International Journal of Food Microbiology 282 (2018) 66–70

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Penicillium gravinicasei, a new species isolated from cave cheese in Apulia, Italy

Pamela Anelli[a], Steve W. Peterson[b], Miriam Haidukowski[a], Antonio F. Logrieco[a], Antonio Moretti[a], Filomena Epifani[a], Antonia Susca[a,*]

[a] Institute of Sciences of Food Production, National Research Council (CNR), Via Amendola 122/O, 70126 Bari, Italy
[b] Mycotoxin Prevention and Applied Microbiology Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, 1815 North University Street, Peoria, IL 61604, USA

ARTICLE INFO

Keywords:
Penicillium
Cheese
Cave

ABSTRACT

Several species of the genus *Penicillium* were isolated during a survey of the mycobiota of Apulian cave cheeses ripened in a cave in Gravina di Puglia, Italy. A novel species, *Penicillium gravinicasei*, is described in *Penicillium* section *Cinnamopurpurea*. Its taxonomic novelty was determined using a polyphasic approach, combining phenotypic, molecular (β-tubulin, calmodulin, ITS and DNA dependent RNA polymerase) DNA sequences and mycotoxin production data. Phylogenetic analyses of the *RPB2* data showed that isolates of the novel species form a clade most closely related to *Penicillium cinnamopurpureum* and *P. parvulum* with high bootstrap support. The fungus did not produce ochratoxin A, citrinin, patulin, sterigmatocystin or aflatoxin B_1 on standard agar media. The novel species had a high growth rate on agar media supplemented with 5% NaCl, and could be distinguished from other *Penicillium* section *Cinnamopurpurea* species by phenotypic and molecular characteristics.

1. Introduction

Numerous microfungi belonging to the genus *Penicillium* were isolated during a survey of the mycobiota of Apulian cave cheeses. These cheeses are traditional Italian semi-hard cheese, widespread in the Apulian area, made from raw milk of sheep or cow and aged in natural cellars or tufa caves, where they are spontaneously colonized by an autochthonous mycobiota, mainly belonging to the genera *Aspergillus* and *Penicillium*. In these cheeses, the mycobiota plays an important role during ripening, conferring particular organoleptic characteristics to the cheese and representing “added value” to the food product.

Among the penicillia recovered from a cheese bought in an artisan cheese factory in Gravina di Puglia was one not assignable to any described species. The taxonomic and phylogenetic position of the fungal isolates was investigated utilizing β-tubulin, calmodulin, ITS and RPB2 DNA sequences. The phenotype, phylogenetic position and mycotoxigenic potential are described here for the new species *Penicillium gravinicasei*.

2. Material and methods

2.1. Strains

The strains included in the study were retrieved from ITEM (Agri-Food Toxigenic Fungi Culture Collection, Bari, Italy) and are also deposited in the Agricultural Research Service Culture Collection (NRRL), Peoria, IL USA.

2.2. Morphology

For morphological observations we followed Samson and Frisvad (2004) and Visagie et al. (2014). Malt extract and yeast extract were purchased from Difco. Culture were grown on Czapek's agar (CZA), Czapek yeast autolysate agar (CYA), Blakeslee's malt extract (MEA), CYA with 5% NaCl (CYAS), yeast extract agar (YES), oat meal agar (OA) and creatine agar with bromcresol purple (CREA), prepared using Difco malt extract according to the formulations given by Samson et al. (2014). Cultures were incubated at 25 ± 0.5 °C and on CYA at 37 °C, for 7 days. Macro photography was conducted using a Nikon D7100 digital camera. Photographs were optimized for contrast, resized and fitted into composite plates using Photoshop Elements 10.

* Corresponding author.
E-mail address: antonella.susca@ispa.cnr.it (A. Susca).

https://doi.org/10.1016/j.ijfoodmicro.2018.06.006
Received 8 February 2018; Received in revised form 30 May 2018; Accepted 8 June 2018
Available online 08 June 2018
0168-1605/

2.3. Molecular studies: DNA extraction, PCR and sequencing and phylogenetic analysis

DNA was isolated from Petri plate cultures using an Ultraclean microbial DNA isolation kit (MoBio, Carlsbad, CA, USA). The loci β-tubulin (*benA*), calmodulin (*CaM*), ITS1–5.8S-ITS2 (ITS) and DNA dependent RNA polymerase beta second largest subunit (*RPB2*) were amplified using primers and protocols listed previously (Peterson, 2008). Loci were bidirectionally sequenced using the amplification primers. Sequences were viewed and corrected using SEQUENCHER 5.1 (Gene Code Corporation, Ann Arbor, MI, USA). Completed sequences were aligned using MAFFT 7.217 (Katoh and Standley, 2013), and analyzed with IQTREE 1.3.11.1 (Hoang et al., 2017; Nguyen et al., 2015). Sequences were deposited in GenBank or the European Nucleotide Archive (ENA).

2.4. Secondary metabolites analysis

Ethanol and mycotoxin standards (purity > 99%) ochratoxin A (OTA), patulin (PAT), aflatoxin B_1 (AfB_1), citrinin (CIT), sterigmatocystin (STC) were obtained from Sigma-Aldrich (Milan, Italy). RC 0.2 μm filter (regenerated cellulose membranes) was obtained from Phenomenex (Phenomenex, USA). Mycotoxins stock were reconstituted with appropriate solutions: OTA with acetonitrile/water/acetic acid (99:99:2, *v/v/v*), PAT with water/acetonitrile (85:15, v/v), AFB_1, with water/methanol (60:40, *v*/v), STC with water/acetonitrile (75:25, v/v), CIT with acetonitrile/water/acetic acid (99:99:2, v/v/v). Appropriate ranges of standard solutions were then obtained: 50–100 ng/mL(OTA), 80–800 ng/mL (PAT), 0.4–10.0 ng/mL (AFB1), 500–5000 ng/mL (STC), and 100–2000 ng/mL(CIT).One gram of agar with fungal culture was extract with 5 mL of acetonitrile/methanol/water (90:90:80, *v/v/v*) for OTA, PAT and CIT, and with 5 mL of methanol/water (80:20, v/v) for AFB_1, and STC, on orbital shaker for 60 min. Residues were dissolved in 1 mL of appropriate solutions and filtered using RC 0.20 μm regenerated cellulose filter: acetonitrile/water/glacial acetic acid (99:99:2, *v/v/v*) for OTA and CIT, water/acetonitrile (85:15, v/v) for PAT, water for AFB_1, water/acetonitrile (75:25, v/v) for STC. Fifty microliter of extracts was injected into to HPLC apparatus (Agilent 1260 Series, Agilent Technology, Santa Clara, CA, USA). The quantification of mycotoxins were performed by HPLC/FLD according to Susca et al. (2016) for OTA, Sewram et al. (2000) for PAT, Fani et al. (2014) for AFB_1, Veršilovskis et al. (2008) for STC and Lee et al. (2006) for CIT. The detection limits, based on a signal to noise ratio of 3:1, were as following: 2 μg/kg (OTA), 5 μg/kg (PAT), 0.2 μg/kg (AFB1), 4 μg/kg (STC), 5 μg/kg (CIT).

3. Results

3.1. Isolates

A set of 5 similar strains isolated from the surface of a cave cheese sample could not be unambiguously identified using morphological observations and therefore was subjected to a more detailed taxonomic study. These isolates were compared with species belonging to *Penicillium* section *Cinnamopurpurea*. The strains are permanently preserved in culture collections as cultures ITEM 17409–ITEM 17412 and NRRL as cultures NRRL 66730–NRRL 66734.

3.2. Taxonomy

Penicillium gravinicasei S.W. Peterson, P. Anelli, M. Haidukowski, & A. Susca sp. nov. Fig. 1 Mycobank number MB 823510.

Typification: Italy, Gravina di Puglia (province Bari), isolated from "Pallone di Gravina" cave-cheese on 31 Aug 2016 by Filomena Epifani, holotype = BPI 910534. Ex type cultures are ITEM 17411, deposited in the ITEM culture collection, Institute of Sciences of Food Production, National Council of Researches (CNR), Bari, Italy = NRRL 66733. ITS barcode = MG600580, alternative markers *benA* = MG600656, *CaM* = MG600570, *RPB2* = MG600575.

Etymology: The species is named for the geographic locale Gravina di Puglia and the substrate cheese.

Descriptions are prepared from colonies grown for 7d at 25 °C, except where noted.

CZA colonies attain 10–12 mm diam., thin and low, margin entire and on the surface, moderate sporulation, centrally slight aerial growth, no exudate, aerial hyphae hyaline, submerged hyphae are darkly pigmented in a purple-brown color, deep slate violet (R-44), colony reverse dark slate violet centrally to slate violet peripherally (R-44). **CYA** colonies attaining 17–18 mm diam., rising 2–3 mm centrally, moderate sporulation, hyphae hyaline, exudate plentiful, yellow droplets covering entire colony giving a yellow color, margin sunken, reverse raisin black (R-44), conidia gnaphalium green (R-47). **MEA** colonies attaining 14–16 mm diam., velutinous, prolific sporulation, conidia *en mass* medici blue (R-48), margin ca 2 mm submerged growth of hyphae, no exudate, reverse olive-buff (R-40). **YES** colonies attaining 20–21 mm diam., margin sunk in agar, buckled rising 3–4 mm from agar, prolific sporulation in light celandine (R-47), concentric grooves, no exudate, reverse Mikado brown to bister brown (R-29). **CYAS** colonies attaining 18 mm diam., velutinous to mealy, central 1/3 of colony raised 1–2 mm, prolific sporulation in light celandine green (R-47), no exudate, reverse raisin brown (R-44) centrally to buff near edges. **OA** colonies attaining 11–12 mm diam., velutinous, low, margin entire, heavy yellow exudate, sporulation sparse in celandine green (R-47). **CREA** attaining 8–9 mm diam., velutinous, good sporulation in celandine green (R-47), no acid production. On CYA at **37 °C** no growth. No soluble pigment, sclerotia or ascomata were produced on these media.

Conidiophores monoverticillate, (10)60–80(130) × 2–3 μm with occasional subapical branching, enlarging apically and forming a vesicle 4–5 μm diam., supporting a whorl of 2–5 ampulliform *phialides* 6–8 × 2.0–2.5 μm, producing smooth, globose to subglobose conidia, 2.0–2.5 μm diam. Conidia form short disordered chains.

Additional isolates examined. Italy, Gravina di Puglia isolated from cave-cheese, *Filomena Epifani*, Aug 2016, NRRL 66730, NRRL 66731, NRRL 66732 and NRRL 66734.

The phenotypic characters were consistent with placement in *Penicillium* section *Cinnamopurpurea*. *Penicillium gravinicasei* can be distinguished from other species in *P.* section *Cinnamopurpurea* on the basis of growth rate on various media and conidium size and shape. Growth of *Penicillium* sect. *Cinnamopurpurea* species is generally slow. Among the species that grow > 14 mm in 7 d are *P. infrapurpurea* and *P. gravinicasei*. *Penicillium gravinicasei* grows 17–18 mm on CYA, 11–12 mm on OA, 18 mm on CYAS and 8–9 mm on CREA after 7 d, while *P. infrapurpureum* grows 14–17 mm on CYA, 17–22 mm on OA, 16–18 mm on CYAS and 4–5 mm on CREA (Visagie et al., 2014). The conidia of *P. gravinicasei* are spherical 2–2.5 μm diam., while *P. infrapurpureum* has ellipsoidal conidia 2.5–3.5 (−5.5) × 2.5–3.5 μm.

3.2.1. Sequencing and phylogenetic analysis

The sequences were from the type and other strains of *Penicillium gravinicasei* were deposited in GenBank (MG600562–MG600581). Additional sequences from species of *Penicillium* sect. *Cinnamopurpurea* were downloaded from GenBank. Single and combined locus maximum likelihood analyses were conducted (see Supplemental Data). *Penicillium gravinicasei* is placed, with < 50% bootstrap support, as a basal taxon in those trees, leaving uncertainty about its placement. In the single locus *RPB2* tree (Fig. 2), *P. gravinicasei* is included in a clade that also contains *P. parvulum* and *P. cinnamopurpureum*. This clade has 92% bootstrap support so we can be fairly certain that *P. gravinicasei* belongs in *P.* section *Cinnamopurpurea*. *Penicillium infrapurpureum* was not included in the tree because no *RPB2* sequences for it were available in GenBank but as can be seen in the supplemental data it is closely related to *P. cvjetkovicii* with high bootstrap support.