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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 the chestnut mills. Sequencing of ITS region, β-tubulin and calmodulin, macro-morphology and secondary metabolite production permitted to determine 20 species of *Penicillium*. In fresh chestnuts, *P. bialowiezense* was dominant, while *P. crustosum* was more frequent in the other sources. Pathogenicity test on chestnut showed that around 70% 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 for 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 C. Mycophenolic acid was produced by *P. bialowiezense*. Cyclopenins and viridicatins were produced by most *P. crustosum*, *P. polonicum*, *P. solitum* and *P. discolor*. Some isolates of *P. crustosum* were also able to produce roquefortine C or penitrem A. Information about the occurrence of *Penicillium* spp. and their mycotoxins will help to set up chestnut management procedures, to control the fungal growth and the mycotoxin production.

Keywords: Penicillium crustosum, mycotoxins, *Castanea sativa*, indoor, pathogenicity, polyphasic identification.

1. Introduction

Chestnut is the most popular nut-bearing tree in several European and Asian countries, with new productions in 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 marrons glacés represents 20% of the total production. Moulds contaminate chestnut before harvest, but also during transportation, storage and processing. Fungal spoilage can be responsible for significant economic losses. Moreover, a number of fungi isolated from chestnuts are well-known mycotoxin producers. The occurrence of toxigenic *Fusarium* spp., *Penicillium* spp. and *Aspergillus* spp. and 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. are ubiquitously causing decay on fruit (Washington et al., 1997), as well as contaminant in post-harvest and indoor (Nielsen, 2003). The most frequent *Penicillium* species reported in nuts belong to the series *Camemberti* and *Solita*, except for *P. nordicum* (Frisvad and Samson, 2004). A few papers reported the species of *Penicillium* 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) predominantly isolated *P. expansum* and *P. crustosum*, while Donis-Gonzales and collegues (2016) stated that *P. expansum*, *P. griseofulvum* and *P. chrysogenum* were the main species. *Penicillium* spp. (about 10^4 CFU/g) have been reported also on dried chestnuts and chestnut flour (Pietri et al., 2012).

Mycotoxins are secondary metabolites 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 in foodstuffs have been established for some of these molecules in Europe. The

57 occurrence of ochratoxin A (OTA), penitrem A (PenA), chaetoglobosin A (ChA) and C,
58 deoxynivalenol and zearalenone in fresh chestnuts has been reported by Donis-Gonzales et al. (2009)
59 and Overy et al. (2003). Pietri et al. (2012) and Bertuzzi et al. (2015) reported the presence of
60 aflatoxins (AFs), OTA, citrinin, roquefortine C (RoqC) and mycophenolic acid (MPA) in industrial
61 chestnut products. For chestnuts, limits have been established only for AFs, and they are specified by
62 Commission Regulation (EU) No. 165/2010. Furthermore, several studies have reported the
63 production by *Penicillium* species of different secondary metabolites, such as mycotoxins, alkaloids,
64 antibiotics and allergens, with negative effects on human health (Barkai-Golan, 2008). Patulin (PAT)
65 and OTA levels, which are produced by *Penicillium* spp., are regulated on certain foodstuffs, but not
66 on chestnuts.
67 *Penicillium* species are identified through a polyphasic approach, where the traditional methods of
68 identification, i.e. micro and macro-morphological analyses (colony diameter and colour, growth rate,
69 texture of conidia), are combined with molecular and secondary metabolite analysis (Visagie et al.,
70 2014).
71 Information about the occurrence of *Penicillium* spp. and their food-borne mycotoxins is incomplete,
72 underlining the need to set up chestnut management procedures from the orchard to the commercial
73 product, since these species could represent a serious human health risk and cause significant
74 economic losses.
75 A monitoring was carried out on fresh chestnuts from orchards, on dried chestnuts, chestnut
76 granulates and chestnut flour, taken during processing, and during an indoor monitoring inside the
77 chestnut mills. This study aimed to determine the species of *Penicillium* through molecular and
78 macromorphological analyses. The isolates were also characterized for their virulence on chestnuts
79 and for their ability to produce 14 toxic metabolites *in vivo.*
80
81 **2. Material and methods**
82

83 *2.1 Fungal strains and sampling*

84 One hundred and twenty-four strains of *Penicillium* spp. were isolated during 2015 from different
85 sources: i) fresh chestnuts from three chestnut orchards; ii) samples of dried chestnuts, chestnut
86 granulates and chestnut flour, taken during processing of chestnuts from three countries; iii)
87 moreover, an indoor monitoring was performed inside the production mills. Sampling on fresh
88 chestnuts was conducted on fruits harvested in orchards located in three villages (Ormea, Perlo and
89 Viola) located in Piedmont, north-west Italy, with five replicates (50 chestnuts per orchard). Sampling
90 during processing was conducted on each chestnut processing phase (dried chestnuts, chestnut
91 granulates and chestnut flour) from three different countries (Parenti, Calabria, Italy; Tropoje, Scutari,
92 Albania; Ourense, Galicia, Spain) with three replicates (60 g per processing phase and country). The
93 surfaces of fresh chestnuts, dried chestnuts, and chestnut granulates were disinfected with 1% sodium
94 hypochlorite, washed in sterile deionized water and air dried, as described by Rodrigues et al. (2012).
95 Fresh chestnuts (four fragments per chestnut) were then plated onto Potato Dextrose Agar (PDA,
96 Merck, Germany). Fungi were recovered from dried chestnuts, chestnut granulates and chestnut flour
97 using dilution plate technique. Briefly, 20 g each sample, were homogenized in distilled water for 5
98 min at 300 rpm by using a stomacher, and three homogenates were taken and plated, after serial
99 dilution, onto PDA in triplicate. For indoor sampling, 20 Rose Bengal Chloramphenicol agar (Fluka,
100 Germany) Petri dishes were placed, as spore traps, in the processing mills areas for 24 h. Fungal
101 growth was observed after 3 to 7 days of incubation at 26 °C, and representative colonies from each
102 morphotype and source were re-isolated and maintained as monosporic cultures in Yeast Extract
103 Sucrose Agar tubes (Visagie et al., 2014) for identification, pathogenicity tests and chemical analyses.
104 All the isolates are listed in Table 1.
105
106 *2.2 Molecular identification*

107 Genomic DNA was extracted from representative monosporic isolates grown for 7 days in Czapek-
108 Dox broth (Fluka) at 26 °C in the dark using Omega E.Z.N.A Fungal DNA Mini Kit (VWR, USA),

according to the manufacturer's instructions. Species were assigned by analysing the internal transcribed spacers of the rDNA region (ITS) using ITS1 and ITS4 primers (White et al. 1990), the β-tubulin gene (*BenA*) using Bt2a and Bt2b primers (Glass and Donaldson, 1995), and the calmodulin gene (*CaM*) using CMD5 and CMD6 primers (Hong et al., 2006). PCR was carried out in a total volume of 25 µL which contained: 2 µL buffer 10x, 0.8 µL $MgCl_2$, 1 µL dNTPs (10 mM), 1 µL each primer (10 mM), 0.2 µL of Taq Platinum Pfx DNA polymerase (Invitrogen, USA) and 40 ng of template DNA. Thermal cycling programs were performed according to Visagie et al. (2014). The PCR products were run on a 1% agarose gel with 1 µL GelRed™ (VWR) at 100V/cm for 30 minutes, and compared with a positive control, that is the *P. griseofulvum* strain PG3 from the Agroinnova collection (Banani et al., 2016). Get Pilot Wide range Ladder (Qiagen, Germany) was used as molecular marker. The amplified DNA fragments were purified for both genes using QIAquick© PCR purification Kit (Qiagen) and sequenced in both directions by Macrogen, Inc. (The Netherlands). The consensus sequences were assembled using DNA Baser program (Heracle Biosoft S.R.L., Romania). After cutting the trimmed regions and manual correction, a dataset of 465 bp for the ITS region, 369 bp for the β-tubulin gene and 400 bp for the calmodulin gene was obtained. All the obtained sequences were compared with those deposited in the reference database using the BLAST program, including verified RefSeq sequence in public datasets from the National Centre for Biotechnology Information (NCBI) to identify the isolates. Furthermore the sequences were compared to references accession number of the current accepted *Penicillium* species reported by the International Commission of *Penicillium* and *Aspergillus* (ICPA). Representative sequences of each species were deposited in GenBank with the accession numbers listed in Table 2.

Additionally, the consensus sequences were aligned using CLUSTALW through Molecular Evolutionary Genetics Analysis (MEGA6) software, version 6.0, and concatenated by using Sequence Matrix Species identifier version 1.8. For phylogeny the best fit model was determined using MEGA, based on the lowest Bayesian Information Criterion (BIC) by using the 3 genes combined dataset. The phylogenetic tree was built through MEGA using the Maximum Likelihood

(ML) methods using the K2+G model with 1,000 bootstrap replicates. The Bio-Neighbour-Joining (BioNJ) option, and the heuristic search with the Nearest-Neighbour-Interchange (NNI) options were used to calculate the initial tree for the ML analyses. All the reference sequences from ICPA used for phylogeny are reported in Suppl. Table 1.

2.3 Macro-morphology characterization

A macro-morphological identification of all the isolates was performed according to Visagie et al. (2014). The isolates were 3-point inoculated with a spore suspension on Yeast Extract Sucrose Agar (YES agar), Czapek Yeast Autolysate agar (CYA) and Malt extract agar (MEA), and they were observed for growth (mean diameter) and colony characters (colony texture, texture and colour of mycelium, colony reverse colours, presence and colour of the soluble pigments and exudates) after 7 days of incubation at 25 °C in the dark. The colour standards and nomenclature by Robert Ridgway (1912) were used for morphological description.

2.4 Pathogenicity assay

In order to evaluate the pathogenicity of the *Penicillium* spp. isolates on chestnuts, the nut surface was disinfected with a 10% solution of sodium hypochlorite, washed by immersion in sterile deionized water and air dried. The nuts were wounded (3 injuries of 1 cm each) and inoculated with a spore suspension of each isolate (1×10^5 cfu/mL), except for the species *P. crustosum*, as 16 isolates were selected out of 40 strains. The chestnuts were kept in 15 cm boxes for 7 days at 26±1 °C in the dark. Control chestnuts were prepared with sterile deionized water. The symptoms were observed, and a disease index (D.I) was calculated on a scale ranging from 0 to 100: non-pathogenic (NP) = no symptoms; slightly virulent (SV) = 1-30% infected area; moderately virulent (MV) = 31-50% infected area; highly virulent (HV) = 51-100% infected area. White mycelial growth, which turned into different shades of green and blue, depending on the inoculated species, was observed on symptomatic chestnuts, as a result of conidia production. Dried pulp and chalky tissues were observed

in symptomatic nuts. No symptoms were observed in the controls. The experiment was performed with six replicates for each strain. The pathogenicity assay was performed twice.

2.5 Mycotoxin production on chestnuts

Penicillium metabolites were extracted from the chestnuts used in the pathogenicity tests for 70 isolates, with at least one isolate per species (Table 2). The chestnuts were divided into three subsamples for each isolate and extracted twice by means of solid-liquid extraction with 10 mL of ethyl acetate. The samples were shaken for 1 min and the organic phases were then collected in a flask. The extract was evaporated to dryness in a rotary evaporator at 35 °C. The residue was dissolved in 500 µL of H_2O:CH_3CN 1:1 for HPLC-DAD analysis.

The HPLC apparatus was an Agilent 1100 series equipped with a G1379 degasser, a G1313A autosampler, a G1316A column thermostat set at 30 °C, a G1315B UV diode array detector set at 230, 276, 300 and 330 nm, a G1311 quaternary pump and an Agilent Chemstation G2170AA Windows XP operating system (Agilent®, Germany). A Luna C18 analytical column (150x4.6 mm i.d., 3 µm, Phenomenex®, USA), preceded by a guard column (4x3mm i.d.) with the same stationary phase, was used for the HPLC procedure. The mobile phases consisted of water acidified with formic acid 0.05 % (A) and acetonitrile (B), at a flow rate of 0.800 mL/min in gradient mode, 0-5 min: 5% of B, 5-45 min: from 5 to 50% B, 45-60 min: from 50 to 80% B, 60-70 min: from 80 to 100% B. Twenty µL of the samples was injected. UV spectra were collected, by means of DAD, every 0.4 s, from 190 to 700 nm, with a resolution of 2 nm. Authentic mycophenolic acid (MPA), meleagrine (MEL), andrastin A (AndA), roquefortine C (RoqC), patulin (PAT), chaetoglobosin A (ChA), cyclopenin (CPN), cyclopenol (CPL), viridicatin (VIR), viridicatol (VOL), penitrem A (PenA), cyclopiazonic acid (CPA), verrucosidin (VER) and penicillic acid (PA) standards were used for the identification by comparing their retention times with the UV spectra.

3. Results

187

188 *3.1 Molecular identification*

189 One hundred and twenty-four isolates of *Penicillium* spp. were collected: 29 from fresh chestnuts in
190 orchards, 32 during processing of chestnuts (dried chestnuts and chestnut granulates), 41 from flour
191 and 22 from indoor sampling inside the production mills (Table 2). A total of 20 species, divided into
192 2 subgenera and 8 sections, were identified according to the classification reported by Houbraken and
193 Samson (2011), Visagie et al. (2014) and Houbraken et al. (2016). For every species, one sequence
194 of the ITS region, one of β-tubulin gene and one of calmodulin gene were deposited in GenBank with
195 the accession numbers listed in Table 2.

196 The ITS region was able to identify the isolates at species level only for 6 out of 20 species (Suppl.
197 Table 2). Seventeen out of 20 species were confirmed through the analysis of the β-tubulin gene
198 (Suppl. Table 3). Uncertain identification was obtained for the following isolates belonging to three
199 series: Cas10, Cas40, Cal3F and CalC (series *Camemberti)*; 3B1, 3B4, 3B5, 3B6, F3A, Cas13, Cas9
200 and 3B30 (series *Camemberti* – ex series *Solita* (Frisvad and Samson, 2004)); XA, XC, XO and XP
201 (series *Viridicata*).

202 The calmodulin gene assigned univocal results for one out of three uncertain identifications: the
203 isolates of *P. viridicatum* (XA, XC, XO and XP) (Suppl. Table 4). The isolates associated to series
204 *Camemberti* and series *Camemberti* – ex series *Solita* (Frisvad and Samson, 2004) remained
205 ambiguous, with 100% homology with *P.commune, P. camemberti, P. caseifulvum* and *P. palitans*
206 for series *Camemberti*, and *P. solitum* and *P. discolor* for series *Camemberti* – ex series *Solita*
207 (Frisvad and Samson, 2004) (Suppl. Table 4).

208 Moreover, the phylogenetic analysis based on the 3 concatenated genes, and performed in order to
209 classified the strains, confirmed the results obtained by BLAST search, by giving univocal
210 identification for eighteen species out of twenty (Fig.1). For each identified species the strains
211 clustered together with reference strains with high bootstrap values (>80%), with the exception of the
212 strains belonging to series *Camemberti* and series *Camemberti* – ex serie *Solita* (Fig. 1 and Suppl.

213 Fig. 1). In particular, one group clustered with *P. caseifulvum*, *P. commune* and *P. camemberti*
214 (bootstrap 98), whilea second group clustered with *P. solitum* and *P. discolor* (bootstrap 97) (Suppl.
215 Fig. 1).
216 Further macro-morphological identification and secondary metabolite production, together with
217 molecular analysis, permitted to determine the species.
218 By considering the isolation sources, isolates from fresh chestnuts were found to belong to eight
219 species (Table 2): *Penicillium* sp. (series *Camemberti)*, *P. crustosum*, *P. expansum*, *P. glabrum*, *P.*
220 *manginii*, *P. pancosmium* and *P. yezoense*, with *P. bialowiezense* as the dominant species (10/30).
221 Twelve species were found during chestnuts processing (dried chestnuts and chestnut granulates): *P.*
222 *brevicompactum*, *P. citrinum*, *Penicillium* sp. (series *Camemberti*), *P. expansum*, *P. glabrum*, *P.*
223 *glandicola*, *P. palitans*, *P. polonicum*, *P. solitum*, *Penicillium viridicatum* and *P. yezoense*, with *P.*
224 *crustosum* as the dominant species (11/32).
225 The isolates from chestnut flour belonged to 9 species (Table 2): *Penicillium* sp. (series *Camemberti*),
226 *P. corylophilum*, *Penicillium* sp. (series *Camemberti* – ex series *Solita* (Frisvad and Samson, 2004)),
227 *P. expansum*, *P. glabrum*, *P. polonicum*, *P. verrucosum* and the majority of isolates (24/41) were *P.*
228 *crustosum.*
229 Indoor sampling was mainly represented by *Penicillium* sp. (series *Camemberti* – ex series *Solita*
230 (Frisvad and Samson 2004)), *P. bialowiezense*, *P. brevicompactum*, *P. chrysogenum*, *P. expansum*,
231 *P. glandicola* and *P. polonicum*, with *P. crustosum* as dominant species (14/22).
232
233 *3.2 Morphological identification*
234 After molecular identification, the strains were examined to establish their macro-morphological
235 characteristics in order to confirm the species on the basis of phenotypic criteria.
236 The isolates showed uniform characteristics, with typical morphology and growth rate on the analysed
237 media, similar to those reported in literature (Table 3, Fig. 2). Members of the *P. glabrum*-clade

showed variability in shade and intensity of the reverse colours on CYA and YES media, and the colours ranged from pale to vivid orange or to a pinkish colour.

The morphology of Cas10, Cas40, Cal3F and CalC strains belonging to *Penicillium* series *Camemberti* permitted the assignation to the species *P. commune*, with a velutinous to fasciculate texture, cream colour to beige reverse on CYA and cream colour to yellow reverse on YES (Table 3, Fig. 2). These isolates showed visible differences from *P. palitans* (Fig. 2).

The 3B1, 3B4, 3B5, 3B6, F3A, Cas13, Cas9 and 3B30 strains belonging to *Penicillium* series *Camemberti* – ex series *Solita* (Frisvad and Samson, 2004*)* were identified as *P. discolor,* with a velutinous to fasciculate texture, and were cream yellow reverse on CYA, bright strong yellow reverse on YES, and deep primuline yellow colour on MEA (Table 3, Fig.2). These strains were morphologically different from *P. solitum* (Fig. 2).

3.3 *Pathogenicity*

According to the observed symptoms (Figure 2), the pathogenicity test divided the strains into four categories (Table 4): 35 % were highly virulent (HV), 22% moderately virulent (MV), 15% slightly virulent (SV), and 28% non-pathogenic (NP). The symptoms typical of each isolated species are shown in Figure 2. The results of the 96 analysed strains are reported in Table 1. Glabrum pancosmium solitum

The non-pathogenic strains included all the isolates of *P. corylophilum* and *P. yezoense,* and 3 out of 4 isolates of *P. glandicola. P. bialowiezense, P. brevicompactum, P. crustosum, P.glabrum, P. pancosmium* and *P. palitans* included both non-pathogenic and pathogenic strains. On the contrary, all the strains of *P. citrinum P. commune, P. chrysogenum P. discolor, P. expansum, P. manginii P. nordicum, P. polonicum, P. solitum, P. verrucosum* and *P. viridicatum* were pathogenic.

By considering the most frequently isolated species, *P. bialowiezense* strains were non-pathogenic (5/12), moderately virulent (4/12) or highly virulent (3/12). All the strains belonging to the *P. crustosum* species were pathogenic (3 SV, 2 MV, 8 HV), except for one (Cas17). *P. glabrum* strains

were predominantly non-pathogenic (11/15), except for two SV (E2 and B3), one MV (E3) and one HV (E7). The *P. expansum* strains were all highly virulent, except for one MV (POX1).

3.4 *Mycotoxin production on chestnuts*

The mycotoxins detected on the inoculated chestnuts are reported in Table 1. The produced metabolites differed based on the *Penicillium* species. All the *P. expansum* isolates were able to produce PAT; four isolates (POX1, POX2, X5 and PF1) produced ChA and 3D, X5 and PF1 produced RoqC. Cyclopenins (CPN and CPL) and viridicatins (VIR and VOL) were produced by most of the isolates belonging to *P. crustosum*, *P. polonicum*, *P. solitum* and *P. discolor*. Some isolates of *P. crustosum* were also able to produce RoqC and/or PenA. The isolates of *P. discolour* were also able to produce ChA, while the isolates of *P. polonicum* could also produce VER. MPA was produced by three *P. bialowiezense* isolates. One isolate of *P. viridicatum* was producer of PA, while CPA was produced by two strains of *P. commune*. *P. glandicola* was able to produce MEL and AndA. None of the analysed metabolites was detected for any of the isolates of *P. chrysogenum*, *P. brevicompactum*, *P. palitans*, *P. verrucosum*, *P. nordicum*, *P. glabrum*, *P. pancosmium*, *P. manginii* or *P. citrinum*.

4. Discussion

Fungal contamination of chestnuts was monitored from harvest to storage in previous studies (Overy et al., 2003; Rodrigues et al., 2012). Some authors reported the presence of *Penicillium* spp. in fresh chestnuts or in commercial products (Wells and Payne, 1975; Rodrigues et al., 2013), while Bertuzzi et al. (2015) focused on the occurrence of some Penicillium-toxins. The occurrence of different species of *Penicillium* and their mycotoxin production in chestnuts was investigated for the first time in this work. *Penicillium* were isolated from different sources, from chestnut orchard and throughout the flour processing phases, including the indoor environment of chestnut processing. One hundred and twenty-four *Penicillium* isolates were collected from fresh chestnuts, dried chestnuts, chestnut granulates, chestnut flour and indoor sampling inside the production mills. The isolates were then

290 characterized through biological, molecular and chemical tools, focusing on the pathogenicity on
291 chestnuts and on the potential production of mycotoxins, noxious to human health.
292 As reported by Visagie and colleagues (2014), the identification of the species requires a
293 multidisciplinary approach that considers the morphological characteristics, and which includes
294 molecular analyses in combination with secondary metabolite production in order to avoid species
295 misidentification. The β-tubulin gene has been recommended as a specific barcode for species
296 identification (Samson et al. 2010; Visagie et al. 2014), and 108 out of 124 isolates were
297 unambiguously identified through the *BenA* sequences. The remaining 16 isolates were determined
298 through the analysis of calmodulin gene sequences and the colony morphology. Furthermore, the
299 molecular phylogeny obtained by the concatenated datasets confirmed species identification, and
300 highlighted an intraspecific variability for some species i.e. *P. bialowiezense*, *P. commune* and *P.*
301 *glabrum*, as observed in the phylogenetic studies of Barreto et al. (2011), Houbraken et al. (2012) and
302 Visagie et al. (2014).
303 Twenty species divided into 2 subgenera (*Aspergilloides* and *Penicillium*) and 8 sections
304 (*Aspergilloides, Brevicompacta, Chrysogena, Citrina, Exicauilis, Fasciculata, Penicillium* and
305 *Robsamsonia*) were identified on the basis of the accepted taxonomy reported in Houbraken and
306 Samson (2011), Visagie et al. (2014) and Houbraken et al. (2016). Depending on the source of
307 isolation, we found 8 species from orchard sampling, 12 species from chestnut processing, 9 species
308 from flour and 8 species from indoor sampling, similarly to the number of species reported by
309 Filtenborg et al. (2004) in food commodities.
310 The *Penicillium* genus includes over 200 species with different eco-physiological adaptations,
311 including tolerance to cold and to low water activity (Filtenborg et. al, 2004; Pitt and Hocking 2009;
312 Rosso and Robinson 2001). In our study, we were able to isolate different *Penicillium* species from
313 both fresh and dry chestnut products. The found species partially confirm previous monitoring
314 performed on fresh chestnuts, commercial products and the indoor environment (Donis-Gonzales et

al., 2016; Magan 2006; Overy et al., 2003; Pietri et al., 2012; Sieber et al., 2007). *P. crustosum, P. glabrum* and *P. bialowiezense* were the predominant species and they represented 57% of the isolates. In agreement with Overy et al. (2003), *P. crustosum* was the predominant species, with 44 isolates from all the sources out of 124, thus confirming *P. crustosum* as a ubiquitous species that is able to survive in different environmental conditions (Domsch et al., 2007; Scholtz and Korsten, 2016).

P. glabrum was the second most frequent species. As reported by several authors, this species has a worldwide distribution and has been isolated from various foods, including fresh chestnuts, but also from soil and indoor environment (Frisvad and Samson, 2004; Houbraken et al., 2014; Samson et al., 2004; Spadaro et al., 2010). Other species, although less frequently, were isolated, including*P. expansum, P. palitans, P. chrysogenum* and *P. discolor,* which have been associated with nuts (Mujica and Vergara, 1945; Frisvad and Samson, 2004; Donis-Gonzalez et al., 2016).

Fruit decay and economic losses caused by different *Penicillium* species, i.e. *P. expansum, P. digitatum, P. italicum* in apple and citrus fruit (Prusky et al., 2014) and *P. expansum, P. griseofulvum* and *P. chrysogenum* in chestnut (Donis-Gonzalez et al., 2016), are well known. Our study highlights the virulence potential of the isolated *Penicillium* spp., with around 70% of pathogenic strains, including the environmental strains, similarly to what previously reported by Louw and Korsten (2014) for apples and pears. Lingling et al. (2013) reported in pathogenicity tests that 60% of isolates of *Penicillium* spp. were pathogenic on *Castanea mollissima*. *P. expansum* and *P. crustosum* were the most virulent species. Both species are important post-harvest pathogens that are particularly virulent and show a high adaptability to the environment (Louw and Korsten 2014; Scholtz and Korsten, 2016). To the best of our knowledge, this is the first report of *P. bialowiezense, P. brevicompactum, P. citrinum, P. commune, P. glandicola, P. manginii, P. pancosmium, P. polonicum, P. solitum, P. viridicatum, P. verrucosum* as agents of moulds on *Castanea sativa*. *P. nordicum* and *P. palitans* were previously reported on nuts, but not as pathogenic on *C. sativa*.

Fifty-nine percent of the analysed strains (41/70) were able to produce at least one mycotoxin on chestnuts. Fourteen secondary metabolites, associated with the isolated species, were evaluated on

the inoculated chestnuts, even though none of these compounds has an established legislative threshold for chestnuts, according to the European legislation (Commission Regulation (EU) No. 165/2010). As reported by Frisvad and Samson (2004), different secondary metabolites are produced by different *Penicillium* spp. and they can often be used as markers to differentiate the species. In particular, mycophenolic acid is mainly produced by *P. bialowiezense* and *P. brevicompactum*, while patulin is mainly produced by *P. expansum*, and cyclopiazonic acid by *P. commune*. Other metabolites, such as roquefortine C, andrastin A, cyclopenine and cyclopenol, are produced by different species. Toxic effects on human health have been reported for all these molecules (Barkai-Golan, 2008).

The isolation of different *Penicillium* species from all the investigated samples and their mycotoxin production are cause of concern because of their effects on human health. The environmental conditions, together with the storage and processing of the material, probably could promote the fungal growth, thus indicating a contamination throughout the chestnut production chain.

To the best of our knowledge, this is the first study focusing on *Penicillium* spp. on fresh chestnuts and the chestnut flour processing phases, which revealed on a relatively limited number of samples, a high diversity in species of *Penicillium* spp., as well as a great virulence and mycotoxin production potential. Further studies are needed to analyse the whole production chain in order to follow chestnut production from the chestnut orchard throughout the processing phases, to understand the critical points of contamination. Information about the occurrence of *Penicillium* spp. and their food-borne mycotoxins will help to set up chestnut management procedures from the orchard to the commercial product, with the aim of controlling the fungal growth and managing the mycotoxin production.

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Tables

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*	-	-

Strain	Source	Species	Virulence	Secondary metabolites
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*	-	-
XM	PP	*P. crustosum*	-	-
XG	PP	*P. crustosum*	-	-
SP1	PP	*P. crustosum*	-	-
SP4	PP	*P. crustosum*	SV	CPN, CPL, VIR, VOL, RoqC, PenA
SP5	PP	*P. crustosum*	HV	CPN, CPL, VIR, VOL, RoqC
Cas10	C	*P. commune*	HV	n.d.
Cas40	C	*P. commune*	MV	n.d.
Cal3f	F	*P. commune*	SV	CPA
CalC	PP	*P. commune*	MV	CPA
X2	PP	*P. palitans*	NP	-
SP2	PP	*P. palitans*	NP	-
SP3	PP	*P. palitans*	MV	n.d.
XL	PP	*P. palitans*	SV	n.d.
XF	PP	*P. solitum*	HV	CPN, CPL, VIR, VOL
3B1	I	*P. discolor*	SV	CPN, CPL, VIR, VOL, ChA
3B4	I	*P. discolor*	MV	CPN, CPL, VIR, VOL, ChA
3B5	I	*P. discolor*	HV	CPN, CPL, VIR, VOL, ChA
3B6	I	*P. discolor*	HV	CPN, CPL, VIR, VOL, ChA
F3A	F	*P. discolor*	HV	CPN, CPL, ChA
Cas13	I	*P. discolor*	SV	ChA
Cas9	I	*P. discolor*	HV	CPN, CPL, VIR, VOL, ChA

Strain	Source	Species	Virulence	Secondary metabolites
3B30	I	*P. discolor*	MV	n.d.
4.4	I	*P. polonicum*	HV	n.d.
MO4	F	*P. polonicum*	SV	CPN, CPL, VIR, VOL, VER
X3	PP	*P. polonicum*	HV	CPN
X6	PP	*P. polonicum*	MV	CPN, CPL, VIR, VOL, VER
X9	PP	*P. polonicum*	MV	CPN, CPL, VER
XE	PP	*P. polonicum*	SV	CPN, CPL, VIR, VOL
XA	PP	*P. viridicatum*	HV	PA
XC	PP	*P. viridicatum*	MV	n.d.
XO	PP	*P. viridicatum*	MV	n.d.
XP	PP	*P. viridicatum*	SV	n.d.
F6	F	*P. verrucosum*	MV	n.d.
F1B	F	*P. verrucosum*	SV	n.d.
F8	F	*P. nordicum*	SV	n.d.
F1A	F	*P. corylophilum*	NP	-
Cas33	C	*P. yezoense*	NP	-
XB	PP	*P. yezoense*	NP	-
E1	C	*P. glabrum*	NP	-
E2	C	*P. glabrum*	SV	n.d.
E3	C	*P. glabrum*	MV	n.d.
E7	C	*P. glabrum*	HV	n.d.
B3	C	*P. glabrum*	SV	n.d.
Cal66	F	*P. glabrum*	NP	-
Cal67	F	*P. glabrum*	NP	-
Cal68	F	*P. glabrum*	NP	-
Cal1f	F	*P. glabrum*	NP	-
Cal2f	F	*P. glabrum*	NP	-
Cal4f	F	*P. glabrum*	NP	-
Cal8f	F	*P. glabrum*	NP	-
Cal10f	F	*P. glabrum*	NP	-
Cal11f	F	*P. glabrum*	NP	-
XD	PP	*P. glabrum*	NP	-
CP2	C	*P.pancosmium*	HV	n.d.
CP3	C	*P.pancosmium*	HV	n.d.
FP10	C	*P.pancosmium*	MV	n.d.
FP20	C	*P.pancosmium*	NP	-
Yell	C	*P.manginii*	HV	n.d.
CalA	PP	*P. citrinum*	MV	n.d.

Source: C: chestnut; F: flour; I: indoors; PP: processing phases. Virulence: NP: non-pathogenic, SV: slightly virulent; MV: moderately virulent; HV: highly virulent; -: not tested. Secondary metabolites: MPA: mycophenolic acid; MEL: meleagrin; AndA: andrastin A; RoqC: roquefortine C; PAT: patulin; CHA: chaetoglobosin A; CIN: cyclopenin; COL: cyclopenol; VIR: viridicatin; VOL: viridicatol; PenA: penitrem A; CPA: cyclopiazonic acid; VER: verrucosidin; PA: penicillic acid; -: not tested; n.d.: not detected.

475 **Table 2** – Number of isolates per source of isolation and species, number of samples (n) and accession numbers of the ITS region, β-tubulin and calmodulin
476 genes for one strain per species (reported in parentheses) found in this study.

	Orchard (n=3)	Processing phases (n=11)	Flour (n=4)	Indoors (n=6)	No. of isolates per species	ITS accession number	β-tubulin accession number	Calmodulin accession number
P. bialowiezense	10	-	-	2	**12**	MG821357 (B1)	MF100873	MF100893
P. brevicompactum	-	1	-	1	**2**	MG821358 (Cas18)	MF100870	MF100890
P. chrysogenum	-	-	-	1	**1**	MG821359 (Cas26)	MF100859	MF100879
P. citrinum	-	1	-	-	**1**	MG821360 (CalA)	MF100868	MF100888
P. commune	2	1	1	-	**4**	MG821361 (CalC)	MF100877	MF100897
P. corylophilum	-	-	1	-	**1**	MG821362 (F1A)	MF100865	MF100885
P. crustosum	2	11	24	7	**44**	MG821363 (5A)	MF100874	MF100894
P. discolor	-	-	1	7	**8**	MG821364 (3B4)	MF100876	MF100896
P. expansum	4	1	1	1	**7**	MG821365 (PCAS)	MF100860	MF100880
P. glabrum	5	1	9	-	**15**	MG821366 (XD)	MF100875	MF100895
P. glandicola	-	2	-	2	**4**	MG821367 (3C)	MF100862	MF100882
P. manginii	1	-	-	-	**1**	MG821368 (YELL)	MF100867	MF100887
P. nordicum	-	-	1	-	**1**	MG821369 (F8)	MF100864	MF100884
P. palitans	-	4	-	-	**4**	MG821370 (SP2)	MF100871	MF100891
P. pancosmium	4	-	-	-	**4**	MG821371 (CP2)	MF100866	MF100886
P. polonicum	-	4	1	1	**6**	MG821372 (MO4)	MF100858	MF100878
P. solitum	-	1	-	-	**1**	MG821373 (XF)	MF100861	MF100881
P. viridicatum	-	4	-	-	**4**	MG821375 (XA)	MF100872	MF100892
P. yezoense	1	1	-	-	**2**	MG821376 (Cas33)	MF100869	MF100889
P. verrucosum	-	-	2	-	**2**	MG821374 (F1B)	MF100863	MF100883
Total	**29**	**32**	**41**	**22**	**124**			

477

478 **Table 3** – Average colony diameter of the *Penicillium* species isolated in this study.

	Culture characteristics	YES*	MEA*	CYA*
P. bialowiezense	Velutinous	2.8 ±0.2	1.6 ±0.1	1.2 ±0.1
P. brevicompactum	Velutinous	1.9 ±0.1	2.1 ±0.1	1.3 ±0.1
P. chrysogenum	Floccose to velutinous	3.6 ±0.1	2.1 ±0.1	2.8 ±0.2
P. citrinum	Velutinous	2.3 ±0.1	1.2 ±0.1	1.8 ±0.1
P. commune	Velutinous to fasciculate	3.2 ±0.1	2.3 ±0.2	2.9 ±0.2
P. corylophilum	Velutinous	1.9 ±0.1	1.6 ±0.2	1.6 ±0.1
P. crustosum	Velutinous to crustose	3.7 ±0.2	3.1 ±0.1	3.0 ±0.1
P. discolor	Velutinous to fasciculate	3.5 ±0.3	3.0 ±0.2	2.8 ±0.1
P. expansum	Velutinous to fasciculate	3.6 ±0.3	2.8 ±0.1	3.3 ±0.3
P. glabrum	Velutinous	3.0 ±0.1	3.0 ±0.3	2.8 ±0.1
P. glandicola	Fasciculate	2.8 ±0.1	2.1 ±0.2	1.1 ±0.2
P. manginii	Velutinous to floccose	3.0 ±0.1	2.5 ±0.1	2.8 ±0.1
P. nordicum	Velutinous to floccose	2.5 ±0.4	2.0 ±0.2	2.0 ±0.1
P. palitans	Velutinous	3.2 ±0.2	2.4 ±0.1	1.4 ±0.1
P. pancosmium	Velutinous to floccose	2.9 ±0.1	2.7 ±0.2	2.3 ±0.3
P. polonicum	Velutinous	2.7 ±0.2	2.2 ±0.1	2.4 ±0.4
P. solitum	Velutinous	3.2 ±0.2	2.7 ±0.2	2.7 ±0.2
P. viridicatum	Velutinous to fasciculate	2.8 ±0.1	2.9 ±0.1	2.7 ±0.2
P. yezoense	Velutinous	3.7 ±0.3	3.4 ±0.1	3.4 ±0.5
P. verrucosum	Velutinous to floccose	2.7 ±0.1	1.4 ±0.1	1.4 ±0.2

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481 *Colony diameters of strains grown on YES, MEA and CYA incubated at 25 °C for 7 days.

Table 4 – *In vivo* pathogenicity assay for the *Penicillium* spp. strains isolated in this study.

	NP	SV	MV	HV	Number of isolates
P. bialowiezense	5	-	4	3	**12**
P. brevicompactum	1	-	-	1	**2**
P. chrysogenum	-	-	-	1	**1**
P. citrinum	-	-	1	-	**1**
P. commune	-	1	2	1	**4**
P. corylophilum	1	-	-	-	**1**
P. crustosum	1	3	2	10	**16**
P. discolor	-	2	2	4	**8**
P. expansum	-	-	1	6	**7**
P. glabrum	11	2	1	1	**15**
P. glandicola	3	-	1		**4**
P. manginii	-	-	-	1	**1**
P. nordicum	-	1	-	-	**1**
P. palitans	2	1	1		**4**
P. pancosmium	1		1	2	**4**
P. polonicum	-	2	2	2	**6**
P. solitum	-	-	-	1	**1**
P. viridicatum	-	1	2	1	**4**
P. yezoense	2	-	-	-	**2**
P. verrucosum	-	1	1	-	**2**
	27	**14**	**21**	**32**	**96**

In vivo severity of symptoms: non-pathogenic (NP) = no symptoms; slightly virulent (SV) = 1-30% infected area; moderately virulent (MV) = 31-50% infected; highly virulent (HV) = 51-100% infected area.

Figure 1 - Best scoring Maximum Likelihood tree based on the concatenated ITS region, β-tubulin and calmodulin sequences datasets. The numbers at major nodes indicate the bootstrap value from 1000 bootstrapped datasets. Branches with bootstrap values lower than 80% are not shown. Phylogeny was rooted by *Fusarium equiseti*. Evolutionary analyses were conducted using MEGA version 6.

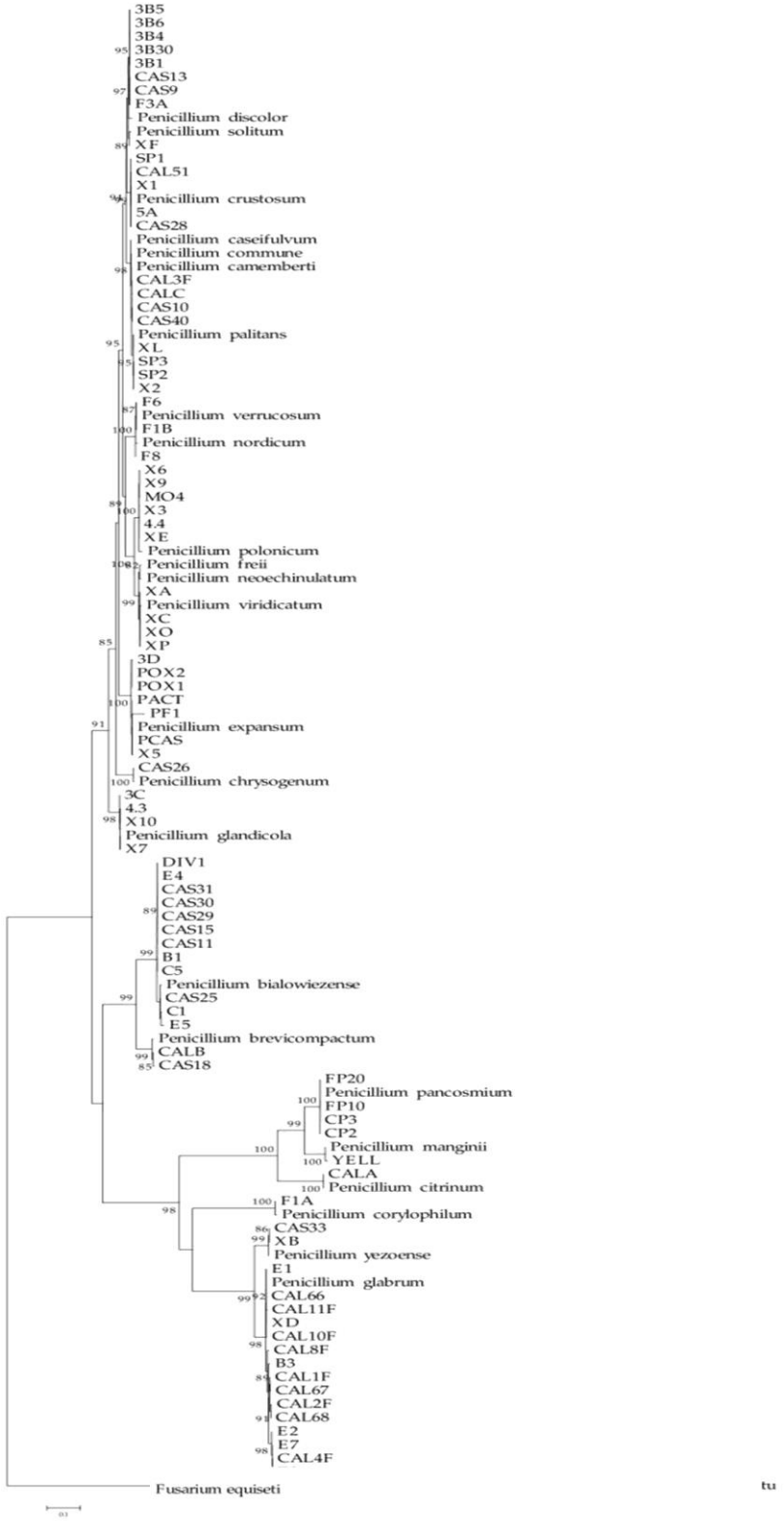

Figure 2 – Colonies morphology (top and reverse) of the *Penicillium* species isolated in this study after 7 days of incubation at 25 °C in the dark on YES (a), MEA (b) and CYA (c). d. Results of the pathogenicity assay of the *Penicillium* species isolated in this study inoculated on chestnut after 7 days at 26±1°C in the dark.

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