Short research communication

Putative neuromycotoxicoses in an adult male following ingestion of moldy walnuts

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Abstract

A tremorgenic syndrome occurs in dogs following ingestion of moldy walnuts and *Penicillium crustosum* has been implicated as the offending fungus. This is the first report of suspected moldy walnut toxicosis in man. An adult male ingested approximately 8 fungal-infected walnut kernels and after 12 hours experienced tremors, generalized pain, incoordination, confusion, anxiety and diaphoresis. Following symptomatic and supportive treatment at a local hospital the man made an uneventful recovery. A batch of walnuts (approximately 20) were submitted for mycological culturing and identification as well as for mycotoxin analysis. *Penicillium crustosum* Thom was the most abundant fungus present on walnut samples, often occurring as monocultures on isolation plates. Identifications were confirmed with DNA sequences. The kernels and shells of the moldy walnuts as well as *P. crustosum* isolates plated on Yeast Extract Sucrose (YES) and Czapek Yeast Autolysate (CYA) agars and incubated in the dark at 25 ºC for 7 days were screened for tremorgenic mycotoxins and known *P. crustosum* metabolites using a liquid chromatography-tandem mass spectrometric (LC-MS/MS) method. A relatively low penitrem A concentration of only 1.9 ng/g was detected on the walnut kernels when compared to roquefortine C concentrations of 21.7 µg/g. A similar result was obtained from *P. crustosum* isolates cultured on YES and CYA, with penitrem A concentrations much lower (0.6–6.4 µg per g mycelium/agar) compared to roquefortine C concentrations (172–1225 µg/g). The authors surmised that besides penitrem A, roquefortine C might also play an additive or synergistic role in intoxication of man.

Keywords Moldy walnuts, *Penicillium crustosum***,** Penitrem, Roquefortine, Tremorgenic mycotoxins

Introduction

Moldy walnut intoxication has been described in dogs and *Penicillium crustosum* has been implicated as the offending fungus (Munday et al. 2008; Richard et al. 1981). In addition, *P. crustosum* is a ubiquitous spoilage organism and various incidences of poisoning in dogs have been reported after ingesting food waste such as rice, apples and cream cheese (Eriksen et al. 2010; Naudé et al. 2002; Young et al. 2003). However, there is a dearth of information on *P. crustosum* poisoning in humans. An elderly couple ingested soup for lunch, prepared from a damaged can, and both were hospitalized after experiencing a sudden onset of generalized muscle tremors (Lewis et al. 2005). Subsequently *P. crustosum* was cultured from the damaged can and the authors surmised that the clinical signs were due to penitrem A. Another case described intoxication in a 44-year-old man 4 hours after consuming beer contaminated by "*P. crustosum"*, with only roquefortine subsequently detected from isolates (Cole et al. 1983). However, the strain (IBT 11188) was later re-identified as *P. carneum* (Frisvad and Samson 2004).

Penicillium crustosum synthesizes various mycotoxins such as penitrem A (Fig. 4) through to G and roquefortine C (Fig. 4) (De Jesus et al. 1983; Eriksen et al. 2010; Frisvad and Samson 2004; Frisvad et al. 2004; Moldes-Anaya et al. 2012). Penitrem A is a tremorgenic mycotoxin produced by at least *Penicillium* species classified in sections *Chrysogena* (*P. flavigenum*), *Fasciculata* (*P. crustosum, P. melanoconidium, P. tulipae*), *Penicillium* (*P. clavigerum*), *Robsamsonia* (*P. glandicola*) and *Roquefortorum* (*P. carneum*) (Frisvad et al. 2004). In dogs, receiving lethal intraperitoneal doses of 0.5 mg penitrem A/kg or higher, tremors were noticeable as early as 10 min after dosing (Hayes et al. 1976). Tremors occurred in mice after oral administration of 0.25-0.5 mg penitrem A/kg (Ciegler et al. 1976; Moldes-Anaya et al. 2012). An intraperitoneal LD_{50} of 1.05 mg penitrem A/kg for mice has been reported (Ciegler et al. 1976). Under acidic conditions penitrem A can be

converted to thomitrem A, which also induces tremors in mice, albeit at oral doses 16 times higher (i.e. 8 mg/kg) (Moldes-Anaya et al. 2012; Rundberget et al. 2004).

Roquefortine C is produced by at least 27 *Penicillium* subgenus *Penicillium* species. Roquefortine C is reported to be less toxic than penitrem A. An LD_{50} for roquefortine C after intraperitoneal administration to two mouse strains ranged from 169 – 189 mg/kg (Arnold et al. 1978). Furthermore, Bünger et al. (2004) also concluded that in spite of blue cheeses, containing roquefortine C (produced by *P. roqueforti* used as starter culture), they are seemingly safe for humans and that the lack of toxicity of roquefortine C in man can be ascribed to its low bioavailability.

The mechanism of action of these tremorgenic mycotoxins on the central nervous system is not yet clear, but penitrem A enhances the release of both excitatory (such as glutamate) and inhibitory (gamma-aminobutyric acid or GABA) neurotransmitters (Norris et al. 1980) and binds to the GABA receptor ($GABA_A$-R) and modulates its function (Moldes-Anaya et al. 2011). In addition, penitrem A also inhibits high-conductance Ca^{2+}-activated K^+ channels *in vitro*, although this interaction may be unrelated to the tremorgenic properties (Knaus et al.1994).

The objective of this study was to investigate if a tremorgenic syndrome reported in a Caucasian male could have been due to ingestion of moldy walnuts contaminated by *P. crustosum*.

Materials and methods

Case report

A 54-year-old Caucasian man was shelling a pile of walnuts (*Juglans regia*), harvested from a tree in the garden with some of the walnuts visibly moldy. Nevertheless, he wiped the fungal growth off and ingested approximately 8 kernels. Roughly 12 hours following

ingestion he woke and experienced intense arthralgia of the knee and elbow joints. He attempted to stand, but was unable to do so and while sitting experienced and described the following symptoms: tremors, generalized pain, inability to control limbs, confusion, anxiety and perspiration. The man was taken to the emergency clinic of a local hospital. There was no history of exposure to pesticides, drugs of abuse or any other pre-existing disease condition. The patient was hypertensive (172/101 mm Hg) and both heart and respiratory rates were marginally increased. No electrocardiographic (ECG) aberrations were detected. Serum electrolytes and haematology parameters were within reference ranges (RR), although the blood oxygen saturation was slightly low (pO_2 5.6 kPa; RR = 11 – 15 kPa and O_2 saturation 78%; RR 95-99%). The patient was placed on an intravenous electrolyte infusion (lactated Ringers solution) and received diazepam, paracetamol and paracoxib intravenously. The clinical signs resolved rapidly and after monitoring for a few hours he was discharged. Based on the history of ingestion of moldy walnuts and the neurological clinical signs a provisional diagnosis of a tremorgenic neuromycotoxicosis was made. The remaining moldy walnuts (approximately 20) were collected (Fig. 1) and sent for fungal isolation and identification as well as for mycotoxin analysis.

Fig. 1 Moldy walnuts (*Juglans regia*) from which an almost pure culture of *Penicillium crustosum* was isolated

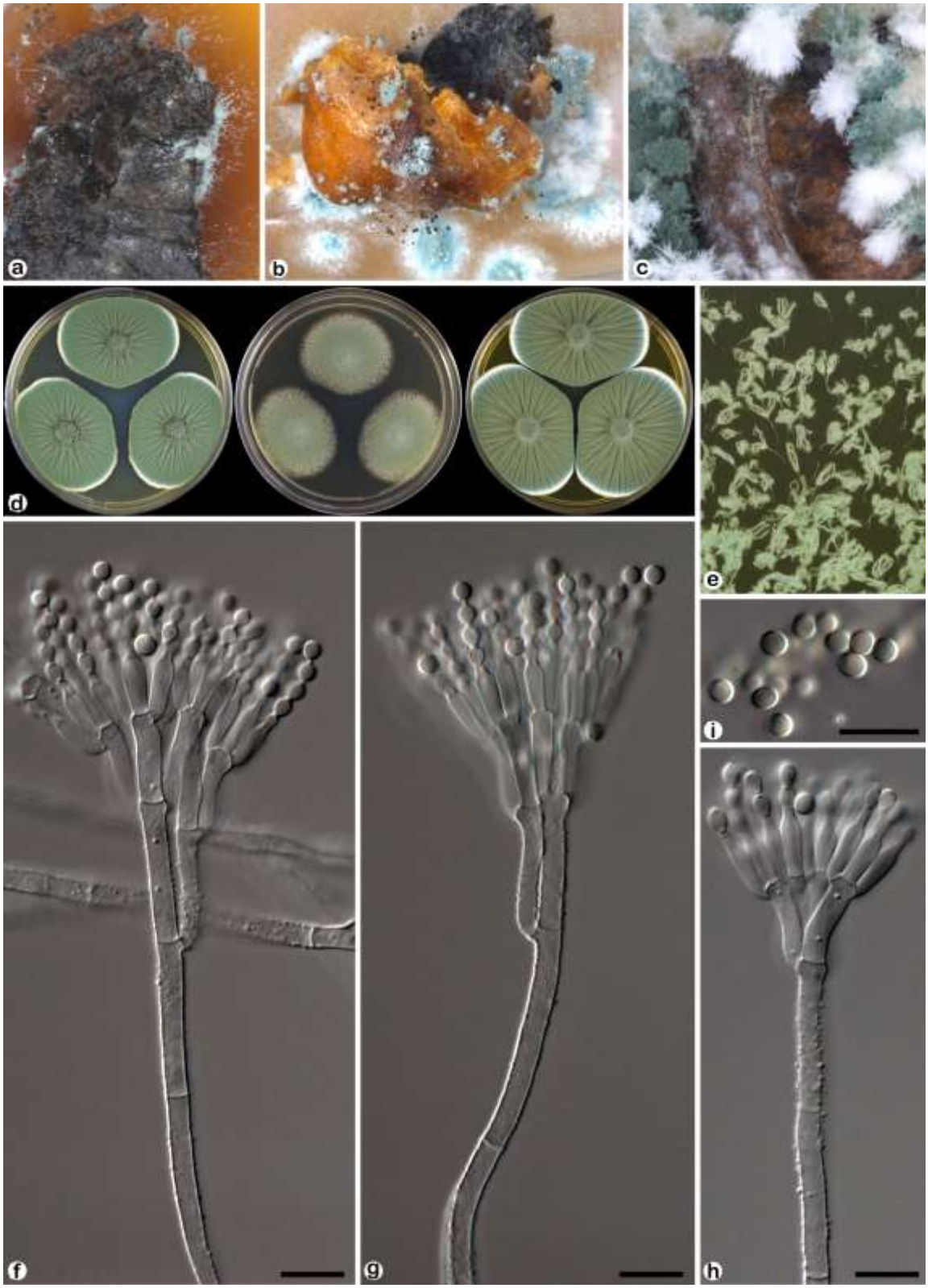


Fig. 2 *Penicillium crustosum*. **a-c** Walnuts incubated for 7–14 days showing fungal growth of mostly *Penicillium*, but also a black *Aspergillus* sect. *Nigri* species, **d** colony morphology after 7 days at 25 ºC, from left to right, CYA, MEA and YES, **e** colony texture on MEA, **f-h** conidiophores and **i** conidia. Scale bars = 10 µm

Fungal isolation and identification

Fungal isolations were made from walnuts by plating kernels and shells onto potato dextrose agar (PDA) amended with 100 ppm chloramphenicol. Plates were inspected for fungal growth after incubation for 7–14 days at 25 ºC (Fig. 2 A-C) using a stereo-microscope. Subsequent isolations, purifications and sub-culturing were done on Blakeslee's (1915) Malt Extract Agar (MEA; malt by Bacto™).

Recovered strains were identified using morphological characterization and subsequent sequencing using the recommended methods of Visagie et al. (2014). In summary, strains were inoculated onto Czapek Yeast Autolysate agar (CYA), MEA and Yeast Extract Sucrose agar (YES), incubated in the dark for 7 days at 25 ºC and characterized using colony and micromorphological characters. For microscopy, mounts were prepared from 7–10 days MEA colonies using lactic acid as mounting fluid, with 90% ethanol used to wash away excess conidia. Characters were captured using a Zeiss AXIO Imager.M2 compound and Zeiss AXIO Zoom.V16 dissecting microscopes equipped with respectively AxioCaM MRc and MRc5 cameras, and Zen Blue v 2.3 software. The photoplate (Fig. 2) was prepared in Affinity Photo v 1.6.6 (Serif Europe Ltd, UK).

For sequencing, DNA was extracted from 10 day old MEA colonies using the Quick-DNA™ Fungal/Bacterial Miniprep kit (Zymo Research, cat. no. D6005, CA, USA). Polymerase Chain Reactions of the partial beta-tubulin gene region (*BenA*) were set up using OneTaq® 2X Master Mix with GC buffer (New England Biolabs Inc., cat. no. M0483S, MA, USA) and primers Bt2a and Bt2b (Glass and Donaldson 1995) using the PCR protocol recommended in Visagie et al. (2014). PCR products were sent to Inqaba Biotec (Pretoria, South Africa) for subsequent PCR-clean-up and sequencing. Sequence contigs were prepared in Geneious v R8.1. BLAST searches against a locally curated database of *Penicillium* were made to make a preliminary identification. A subset of sequences from section *Fasciculata* was subsequently aligned in MAFFT v 7.305b (Katoh and Standley 2013) and alignments manually adjusted in Geneious where needed. A Maximum Likelihood

analysis was done in IQtree v 1.6 (Nguyen et al. 2015) after selecting the most suitable substitution model with the Modelfinder (Kalyaanamoorthy et al 2017) algorithm built into the software. The analysis was executed using the following command: “qtree -s Fasciculata_BenA.nex -pre IQtree_Fasciculata_BenA -nt AUTO -m MFP -bb 1000 –bnni”. The tree was visualized in Figtree v 1.4.3 ((http://tree.bio.ed.ac.uk/software/figtree) and edited for publication in Affinity Designer v 1.6.1 (Serif Europe Ltd, UK).

Mycotoxin analysis

From the batch of moldy walnuts collected, 9 walnuts were shelled and the kernels (32.18 g) and shells (29.77 g) were milled and analysed. The kernels and shells were extracted separately. Five g of each were extracted using 20 ml of acetonitrile/water/acetic acid 79/20/1(v/v/v), followed by a dilution of 1+1 and injection of 5 µl of the diluted extract. Results were corrected for apparent recovery obtained during full method validation for walnuts that proved to be a more difficult matrix in view of matrix effects compared to almonds and pistachio nuts (manuscript in preparation).

Plugs of both agars (0.2 - 0.6 g) containing fungal growth were extracted using acetonitrile/water/acetic acid 79/20/1 (v/v/v). A QTrap 5500 liquid chromatography-mass spectrometry (LC-MS/MS) system (Applied Biosystems, Foster City, CA, USA) equipped with a TurboIon Spray electrospray ionization (ESI) source and a 1290 Series high performance liquid chromatography (HPLC) system (Agilent, Waldbronn, Germany). Chromatographic separation was performed at 25 ºC on a Gemini C_{18}-column, 150x4.6 mm i.d., 5 µm particle size, equipped with a C_{18} 4x3 mm i.d. security guard cartridge (all from Phenomenex, Torrance, CA, USA).

The chromatographic method as well as chromatographic and mass spectrometric parameters are described by Malachova et al. (2014), but the method has in the meantime been expanded to cover more than 650 metabolites (manuscript in preparation). Electrospray

ionization-tandem mass spectrometry (ESI-MS/MS) was performed in the time-scheduled multiple reaction monitoring (MRM) mode both in positive and negative polarities in two separate chromatographic runs per sample by scanning two fragmentation reactions per analyte. The MRM detection window of each analyte was set to its expected retention time ± 27 sec and ± 48 sec in the positive and the negative mode, respectively. Confirmation of positive analyte identification was obtained by the acquisition of two MRMs per analyte, which yielded 4.0 identification points according to commission decision 2002/657/EC. In addition, the liquid chromatography (LC) retention time and the intensity ratio of the two MRM transition agreed with the related values of an authentic standard within 0.1 min and 30 % rel., respectively.

Quantification was performed via external calibration using serial dilutions of a multi-analyte stock solution. Results were corrected for apparent recoveries obtained upon method validation for walnuts (manuscript in preparation). The accuracy of the method is verified on a continuous basis by regular participation in proficiency testing schemes (Malachova et al. 2014, 2015).

The commercial sources of the standards were: Sigma-Aldrich (Vienna, Austria) for penitrem A; Romer Labs (Tulln, Austria) for paxilline; Iris Biotech GmbH (Marktredwitz, Germany) for roquefortine C and Enzo Life Sciences (Lausen, Switzerland) for roquefortine E. Line 151 LC-MS/MS parameters for desoxypaxilline were determined from an extract containing large amounts of paxilline.

Results

Fungal isolation and identification

After 7–14 days incubation, walnut kernels and shells were almost exclusively colonized by a *Penicillium* species (Fig. 2a-c). However, single colonies belonging to *Aspergillus* sect. *Nigri*, later identified as *A. tubingensis*, were also observed on two kernels (Fig. 2a,b). Thirteen

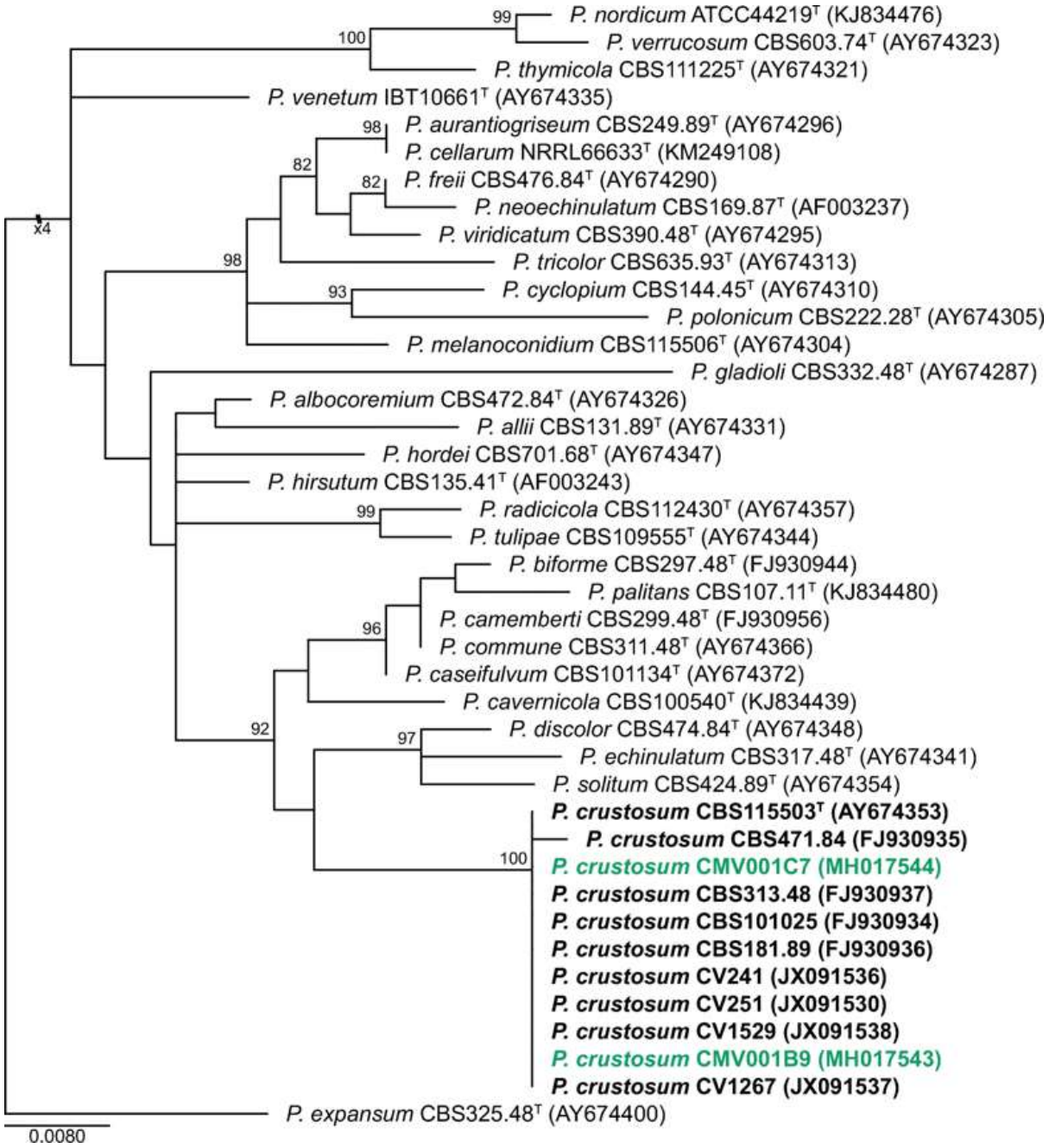
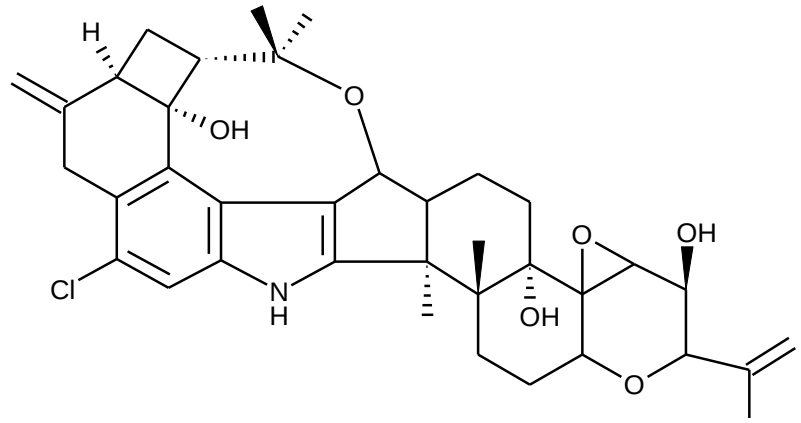


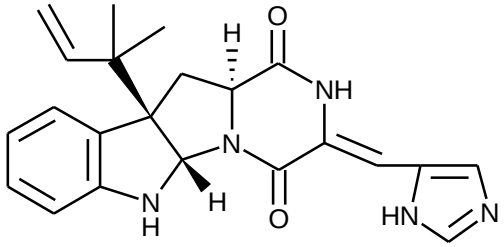
Fig. 3 Maximum likelihood tree of *Penicillium* section *Fasciculata*. Strains from walnuts (bold, green text) resolved in a clade with *P. crustosum* reference sequences (bold text). Bootstrap values higher than 80% are shown above relevant branches. Ex-type strains are indicated by ^{T}. GenBank accession numbers are provided between brackets. The tree was rooted to *P. expansum*

isolates were subsequently purified and plated onto CYA, MEA and YES. Strains shared similar colony and microscopic features resembling those of *Penicillium crustosum* Thom.,

including smooth walled conidia, roughened stipes, and crustose colonies on CYA and MEA. BLAST searches of the *BenA* gene sequences obtained from two representative strains placed them in section *Fasciculata* with 100% matches to the ex-type sequence of *P. crustosum*. The maximum likelihood analysis resolved newly isolated strains in a clade with the *P. crustosum* ex-type and additional reference strains (Fig. 3).



Penitrem A



Roquefortine C

Fig. 4 The chemical structures of the common tremorgenic mycotoxins detected after screening of the mouldy walnut kernels and shells as well as the *P. crustosum* isolates with liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Mycotoxin analysis

The common metabolites synthesized by *P. crustosum*, of which the concentrations could be quantified, are listed in Table 1. A relatively low penitrem A concentration of 1.9 ng/g was

Table 1 Commonly occurring *Penicillium crustosum* metabolites and estimated median concentrations (ng/g) detected in milled walnut (n = 9) kernels and shells and synthesized by isolates (n = 4) plated on Yeast Extract Sucrose agar (YES) and Czapek Yeast Autolysate agar (CYA) and incubated in the dark at 25 ºC for 7 days. The isolates were deposited in the National Collection of Fungi, Biosystematics Division, Plant Protection Research Institute, Agricultural Research Council (ARC-PPRI), Pretoria, South Africa. LOD = limit of detection

Samples		Penitrem A	Roquefortine C	Roquefortine D	Paxilline
Walnut kernels		1.9	21700	<LOD	<LOD
Walnut shells		3.1	16300	<LOD	<LOD
	PPRI isolate no.				
YES agar	C1	3820	1012800	2610	7430
	C5	1300	530500	480	7890
	C7	560	222200	280	3820
	B9	680	171500	500	1540
Median		990	376350	490	5625
Standard deviation		1521.6	386431	1099.5	3027.9
CYA agar	C1	5670	1116800	5880	9970
	C5	5500	1224800	1400	19980
	C7	5000	1145600	1060	19510
	B9	6380	1205600	12710	9380
Median		5585	1175600	3640	14740
Standard deviation		571	50514	5429.1	5822.1

Penitrem A (LOD = 0.5 ng/g)
Roquefortine C (LOD = 10 ng/g)
Roquefortine D (LOD = 0.6 ng/g)
Paxilline (LOD = 12 ng/g)

detected on the walnut kernels as compared to roquefortine C concentrations of 21.7 µg/g (Table 1). The same trend was observed with respect to the synthesis of these two metabolites by *P. crustosum* isolates grown on YES and CYA agars. Penitrem A concentrations ranged from 0.6 – 6.4 µg per g mycelium/agar, whereas roquefortine C concentrations were much higher, ranging from 172 – 1225 µg/g. No paxilline nor roquefortine D was detected on the walnut shells or kernels and the concentrations that were synthesized by *P. crustosum* isolates on YES and CYA were also much lower (Table 1). Desoxypaxilline was also synthesized by the isolates growing on the agars, but quantification was not possible due to a lack of a standard, and none was detected on the walnut kernels or shells.

Discussion

This is the first report of suspected penitrem A and roquefortine C poisoning in man following ingestion of moldy walnuts. The signs of intoxication were similar to the two cases reported in literature i.e. generalised weakness and tremor, incoordination, anxiety and diaphoresis (profuse sweating) (Cole et al. 1983; Lewis et al. 2005). In the current case the onset of clinical signs developed after approximately 12 hours and the recovery was fairly quick compared to the case of the elderly couple who experienced severe muscle tremors after an hour and only recovered the next day following hospitalization (Lewis et al. 2005). In the man that consumed *P. carneum*-contaminated beer, the first clinical signs were noticed after 4 hours and at 12 hours his handwriting was illegible due to severe tremor, but all the clinical signs abated after 30 hours.

Relatively high concentrations of roquefortine C were detected on the walnut kernels and shells and synthesized by the *P. crustosum* isolates (Table 1). Rundberget et al. (2004) reported penitrem A and roquefortine C concentrations of 6.5 and 3.3 µg/g synthesized by a Norwegian *P. crustosum* isolate (1590P5) and 5.8 and 4.8 µg/g synthesized by a South

African *P. crustosum* isolate (PPRI 6859), respectively, when grown on rice at 25 °C for 1 week. In the current experiment, the *P. crustosum* isolated from walnuts and incubated on CYA at 25 °C for 1 week synthesized a similar quantity penitrem A (median of 5.6 µg/g agar), but much higher roquefortine C concentrations (median of 1175.6 µg/g agar) (Table 1). On the other hand, the South African isolate from this study incubated on YES at 25 °C for 1 week synthesized much lower penitrem A (median of 1.0 µg/g agar) and roquefortine C (median of 376.3 µg/g agar) concentrations. Although other penitrems have also been detected in moldy food waste which resulted in intoxication in dogs (Eriksen et al. 2010) as well as in cheeses, especially those containing nuts, and intended for human consumption in Europe (Kalinina et al. 2018), no quantitative reference standards were available and the mycotoxin analysis did not include these potential tremorgens. Other tremorgenic mycotoxins i.e. paxilline and desoxypaxilline were synthesized by the isolates, but they were not detected on the walnut kernels or shells.

In light of Cole et al. (1983) detecting only roquefortine from the *P. carneum* (reported as *P. crustosum*) isolated from the beer that poisoned a man (the beer was not available for analysis); the occurrence of Norwegian *P. crustosum* strains that did not synthesize penitrem A (Rundberget et al. 2004) as well as considerably higher roquefortine C concentrations detected in the walnut kernels in the current case (21.7 µg/g), the question is raised if roquefortine C might have a synergistic or additive effect with the highly toxic penitrem A in intoxication of man. Other authors have also speculated on a possible synergistic action of these two toxins (Braselton and Rumler 1996; Naudé et al. 2002). Even though roquefortine was suggested as a cause of poisoning in dogs (Puls and Ladyman 1988; Lowes et al. 1992), the current scientific opinion is that penitrem A was probably always present although it could not be detected in some of the samples previously analysed (Naudé et al. 2002; Tiwary et al. 2009).

Penicillium crustosum was the most abundant fungus found on walnut samples, often occurring as monocultures on isolation plates, but single colonies of *A. tubingensis* were

visible on a few kernels (Fig 2a,b). In a study where *A. tubingensis*, among other Aspergilli, was isolated from raw cashew nuts, no known potential tremorgenic mycotoxin was detected following ultra-high-performance liquid chromatography-mass spectrometer (UHPLC-QTOF-MS) analysis and data processing using huge databases (Lamboni et al. 2016).

A definitive diagnosis of penitrem A and roquefortine C poisoning in man will depend on detecting these mycotoxins in serum and/or urine of the patient, unfortunately no biological fluids were available to analyse in this case. This case highlights the potential effects of fungal-infected foodstuffs on human health.

Conflicts of Interest

None.

Acknowledgements

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