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Several species of *Penicillium* isolated from chestnut flour processing are pathogenic on fresh chestnuts and produce mycotoxins

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ABSTRACT

A collection of 124 isolates of *Penicillium* spp. was created by monitoring fresh chestnuts, dried chestnuts, chestnut granulates, chestnut flour and indoor chestnut mills. Sequencing of the ITS region, β-tubulin and calmodulin, macro-morphology and secondary metabolite production made it possible to determine 20 species of *Penicillium*. *P. bialowiezense* was dominant in the fresh chestnuts, while *P. crustosum* was more frequent in the other sources. A pathogenicity test on chestnut showed that around 70% of the isolates were virulent. *P. corylophilum* and *P. yezoense* were not pathogenic, while the other 18 species had at least one virulent isolate. *P. expansum* and *P. crustosum* were the most virulent. The isolates were characterized to establish their ability to produce 14 toxic metabolites *in vivo*: 59% were able to produce at least one mycotoxin. *P. expansum* was able to produce patulin, chaetoglobosin A and roquefortine, while *P. bialowiezense* produced C. Mycophenolic acid. Cyclopenins and viridicatins were produced by most of the *P. crustosum*, *P. polonicum, P. solitum* and *P. discolour* isolates. Some of the *P. crustosum* isolates were also able to produce roquefortine C or penitrem A. Information about the occurrence of *Penicillium* spp. and their mycotoxins will help producers to set up management procedures that can help to control the fungal growth and the mycotoxin production of chestnuts.

1. Introduction

The chestnut tree is the most popular nut-bearing tree in several European and Asian countries, with new productions in the United States, Australia, New Zealand and Chile. Italy is the second sweet chestnut (*Castanea sativa* Mill.) producer in Europe, with 52,000 tons, and a cultivated area of 21,500 ha in 2014 (FAOSTAT, 2014; Livre Blanc Châtaigne, 2012). The industrial preparation of chestnut flour, dried chestnuts and marron glacés represents 20% of the total production. Chestnuts can be contaminated by moulds before their harvest, but also during transportation, storage and processing. Fungal spoilage can be responsible for significant economic losses. Moreover, a number of fungi that have been isolated from chestnuts are well-known mycotoxin producers. The occurrence of toxigenic *Fusarium* spp., *Penicillium* spp. and *Aspergillus* spp., as well as their associated mycotoxin contaminations on chestnuts and derived commercial products, have been reported in different countries (Abdel-Gawad and Zohri, 1993; Bertuzzi et al., 2015; Jermini et al., 2006; Overy et al., 2003; Pietri et al., 2012; Prencipe et al., 2018; Rodrigues et al., 2013; Wells and Payne, 1975).

Penicillium spp. cause various decays on fruit (Washington et al., 1997), and act as a contaminant in the post-harvest phase and indoor (Nielsen, 2003). The most frequent *Penicillium* species reported in nuts, with the exception of *P. nordicum,* belong to the series *Camemberti* and *Solita* (Frisvad and Samson, 2004). A few papers have reported *Penicillium* species isolated from chestnuts. Overy et al. (2003) stated that *P. crustosum, P. glabrum*-clade and *P. discolor* were the dominant species in fresh chestnuts. Sieber et al. (2007) isolated *P. expansum* and *P. crustosum* in particular, while Donis-González et al. (2016) stated that *P. expansum, P. griseofulvum* and *P. chrysogenum* were the main species. *Penicillium* spp. (about 10^4 CFU/g) have also been reported on dried chestnuts and chestnut flour (Pietri et al., 2012).

Mycotoxins are secondary metabolites that are produced by moulds, which show toxic, mutagenic and teratogenic effects, including potential immunosuppressive activity and carcinogenic effects (Milicevic et al., 2010). Because of their long-term chronic or cumulative effects on human health, maximum levels have been established for some of these molecules in foodstuffs in Europe. The occurrence of ochratoxin A (OTA), penitrem A (PenA), chaetoglobosin A (ChA) and C,

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deoxynivalenol and zearalenone in fresh chestnuts has been reported by Donis-Gonzalez et al. (2009) and by Overy et al. (2003). Pietri et al. (2012) and Bertuzzi et al. (2015) reported the presence of aflatoxins (AFs), OTA, citrinin, roquefortine C (RoqC) and mycophenolic acid (MPA) in industrial chestnut products. Limits have only been established for AFs in chestnuts, and they are specified by Commission Regulation (EU) No. 165/2010. Furthermore, several studies have reported the production of different secondary metabolites, such as mycotoxins, alkaloids, antibiotics and allergens, by *Penicillium*, with negative effects on human health (Barkai-Golan, 2008). The levels of patulin (PAT) and OTA, which are produced by *Penicillium* spp., are currently regulated for certain foodstuffs, but not for chestnuts.

The *Penicillium* species are generally identified through a polyphasic approach, in which the traditional identification methods, i.e. micro and macro-morphological analyses (colony diameter and colour, growth rate, texture of conidia), are combined with molecular and secondary metabolite analysis (Visagie et al., 2014a).

The information available about the occurrence of *Penicillium* spp. and their food-borne mycotoxins is still incomplete, thus underlining the need to set up chestnut management procedures from the orchard to the commercial product, since these species could represent a serious human health risk and cause significant economic losses.

A monitoring process was carried out on fresh chestnuts from orchards, on dried chestnuts, chestnut granulates and chestnut flour, collected during processing and during indoor monitoring inside chestnut mills. This study was aimed at determining the species of *Penicillium* through molecular and macromorphological analyses. The isolates were also characterized to establish their virulence on chestnuts and their ability to produce 14 toxic metabolites *in vivo*.

2. Material and methods

2.1. Fungal strains and sampling

One hundred and twenty-four strains of *Penicillium* spp. were isolated during 2015 from different sources: i) fresh chestnuts from three chestnut orchards; ii) samples of dried chestnuts, chestnut granulates and chestnut flour, collected during the processing of chestnuts from three countries; iii) an indoor monitoring inside the considered production mills. The sampling of fresh chestnuts was conducted on fruit harvested in orchards located in three villages (Ormea, Perlo and Viola) in Piedmont, in the north-west of Italy, with five replicates (50 chestnuts per orchard). Sampling was conducted during processing for each chestnut processing phase (dried chestnuts, chestnut granulates and chestnut flour) from three different countries (Parenti, Calabria, Italy; Tropoje, Scutari, Albania; Ourense, Galicia, Spain) with three replicates (60 g per processing phase and country). The surfaces of the fresh chestnuts, dried chestnuts and chestnut granulates were disinfected with 1% sodium hypochlorite, and the chestnuts were then washed in sterile deionized water and air dried, as described by Rodrigues et al. (2012). Fresh chestnuts (four fragments per chestnut) were then plated onto Potato Dextrose Agar (PDA, Merck, Germany). Fungi were recovered from the dried chestnuts, chestnut granulates and chestnut flour using the dilution plate technique. In short, 20 g of each sample was homogenized in distilled water for 5 min at 300 rpm, using a stomacher, and three homogenates were taken and plated, after serial dilution, onto PDA in triplicate. Instead, 20 Rose Bengal Chloramphenicol agar (Fluka, Germany) Petri dishes were placed, as spore traps, in the processing mill areas for 24 h for the indoor sampling. Fungal growth was observed after 3 to 7 days of incubation at 26 °C, and representative colonies from each morphotype and source were re-isolated and maintained as monosporic cultures in Yeast Extract Sucrose Agar tubes (Visagie et al., 2014a) for identification, pathogenicity tests and chemical analyses. All the isolates are listed in Table 1.

Table 1
Strain name, source of isolation, molecular identification, virulence results and secondary metabolite production of the *Penicillium* spp. strains isolated in this study.

Strain	Source	Species	Virulence	Secondary metabolites
Cas26	I	*P. chrysogenum*	HV	n.d.
Cas11	I	*P. bialowiezense*	NP	–
Cas31	C	*P. bialowiezense*	NP	–
Cas30	C	*P. bialowiezense*	HV	n.d.
Cas29	I	*P. bialowiezense*	MV	n.d.
Cas15	C	*P. bialowiezense*	NP	–
Cas25	C	*P. bialowiezense*	HV	n.d.
E4	C	*P. bialowiezense*	MV	n.d.
E5	C	*P. bialowiezense*	SV	n.d.
B1	C	*P. bialowiezense*	HV	MPA
C1	C	*P. bialowiezense*	MV	MPA
C5	C	*P. bialowiezense*	MV	MPA
DIV1	C	*P. bialowiezense*	NP	–
Cas18	I	*P. brevicompactum*	HV	n.d.
CalB	PP	*P. brevicompactum*	NP	–
3C	I	*P. glandicola*	MV	MEL, AndA
4.3	I	*P. glandicola*	NP	–
X7	PP	*P. glandicola*	NP	–
X10	PP	*P. glandicola*	NP	–
3D	I	*P. expansum*	HV	PAT, RoqC
PACT	C	*P. expansum*	HV	PAT
PCAS	C	*P. expansum*	HV	PAT
POX1	C	*P. expansum*	MV	PAT, ChA
POX2	C	*P. expansum*	HV	PAT, ChA
X5	PP	*P. expansum*	HV	PAT, ChA, RoqC
PF1	F	*P. expansum*	HV	PAT, ChA, RoqC
4A	C	*P. crustosum*	HV	CPN, CPL, VIR, VOL, PenA
Cas12	I	*P. crustosum*	HV	CPN, CPL, VIR, VOL, RoqC
Cas14	I	*P. crustosum*	HV	CPN, CPL, VIR, VOL, RoqC, PenA
Cas28	I	*P. crustosum*	MV	CPN, CPL
Cas34	I	*P. crustosum*	HV	CPN, CPL, VIR, VOL
Cas17	I	*P. crustosum*	NP	–
3.3	I	*P. crustosum*	HV	n.d.
5A	C	*P. crustosum*	MV	CPN, CPL, VIR, VOL, RoqC, PenA
3.2	I	*P. crustosum*	SV	CPN, CPL, VIR, VOL, PenA
Cal51	F	*P. crustosum*	SV	CPN, CPL, VIR, VOL, RoqC, PenA
Cal52	F	*P. crustosum*	–	–
Cal53	F	*P. crustosum*	–	–
Cal54	F	*P. crustosum*	–	–
Cal55	F	*P. crustosum*	–	–
Cal56	F	*P. crustosum*	–	–
Cal57	F	*P. crustosum*	–	–
Cal58	F	*P. crustosum*	–	–
Cal59	F	*P. crustosum*	–	–
Cal60	F	*P. crustosum*	–	–
Cal61	F	*P. crustosum*	–	–
Cal62	F	*P. crustosum*	–	–
Cal63	F	*P. crustosum*	–	–
Cal64	F	*P. crustosum*	HV	CPN, CPL, VIR, VOL, RoqC, PenA
Cal65	F	*P. crustosum*	–	–
Cal69	F	*P. crustosum*	–	–
Cal70	F	*P. crustosum*	–	–
Cal5f	F	*P. crustosum*	–	–
Cal6f	F	*P. crustosum*	–	–
Cal7f	F	*P. crustosum*	–	–
Cal9f	F	*P. crustosum*	–	–
Cal12f	F	*P. crustosum*	HV	CPN, CPL, VIR, VOL, RoqC, PenA
X1	PP	*P. crustosum*	HV	CPN, CPL, VIR, VOL, RoqC, PenA
X4	PP	*P. crustosum*	HV	CPN, CPL, VIR, VOL, RoqC, PenA
XF1	F	*P. crustosum*	-	–
XF2	F	*P. crustosum*	-	–
PLX1	PP	*P. crustosum*	-	–
PLX2	PP	*P. crustosum*	-	–
PLX3	PP	*P. crustosum*	-	–
PLX4	PP	*P. crustosum*	-	–

(*continued on next page*)