural mortality following Abbot's formula: $p = p_i - C / 1 - C$, where p_i denotes the observed non-zero dose response and C represents the natural mortality of controls.

Statistical analysis. Data from the antifungal activity assay were analysed with the XLSTAT software (Addinsoft). Means for each experiment were compared by using Duncan's multiple range test ($P \leq 0.05$).

Mortality data from three independent experiments were used to construct the dose–response curve and determine the LC_{50} to *Artemia salina*. In the dose/response curve, the concentrations tested were plotted against the percentage mortality (means $\pm$ SE). The LC_{50} was calculated with data from three independent experiments by using Probit analysis performed with the software XLSTAT-Dose (Addinsoft).

RESULTS

Extraction and purification of the peptide mixture

The culture filtrate of *T. citrinoviride* was extracted exhaustively with ethyl acetate, yielding a red-brown oily residue (1.8 g) that was highly active against *Biscogniauxia mediterranea* and *Artemisia salina*. The crude extract was fractionated by column chromatography as described in Methods, and the fractions obtained (T_1 – T_{13}) were tested for their biological activity on *B. mediterranea* and *A. salina*. The most active fraction, T_{12} , was further purified by a combination of column chromatography and TLC, resulting in a complex peptide mixture that was separated by analytical HPLC. The HPLC elution profile of the isolated peptide mixture (Fig. 1) revealed a mixture of at least 14 components, and it is dominated by four major peaks. The fractions were manually collected and used for mass spectrometry experiments.

Structural analysis of the peptide mixture

We analysed the peptide mixture by MALDI-TOF MS to determine the monoisotopic mass values. It mainly appeared as sodium adduct $[M + \text{Na}]^+$ ions at m/z 1931.12 (3 %), 1945.14 (36 %), 1959.13 (37 %), 1973.14

Fig. 1. HPLC elution profile of peptide mixture. Numbers assigned to peaks in the chromatogram correspond to peptide sequences presented in Table 1. For chromatographic conditions see Methods.

(17%), 1987.15 (6%) and 2002.15 (1%) as shown in Fig. 2. A careful examination of the mass spectrum revealed the presence of some characteristic fragment ions, which suggested that the compounds belonged to the peptaibol class of peptides having 20 amino acid residues (paracelsin type).

All fractions obtained from HPLC were screened by MALDI-TOF MS, and then analysed in acidic conditions by direct infusion in the nano-electrospray ionization quadrupole time-of-flight (nano-ESI-QTOF) system in the MS mode to identify the fragmentation pattern.

In nano-ESI-QTOF all components appeared in the full-scan mass spectra predominantly as doubly and triply pseudomolecular ions $[M + 2H]^{2+}$ and $[M + 3H]^{3+}$. Peaks of triply charged ions appeared around m/z 1273.07–1319.83, which strongly suggested the existence of non-covalent dimeric associations of the various peptaibol species. The assigned $[M_2 + 3H]^{3+}$ species indicated a relative molecular mass exactly twice that of the monomeric molecular ions. This result greatly surprised us, because there have not been any studies on the release *in vivo* of peptaibols as a dimeric form (homo and hetero). On the other hand, it is known that the 'soft' ionization feature of electrospray ionization (ESI) allows the detection of non-covalent complexes of proteins (Loo, 2000). Tandem MS experiments were performed on selected doubly charged molecular and fragment ions produced in the

ESI source under low-energy collision conditions. As observed for other peptaibols, all components showed a minimal fragmentation in source into two complementary singly charged fragment ions of the b and y type. This characteristic fragmentation is well known and it is due to the particular lability of the secondary amine bond $^{13}\text{Aib}-^{14}\text{Pro}$ (Rebuffat *et al.*, 1995). Five b_{13} fragments (all having consecutively 14 Da different masses) were detected at different m/z values (1135.6, 1149.6, 1163.6, 1177.7 and 1191.7), while the fragments y_7 were observed only at m/z 774.4 and 788.5. All these fragments were the precursor ions for MS/MS experiments. In the fragmentation of the y_7 ion at m/z 774, the full series of b ions was interpreted as having been generated by successive losses of Pheol, Gln, Gln, Aib and Aib (Fig. 3a). Given that the N-terminal amino acid was considered to be Pro in the C-terminal peptide fragment, the smallest fragment at m/z 197 was ascribed to $^{14}\text{Pro}-^{15}\text{Val}$. Thus, the C-terminal amino acid sequence was determined as $^{14}\text{Pro}-^{15}\text{Vxx}-^{16}\text{Aib}-^{17}\text{Aib}-^{18}\text{Gln}-^{19}\text{Gln}-^{20}\text{Pheol}$. There are no satellite peaks within the spectrum, showing that there is only one sequence corresponding to y_7 at m/z 774. Side-chain fragments as d_n -type ions were also observed, in particular, d_5 at m/z 424 and d_6 at m/z 552, identifying ^{18}Gln and ^{19}Gln . Fragments such as a_4 , a_5 and a_6 appeared in the spectrum and confirmed the $^{18}\text{Gln}^{19}\text{Gln}^{20}\text{Pheol}$ sequence. Similarly, the tandem mass experiments of the fragment at m/z 788 afforded a series of b ions, suggesting the amino acid

Fig. 2. MALDI-TOF MS analysis of peptide mixture from *Trichoderma citrinoviride* showing singly charged pseudomolecular adducts of Na^+ and K^+ around m/z 1800–2040.

Fig. 3. ESI-tandem mass spectra of the two singly protonated y_7 (C-terminal) fragments into b ion series. (a) Fragment 774; (b) fragment 788.

sequence of the C-terminal fragment to be ^{14}Pro - ^{15}Vxx - ^{16}Aib - ^{17}Vxx - ^{18}Gln - ^{19}Gln - $^{20}\text{Pheol}$ (Fig. 3b). Since there is no peak due to d_4 side-chain loss from the ^{17}Val residue, it is probable that the ^{17}Val residue is ^{17}Iva . On the other hand, the known peptaibols have Iva residues at this position, while a peptaibol structure with Val at this position has been reported just once to our knowledge (Pócsfalvi *et al.*, 1998).

The fragmentation of the five b_{13} ions gave only b ion series and, for the majority of fractions, yielded satellite peaks at ± 14 Da distance.

As can be seen from the HPLC elution profile (Fig. 1), peak 2 represents the major peptide of the mixture. In MALDI-TOF MS this fraction resulted mainly in singly charged molecular ions with a sodium cation $[\text{M} + \text{Na}]^+$ at m/z 1945.07 and a potassium cation $[\text{M} + \text{K}]^+$ at m/z 1961.15. In ESI-QTOF, it occurred predominantly as a doubly charged ion appearing at m/z 962.0 and at a low intensity as

a triply charged ion at m/z 641.7, from which a molecular mass of 1922.09 Da can be deduced (Fig. 4a). In addition to doubly and triply charged pseudomolecular ions, a peak at m/z 1282.41 was observed. The isotopic pattern clearly shows that the signal is generated from a triply charged species whose molecular mass is 3844.23 Da, exactly twice that of the monomer (Fig. 4a, inset). Thus, peak 2 unambiguously represents a homodimer species. The sample showed in source a minimal fragmentation in two complementary singly charged ions, b_{13} at m/z 1149 and y_7 at m/z 774. The b_{13} fragment, used as the precursor ion for MS/MS experiments, gave only b ion series. The characteristic mass spectrum of the b series of peak 2 is shown in Fig. 4(b). The sequence-specific fragments are the peaks at m/z 1064 (b_{12}), 979 (b_{11}), 922 (b_{10}), 837 (b_9), 724 (b_7), 511 (b_6), 440 (b_5) and 355 (b_4). Examination of the m/z 440 ion showed acylium ions at m/z 355, 284, 199 and 128. The presence of these series makes sequencing up to the thirteenth residue very reliable. Therefore, the N-terminal

Fig. 4. Mass spectra of the peptide corresponding to peak 2. (a) Doubly and triply pseudomolecular ions. Inset: isotopic pattern of the triply charged ion corresponding to the dimeric form of peak 2. (b) ESI-tandem mass spectrum of singly protonated b_{13} precursor ion fragmented into b ions. The sequence of the fragment is written on its spectrum.

amino acid sequence was determined as AcAib-Ala-Aib-Ala-Aib-Ala-Gln-Aib-Lxx-Aib-Gly-Aib-Aib. Thus, the entire molecule can be obtained by combining the N- and C-terminal sequences. This most likely corresponds to the known paracelsin B molecule as found in the peptaibol database (<http://www.cryst.bbk.ac.uk/peptaibol>) and in the literature (Pócsfalvi *et al.*, 1997). Ambiguity remained as regards the isomeric amino acids Val/Iva and Leu/Ile, which are not distinguishable under our experimental conditions. With some exceptions (peaks 2, 4, 7, 9 and 11), b_{13} fragment ions were accompanied by satellite peaks at a difference in mass of ± 14 Da, indicating that there is more than one sequence corresponding to these fragments. The amino acid sequences, as determined by nano-ESI-QTOF analysis, and the theoretical masses are summarized in Table 1. From the data, 28 individual sequences of peptides were deduced. The analysis indicates exchange of Ala with

Aib in position 6, Val/Iva to Leu/Ile at position 9, Ala/Aib to Val/Iva or Leu/Ile in position 12 and Aib to Val/Iva in position 17. Among the 28 identified structures, at least seven sequences are new. The others must correspond to the paracelsin, saturnisporin, trichocellin, longibrachin and suzukacillin types (Rebuffat *et al.*, 1993; Wada *et al.*, 1994; Pócsfalvi *et al.*, 1997; Leclerc *et al.*, 1998; Krause *et al.*, 2006).

Some components having the same molecular mass were not single compounds but a mixture of at least two sequence homologues (for example sequences 3a and 3b; 5a and 5b; 6a and 6c; 10c and 10d; 12a and 12b; 14a and 14b). Furthermore, some sequences of peptides eluting at different retention times seem to be identical (for example sequences 2 and 5a; 5c and 7; 8a and 10b; 6b and 10a; 12a and 14a). For this group of peptides, apart from the

Table 1. Sequences of peptides characterized by nano ESI-QTOF in the micro-heterogeneous mixture produced in liquid culture by *Trichoderma citrinoviride*

Exchanged amino acid positions are highlighted in bold. Abbreviations of the amino acid are according to the one-letter code. Other abbreviations: PA A–F, paracelsin A–F; TC AII–AVIII, trichocellin AII–AVIII; LG AI, longibrachin AI; SA I–IV, sarurnisporin I–IV; SZ, suzukacillin.

Fraction	Theoretical mol. mass (Da)	b ₁₃	y ₇		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Notes
1	1908.06	1135.6	774.4	Ac	U	A	U	A	U	A	Q	U	Vx	U	G	U	U	P	Vx	U	U	Q	Q	Fol	PA A
2	1922.07	1149.6	774.4	Ac	U	A	U	A	U	A	Q	U	Lx	U	G	U	U	P	Vx	U	U	Q	Q	Fol	PA B (SA I)
3a	1922.08	1135.6	788.5	Ac	U	A	U	A	U	A	Q	U	Vx	U	G	U	U	P	Vx	U	Vx	Q	Q	Fol	PA F
3b	1922.08	1135.6	788.5	Ac	U	A	U	A	U	A	Q	U	Lx	U	G	A	U	P	Vx	U	Vx	Q	Q	Fol	New
4	1936.09	1149.6	788.4	Ac	U	A	U	A	U	A	Q	U	Lx	U	G	U	U	P	Vx	U	Vx	Q	Q	Fol	SA II
5a	1922.07	1149.6	774.4	Ac	U	A	U	A	U	A	Q	U	Lx	U	G	U	U	P	Vx	U	U	Q	Q	Fol	=Seq. 2; TC AIII
5b	1922.08	1149.6	774.4	Ac	U	A	U	A	U	U	Q	U	Vx	U	G	U	U	P	Vx	U	U	Q	Q	Fol	PA C
5c	1936.09	1163.7	774.4	Ac	U	A	U	A	U	U	Q	U	Lx	U	G	U	U	P	Vx	U	U	Q	Q	Fol	PA D
6a	1936.09	1149.7	788.4	Ac	U	A	U	A	U	A	Q	U	Lx	U	G	U	U	P	Vx	U	Vx	Q	Q	Fol	TC AII or TC AIV=SA II
6b	1950.10	1163.6	788.4	Ac	U	A	U	A	U	U	Q	U	Lx	U	G	U	U	P	Vx	U	Vx	Q	Q	Fol	SA IV
6c	1936.09	1163.6	774.4	Ac	U	A	U	A	U	A	Q	U	Lx	U	G	Vx	U	P	Vx	U	U	Q	Q	Fol	New
7	1936.10	1163.6	774.4	Ac	U	A	U	A	U	U	Q	U	Lx	U	G	U	U	P	Vx	U	U	Q	Q	Fol	=Seq. 5c; L↔Ile?
8a	1936.09	1163.6	774.4	Ac	U	A	U	A	U	A	Q	U	Vx	U	G	Lx	U	P	Vx	U	U	Q	Q	Fol	LG AI
8b	1950.11	1163.7	788.5	Ac	U	A	U	A	U	U	Q	U	Vx	U	G	Vx	U	P	Vx	U	Vx	Q	Q	Fol	New
9	1950.11	1177.9	774.4	Ac	U	A	U	A	U	A	Q	U	Lx	U	G	Lx	U	P	Vx	U	U	Q	Q	Fol	TC AV or TC AII
10a	1950.10	1163.7	788.5	Ac	U	A	U	A	U	U	Q	U	Lx	U	G	U	U	P	Vx	U	Vx	Q	Q	Fol	=Sequence 6b
10b	1936.09	1163.7	774.4	Ac	U	A	U	A	U	A	Q	U	Vx	U	G	Lx	U	P	Vx	U	U	Q	Q	Fol	=Sequence 8a
10c	1950.11	1177.7	774.4	Ac	U	A	U	A	U	A	Q	U	Lx	U	G	Lx	U	P	Vx	U	U	Q	Q	Fol	TC AVII or TC AV
10d	1950.10	1177.7	774.4	Ac	U	A	U	A	U	U	Q	U	Lx	U	G	Vx	U	P	Vx	U	U	Q	Q	Fol	New
11	1964.12	1177.7	788.4	Ac	U	A	U	A	U	A	Q	U	Lx	U	G	Lx	U	P	Vx	U	Vx	Q	Q	Fol	TC AVI or TC AVIII
12a	1964.12	1177.7	788.5	Ac	U	A	U	A	U	A	Q	U	Lx	U	G	Lx	U	P	Vx	U	Vx	Q	Q	Fol	TC AVI or TC AVIII
12b	1964.12	1177.7	788.5	Ac	U	A	U	A	U	U	Q	U	Lx	U	G	Vx	U	P	Vx	U	Vx	Q	Q	Fol	New
13a	1950.10	1177.7	774.5	Ac	U	A	U	A	U	A	Q	U	Lx	U	G	Lx	U	P	Vx	U	U	Q	Q	Fol	TC AV or TC AVII
13b	1964.12	1177.7	788.5	Ac	U	A	U	A	U	U	Q	U	Lx	U	G	Vx	U	P	Vx	U	Vx	Q	Q	Fol	New
13c	1964.12	1191.7	774.5	Ac	U	A	U	A	U	U	Q	U	Lx	U	G	Lx	U	P	Vx	U	U	Q	Q	Fol	New
14a	1964.13	1177.7	788.5	Ac	U	A	U	A	U	A	Q	U	Lx	U	G	Lx	U	P	Vx	U	Vx	Q	Q	Fol	=Sequences 11 and 12a
14b	1964.12	1177.7	788.5	Ac	U	A	U	A	U	U	Q	U	Lx	U	G	Vx	U	P	Vx	U	Vx	Q	Q	Fol	=Sequence 13b
14c	1978.14	1191.7	788.5	Ac	U	A	U	A	U	U	Q	U	Lx	U	G	Lx	U	P	Vx	U	Vx	Q	Q	Fol	=SZ (Seq. 12; Krause <i>et al.</i> , 2006)

exchange of isobaric Val/Iva or Leu/Ile, we presume that they represent different dimeric combinations of the peptides. Thus, sequences 3a and 3b could be seen as the monomeric form of the heterodimer (peak 3 in the chromatogram) identifiable by a triply charged ion at m/z 1273.07.

Biological activity

The antifungal activity of the purified peptide mixture was assayed against seven species of fungi (Table 2). It showed strong inhibitory effects against all tested fungi up to 50 $\mu\text{g ml}^{-1}$ (>70 % inhibition). At the lower concentration assayed (10 $\mu\text{g ml}^{-1}$), the peptide mixture was still highly active against all fungi except *Apiognomonia quercina* and *Biscogniauxia mediterranea* with a percentage growth inhibition between 45.6 and 78.7 %. The results of the bioassays performed in the *Artemisia salina* model are shown in Fig. 5. Probit analysis of the means of three independent quadruplicate experiments allowed the calculation of an LC_{50} of 1.24 $\mu\text{g ml}^{-1}$.

DISCUSSION

Fungi of the genus *Trichoderma* have been extensively studied for their biocontrol potential and they are among the most commercially marketed (about 60 % of all fungal biological control agents) as biopesticides, biofertilizers and soil amendments (Harman, 2000; Howell, 2003; Harman *et al.*, 2004; Verma *et al.*, 2007). For many years, almost all research has focused on *Trichoderma* species as common soil inhabitants (Klein & Eveleigh, 1998; Harman *et al.*, 2004); only recently has attention turned to those *Trichoderma* species that exist as endophytes in grassy and woody plant tissues (Evans *et al.*, 2003; Holmes *et al.*, 2004; Bailey *et al.*, 2008), and especially to those that can be utilized as potential biocontrol agents (Mejia *et al.*, 2008).

Traditionally, the biocontrol mechanisms proposed for *Trichoderma* species are those that act on pathogens, such as mycoparasitism, antibiosis, and competition for nutri-

ents and space (Howell, 2003). Recent findings showed that some *Trichoderma* species (especially *T. harzianum*, *T. hamatum* and *T. asperellum*) are also able to elicit systemic plant-defence responses (Yedidia *et al.*, 2003; Harman *et al.*, 2004; Khan *et al.*, 2004; Horst *et al.*, 2005), and the disease suppression can be seen as the result of the interaction among the plant, the pathogens and the microbial community (Vinale *et al.*, 2008).

Many species of *Trichoderma* have been shown to produce a wide heterogeneous range of bioactive metabolites that may contribute to their mycoparasitic and antibiotic action (Reino *et al.*, 2008). Those exhibiting antibiotic activity are grouped into two main types: low molecular mass and volatile metabolites (such as polyketides and volatile terpenes), and high molecular mass metabolites such as peptaibols.

In this study, we isolated and characterized a micro-heterogeneous peptide mixture belonging to the group of peptaibiotics from the endophyte strain (S25) of *T. citrinoviride*, a species for which production of antifungal metabolites does not appear to have been described previously.

As is commonly known, peptaibols are linear amphipathic polypeptides characterized by the presence of a high proportion of non-coded amino acids (such as α -aminoisobutyric acid and isovaline), the protection of N- and C-termini by an acetyl group and the reduction of the C-terminus to 2-aminoalcohols (Szekeres *et al.*, 2005). To date, more than 300 of these metabolites have been described and presented in the peptaibol database (<http://www.cryst.bbk.ac.uk/peptaibol/home.shtml>), and many of them occur as subfamilies that display a micro-heterogeneity based on small differences in their amino acid sequences at specific positions in the molecule (Chugh & Wallace, 2001).

We also found a high micro-heterogeneity in the peptaibol mixture produced by *T. citrinoviride*, composed of closely related sequence analogues differing from each other by amino acid substitutions only at positions 6, 9, 12 and 17. Furthermore, we obtained experimental evidence that each characterized peptide can exist as a non-covalent homo-

Table 2. Effects of peptide mixture on radial mycelial growth of seven oak-pathogenic fungi

Concn ($\mu\text{g ml}^{-1}$)	Mycelial growth (mm)*						
	<i>Apiognomonia quercina</i>	<i>Botryosphaeria corticola</i>	<i>Biscogniauxia mediterranea</i>	<i>Botryosphaeria parva</i>	<i>Botryosphaeria obtusa</i>	<i>Diplodia pinea</i>	<i>Diplodia scrobiculata</i>
Control	56.7 $\pm$ 0.5	69.2 $\pm$ 0.5	72.0 $\pm$ 0.9	62.7 $\pm$ 0.4	90.0 $\pm$ 0.8	90.0 $\pm$ 0.8	56.5 $\pm$ 0.3
MeOH	56.5 $\pm$ 0.2	69.2 $\pm$ 0.8	73.8 $\pm$ 0.9	65.7 $\pm$ 1.2	85.2 $\pm$ 0.3	85.5 $\pm$ 0.3	49.5 $\pm$ 0.4
200	10.2 $\pm$ 0.3 (82.0)	6.5 $\pm$ 0.0 (90.6)	9.0 $\pm$ 0.1 (87.8)	0.0 $\pm$ 0.0 (100.0)	0.0 $\pm$ 0.0 (100.0)	0.0 $\pm$ 0.0 (100.0)	0.0 $\pm$ 0.0 (100.0)
100	13.3 $\pm$ 0.3 (76.5)	7.0 $\pm$ 0.0 (89.9)	10.3 $\pm$ 0.2 (86.0)	0.0 $\pm$ 0.0 (100.0)	0.0 $\pm$ 0.0 (100.0)	0.0 $\pm$ 0.0 (100.0)	0.0 $\pm$ 0.0 (100.0)
50	31.4 $\pm$ 0.2 (44.4)	12.0 $\pm$ 0.3 (82.7)	16.8 $\pm$ 0.1 (77.2)	7.0 $\pm$ 0.0 (89.3)	0.0 $\pm$ 0.0 (100.0)	11.8 $\pm$ 0.3 (86.3)	0.0 $\pm$ 0.0 (100.0)
10	10.2 $\pm$ 0.3 (27.3)	24.5 $\pm$ 0.3 (64.6)	62.7 $\pm$ 0.1 (15.0)	14.0 $\pm$ 0.7 (78.7)	27.3 $\pm$ 0.8 (67.9)	46.5 $\pm$ 0.4 (45.6)	21.0 $\pm$ 0.3 (57.6)

*Values represent the means $\pm$ SE of three replicates. Values in parentheses indicate growth inhibition (%). All treatment data are statistically different from controls at the 5 % level by Duncan's multiple range test.

Fig. 5. Mortality response of *Artemia salina* larvae exposed to increasing concentrations of peptide mixture for 36 h. Percentage mortalities were corrected for the natural mortality following Abbot's formula. The values are the means $\pm$ SE of three independent experiments, each with four replicates.

and hetero-dimer. The question is: are these dimeric species representative of species naturally present in solution or are they artefacts arising from the experimental conditions? This point, while intriguing, did not represent the objective of this work, and other experiments should be carried out to answer it. Peptaibols exhibit a wide spectrum of biological activities including antibacterial, antifungal and antiviral effects (Szekeres *et al.*, 2005; Daniel & Filho, 2007), induction of systemic plant-defence responses (Viterbo *et al.*, 2007), tissue damage in insect larvae, and cytolytic activity toward mammalian cells (Peltola *et al.*, 2004; Leitgeb *et al.*, 2007).

All the pathogens tested in this study were found to be highly sensitive to the peptide mixture. Among the seven fungal species tested, appreciable differences in antifungal activity were not found at higher concentrations (200, 100 and 50 $\mu\text{g ml}^{-1}$), but significant differences in activity were seen at a lower concentration (10 $\mu\text{g ml}^{-1}$). These results indicate that the peptide mixture is responsible for the antifungal activity of the organic extract, and may be involved in the antagonistic relationship of *T. citrinoviride* towards some important oak pathogens such as *Apiognomonia quercina*, *Biscogniauxia mediterranea* and *Botryosphaeria corticola*.

The potential toxicity of the purified peptide mixture was evaluated *in vitro* using the *Artemia salina* short-term bioassay. The toxicity of the sample in this model (LC_{50} 1.24 $\mu\text{g ml}^{-1}$) was approximately 10 times higher than that reported for paracelsins by other authors (Favilla *et al.*, 2006). We surmise that this difference might be due to the dimerization of the peptides or to synergistic interactions between the different peptide species present in the

mixture. A number of studies on various antimicrobial ribosomal and non-ribosomal peptides have revealed that dimerization leads to the appearance of a more diverse spectrum of antimicrobial activity than is exhibited by monomers (Tencza *et al.*, 1999; Schibli *et al.*, 2002; Dempsey *et al.*, 2003; Lee *et al.*, 2008). In many cases dimerization was closely connected to enhanced antimicrobial activity mediated by the formation of pores or channels in the lipid membranes. For example, in the case of channel-forming peptides such as alamethicin, channels formed by synthesized covalent dimers displayed lifespans at a particular conductance that were up to 170-fold longer than those of monomers (You *et al.*, 1996).

According to Degenkolb *et al.* (2008), the toxicity reported by Favilla *et al.* (2006) could be due to the contamination of the standard samples by trichothecene-type mycotoxins (especially harzianum A). Our strain of *T. citrinoviride* did not produce trichothecenes on different media (both liquid and solid) that we tested (unpublished data). Thus, what is the real significance of this potential toxic effect? Is it theoretical or real? On the other hand, the brine shrimp lethality assay is considered a very useful tool in testing plant extract bioactivity, which often correlates reasonably well with cytotoxic and antitumour properties (McLaughlin *et al.*, 1993). For example, a positive correlation was observed between brine shrimp lethality and cytotoxicity towards 9KB (human nasopharyngeal carcinoma) cells. On the basis of the results of the present study, the peptide mixture could be evaluated in more specific antitumour systems.

Further work should aim at examining whether the peptide mixture is produced *in planta* and what role it could play in the complex interaction between endophyte and host. Since high quantities of this peptide mixture were extracted from culture *in vitro*, it is possible that its production could occur effectively in natural conditions. In this regard, our isolate of *T. citrinoviride* grown in sterilized soil was shown to produce a similar peptide mixture, which was active against several soil-related pathogens (unpublished data).

In conclusion, these results suggest that the endophytic fungi living in healthy tissues of oak species represent an important source of novel bioactive natural products and biocontrol agents, which may have useful applications in agriculture and forestry.

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