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

3

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12

13 **Abstract:**

14 A collection of 124 isolates of *Penicillium* spp. was created by monitoring fresh chestnuts, dried  
15 chestnuts, chestnut granulates, chestnut flour, and indoor the chestnut mills. Sequencing of ITS  
16 region,  $\beta$ -tubulin and calmodulin, macro-morphology and secondary metabolite production permitted  
17 to determine 20 species of *Penicillium*. In fresh chestnuts, *P. bialowiezense* was dominant, while *P.*  
18 *crustosum* was more frequent in the other sources. Pathogenicity test on chestnut showed that around  
19 70% isolates were virulent. *P. corylophilum* and *P. yezoense* were not pathogenic, while the other 18  
20 species had at least one virulent isolate. *P. expansum* and *P. crustosum* were the most virulent. The  
21 isolates were characterized for their ability to produce 14 toxic metabolites *in vivo*: 59% were able to  
22 produce at least one mycotoxin. *P. expansum* was able to produce patulin, chaetoglobosin A and  
23 roquefortine C. Mycophenolic acid was produced by *P. bialowiezense*. Cyclopenins and viridicatin  
24 were produced by most *P. crustosum*, *P. polonicum*, *P. solitum* and *P. discolor*. Some isolates of *P.*  
25 *crustosum* were also able to produce roquefortine C or penitrem A. Information about the occurrence  
26 of *Penicillium* spp. and their mycotoxins will help to set up chestnut management procedures, to  
27 control the fungal growth and the mycotoxin production.

28

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

## 31 **1. Introduction**

32 Chestnut is the most popular nut-bearing tree in several European and Asian countries, with new  
33 productions in United States, Australia, New Zealand, and Chile. Italy is the second sweet chestnut  
34 (*Castanea sativa* Mill.) producer in Europe, with 52,000 tons and a cultivated area of 21,500 ha in  
35 2014 (FAOSTAT, 2014; Livre Blanc Châtaigne, 2012). The industrial preparation of chestnut flour,  
36 dried chestnuts and marrons glacés represents 20% of the total production. Moulds contaminate  
37 chestnut before harvest, but also during transportation, storage and processing. Fungal spoilage can  
38 be responsible for significant economic losses. Moreover, a number of fungi isolated from chestnuts  
39 are well-known mycotoxin producers. The occurrence of toxigenic *Fusarium* spp., *Penicillium* spp.  
40 and *Aspergillus* spp. and their associated mycotoxin contaminations on chestnuts and derived  
41 commercial products have been reported in different countries (Abdel-Gawad and Zohri, 1993;  
42 Bertuzzi et al., 2015; Jermini et. al., 2006; Overy et al., 2003; Pietri et al., 2012; Prencipe et al., 2018;  
43 Rodrigues et al., 2013; Wells and Payne, 1975).

44 *Penicillium* spp. are ubiquitously causing decay on fruit (Washington et al., 1997), as well as  
45 contaminant in post-harvest and indoor (Nielsen, 2003). The most frequent *Penicillium* species  
46 reported in nuts belong to the series *Camemberti* and *Solita*, except for *P. nordicum* (Frisvad and  
47 Samson, 2004). A few papers reported the species of *Penicillium* isolated from chestnuts. Overy et  
48 al. (2003) stated that *P. crustosum*, *P. glabrum*-clade and *P. discolor* were the dominant species in  
49 fresh chestnuts. Sieber et al. (2007) predominantly isolated *P. expansum* and *P. crustosum*, while  
50 Donis-Gonzales and colleagues (2016) stated that *P. expansum*, *P. griseofulvum* and *P. chrysogenum*  
51 were the main species. *Penicillium* spp. (about  $10^4$  CFU/g) have been reported also on dried chestnuts  
52 and chestnut flour (Pietri et al., 2012).

53 Mycotoxins are secondary metabolites produced by moulds which show toxic, mutagenic and  
54 teratogenic effects, including potential immunosuppressive activity and carcinogenic effects  
55 (Milicevic et al., 2010). Because of their long-term chronic or cumulative effects on human health,  
56 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),

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

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

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

139

### 140 *2.3 Macro-morphology characterization*

141 A macro-morphological identification of all the isolates was performed according to Visagie et al.  
142 (2014). The isolates were 3-point inoculated with a spore suspension on Yeast Extract Sucrose Agar  
143 (YES agar), Czapek Yeast Autolysate agar (CYA) and Malt extract agar (MEA), and they were  
144 observed for growth (mean diameter) and colony characters (colony texture, texture and colour of  
145 mycelium, colony reverse colours, presence and colour of the soluble pigments and exudates) after 7  
146 days of incubation at 25 °C in the dark. The colour standards and nomenclature by Robert Ridgway  
147 (1912) were used for morphological description.

148

### 149 *2.4 Pathogenicity assay*

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



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

163

### 164 *2.5 Mycotoxin production on chestnuts*

165 *Penicillium* metabolites were extracted from the chestnuts used in the pathogenicity tests for 70  
166 isolates, with at least one isolate per species (Table 2). The chestnuts were divided into three  
167 subsamples for each isolate and extracted twice by means of solid-liquid extraction with 10 mL of  
168 ethyl acetate. The samples were shaken for 1 min and the organic phases were then collected in a  
169 flask. The extract was evaporated to dryness in a rotary evaporator at 35 °C. The residue was dissolved  
170 in 500 µL of H<sub>2</sub>O:CH<sub>3</sub>CN 1:1 for HPLC-DAD analysis.

171 The HPLC apparatus was an Agilent 1100 series equipped with a G1379 degasser, a G1313A  
172 autosampler, a G1316A column thermostat set at 30 °C, a G1315B UV diode array detector set at  
173 230, 276, 300 and 330 nm, a G1311 quaternary pump and an Agilent Chemstation G2170AA  
174 Windows XP operating system (Agilent®, Germany). A Luna C18 analytical column (150x4.6 mm  
175 i.d., 3 µm, Phenomenex®, USA), preceded by a guard column (4x3mm i.d.) with the same stationary  
176 phase, was used for the HPLC procedure. The mobile phases consisted of water acidified with formic  
177 acid 0.05 % (A) and acetonitrile (B), at a flow rate of 0.800 mL/min in gradient mode, 0-5 min: 5%  
178 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.  
179 Twenty µL of the samples was injected. UV spectra were collected, by means of DAD, every 0.4 s,  
180 from 190 to 700 nm, with a resolution of 2 nm. Authentic mycophenolic acid (MPA), meleagrine  
181 (MEL), andrastin A (AndA), roquefortine C (RoqC), patulin (PAT), chaetoglobosin A (ChA),  
182 cyclopenin (CPN), cyclophenol (CPL), viridicatin (VIR), viridicatol (VOL), penitrem A (PenA),  
183 cyclopiazonic acid (CPA), verrucosidin (VER) and penicillic acid (PA) standards were used for the  
184 identification by comparing their retention times with the UV spectra.

185

## 186 **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  $\beta$ -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  $\beta$ -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), while a 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

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

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

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

249

### 250 3.3 Pathogenicity

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

256 The non-pathogenic strains included all the isolates of *P. corylophilum* and *P. yezoense*, and 3 out of  
257 4 isolates of *P. glandicola*. *P. bialowiezense*, *P. brevicompactum*, *P. crustosum*, *P. glabrum*, *P.*  
258 *pancosmium* and *P. palitans* included both non-pathogenic and pathogenic strains. On the contrary,  
259 all the strains of *P. citrinum* *P. commune*, *P. chrysogenum* *P. discolor*, *P. expansum*, *P. manginii* *P.*  
260 *nordicum*, *P. polonicum*, *P. solitum*, *P. verrucosum* and *P. viridicatum* were pathogenic.

261 By considering the most frequently isolated species, *P. bialowiezense* strains were non-pathogenic  
262 (5/12), moderately virulent (4/12) or highly virulent (3/12). All the strains belonging to the *P.*  
263 *crustosum* species were pathogenic (3 SV, 2 MV, 8 HV), except for one (Cas17). *P. glabrum* strains

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

266

### 267 3.4 Mycotoxin production on chestnuts

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

279

## 280 4. Discussion

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

315 al., 2016; Magan 2006; Overy et al., 2003; Pietri et al., 2012; Sieber et al., 2007). *P. crustosum*, *P.*  
316 *glabrum* and *P. bialowiezense* were the predominant species and they represented 57% of the isolates.  
317 In agreement with Overy et al. (2003), *P. crustosum* was the predominant species, with 44 isolates  
318 from all the sources out of 124, thus confirming *P. crustosum* as a ubiquitous species that is able to  
319 survive in different environmental conditions (Domsch et al., 2007; Scholtz and Korsten, 2016).  
320 *P. glabrum* was the second most frequent species. As reported by several authors, this species has a  
321 worldwide distribution and has been isolated from various foods, including fresh chestnuts, but also  
322 from soil and indoor environment (Frisvad and Samson, 2004; Houbraken et al., 2014; Samson et al.,  
323 2004; Spadaro et al., 2010). Other species, although less frequently, were isolated, including *P.*  
324 *expansum*, *P. palitans*, *P. chrysogenum* and *P. discolor*, which have been associated with nuts  
325 (Mujica and Vergara, 1945; Frisvad and Samson, 2004; Donis-Gonzalez et al., 2016).  
326 Fruit decay and economic losses caused by different *Penicillium* species, i.e. *P. expansum*, *P.*  
327 *digitatum*, *P. italicum* in apple and citrus fruit (Prusky et al., 2014) and *P. expansum*, *P. griseofulvum*  
328 and *P. chrysogenum* in chestnut (Donis-Gonzalez et al., 2016), are well known. Our study highlights  
329 the virulence potential of the isolated *Penicillium* spp., with around 70% of pathogenic strains,  
330 including the environmental strains, similarly to what previously reported by Louw and Korsten  
331 (2014) for apples and pears. Lingling et al. (2013) reported in pathogenicity tests that 60% of isolates  
332 of *Penicillium* spp. were pathogenic on *Castanea mollissima*. *P. expansum* and *P. crustosum* were  
333 the most virulent species. Both species are important post-harvest pathogens that are particularly  
334 virulent and show a high adaptability to the environment (Louw and Korsten 2014; Scholtz and  
335 Korsten, 2016). To the best of our knowledge, this is the first report of *P. bialowiezense*, *P.*  
336 *brevicompactum*, *P. citrinum*, *P. commune*, *P. glandicola*, *P. manginii*, *P. pancosmium*, *P.*  
337 *polonicum*, *P. solitum*, *P. viridicatum*, *P. verrucosum* as agents of moulds on *Castanea sativa*. *P.*  
338 *nordicum* and *P. palitans* were previously reported on nuts, but not as pathogenic on *C. sativa*.  
339 Fifty-nine percent of the analysed strains (41/70) were able to produce at least one mycotoxin on  
340 chestnuts. Fourteen secondary metabolites, associated with the isolated species, were evaluated on

341 the inoculated chestnuts, even though none of these compounds has an established legislative  
342 threshold for chestnuts, according to the European legislation (Commission Regulation (EU) No.  
343 165/2010). As reported by Frisvad and Samson (2004), different secondary metabolites are produced  
344 by different *Penicillium* spp. and they can often be used as markers to differentiate the species. In  
345 particular, mycophenolic acid is mainly produced by *P. bialowiezense* and *P. brevicompactum*, while  
346 patulin is mainly produced by *P. expansum*, and cyclopiazonic acid by *P. commune*. Other  
347 metabolites, such as roquefortine C, andrastin A, cyclophenine and cyclophenol, are produced by  
348 different species. Toxic effects on human health have been reported for all these molecules (Barkai-  
349 Golan, 2008).

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

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

362

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466 **Tables**

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

Strain	Source	Species	Virulence	Secondary metabolites
Cas26	I	<i>P. chrysogenum</i>	HV	n.d.
Cas11	I	<i>P. bialowiezense</i>	NP	-
Cas31	C	<i>P. bialowiezense</i>	NP	-
Cas30	C	<i>P. bialowiezense</i>	HV	n.d.
Cas29	I	<i>P. bialowiezense</i>	MV	n.d.
Cas15	C	<i>P. bialowiezense</i>	NP	-
Cas25	C	<i>P. bialowiezense</i>	HV	n.d.
E4	C	<i>P. bialowiezense</i>	MV	n.d.
E5	C	<i>P. bialowiezense</i>	SV	n.d.
B1	C	<i>P. bialowiezense</i>	HV	MPA
C1	C	<i>P. bialowiezense</i>	MV	MPA
C5	C	<i>P. bialowiezense</i>	MV	MPA
DIV1	C	<i>P. bialowiezense</i>	NP	-
Cas18	I	<i>P.brevicompactum</i>	HV	n.d.
CalB	PP	<i>P.brevicompactum</i>	NP	-
3C	I	<i>P. glandicola</i>	MV	MEL, AndA
4.3	I	<i>P. glandicola</i>	NP	-
X7	PP	<i>P. glandicola</i>	NP	-
X10	PP	<i>P. glandicola</i>	NP	-
3D	I	<i>P. expansum</i>	HV	PAT, RoqC
PACT	C	<i>P. expansum</i>	HV	PAT
PCAS	C	<i>P. expansum</i>	HV	PAT
POX1	C	<i>P. expansum</i>	MV	PAT, ChA
POX2	C	<i>P. expansum</i>	HV	PAT, ChA
X5	PP	<i>P. expansum</i>	HV	PAT, ChA, Roqc
PF1	F	<i>P. expansum</i>	HV	PAT, ChA, Roqc
4A	C	<i>P. crustosum</i>	HV	CPN, CPL, VIR, VOL, PenA
Cas12	I	<i>P. crustosum</i>	HV	CPN, CPL, VIR, VOL, RoqC
Cas14	I	<i>P. crustosum</i>	HV	CPN, CPL, VIR, VOL, RoqC, PenA
Cas28	I	<i>P. crustosum</i>	MV	CPN, CPL
Cas34	I	<i>P. crustosum</i>	HV	CPN, CPL, VIR, VOL
Cas17	I	<i>P. crustosum</i>	NP	-
3.3	I	<i>P. crustosum</i>	HV	n.d.
5A	C	<i>P. crustosum</i>	MV	CPN, CPL, VIR, VOL, RoqC, PenA
3.2	I	<i>P. crustosum</i>	SV	CPN, CPL, VIR, VOL, PenA
Cal51	F	<i>P. crustosum</i>	SV	CPN, CPL, VIR, VOL, RoqC, PenA
Cal52	F	<i>P. crustosum</i>	-	-
Cal53	F	<i>P. crustosum</i>	-	-
Cal54	F	<i>P. crustosum</i>	-	-
Cal55	F	<i>P. crustosum</i>	-	-

Strain	Source	Species	Virulence	Secondary metabolites
Cal56	F	<i>P. crustosum</i>	-	-
Cal57	F	<i>P. crustosum</i>	-	-
Cal58	F	<i>P. crustosum</i>	-	-
Cal59	F	<i>P. crustosum</i>	-	-
Cal60	F	<i>P. crustosum</i>	-	-
Cal61	F	<i>P. crustosum</i>	-	-
Cal62	F	<i>P. crustosum</i>	-	-
Cal63	F	<i>P. crustosum</i>	-	-
Cal64	F	<i>P. crustosum</i>	HV	CPN, CPL, VIR, VOL, RoqC, PenA
Cal65	F	<i>P. crustosum</i>	-	-
Cal69	F	<i>P. crustosum</i>	-	-
Cal70	F	<i>P. crustosum</i>	-	-
Cal5f	F	<i>P. crustosum</i>	-	-
Cal6f	F	<i>P. crustosum</i>	-	-
Cal7f	F	<i>P. crustosum</i>	-	-
Cal9f	F	<i>P. crustosum</i>	-	-
Cal12f	F	<i>P. crustosum</i>	HV	CPN, CPL, VIR, VOL, RoqC, PenA
X1	PP	<i>P. crustosum</i>	HV	CPN, CPL, VIR, VOL, RoqC, PenA
X4	PP	<i>P. crustosum</i>	HV	CPN, CPL, VIR, VOL, RoqC, PenA
XF1	F	<i>P. crustosum</i>	-	-
XF2	F	<i>P. crustosum</i>	-	-
PLX1	PP	<i>P. crustosum</i>	-	-
PLX2	PP	<i>P. crustosum</i>	-	-
PLX3	PP	<i>P. crustosum</i>	-	-
PLX4	PP	<i>P. crustosum</i>	-	-
XM	PP	<i>P. crustosum</i>	-	-
XG	PP	<i>P. crustosum</i>	-	-
SP1	PP	<i>P. crustosum</i>	-	-
SP4	PP	<i>P. crustosum</i>	SV	CPN, CPL, VIR, VOL, RoqC, PenA
SP5	PP	<i>P. crustosum</i>	HV	CPN, CPL, VIR, VOL, RoqC
Cas10	C	<i>P. commune</i>	HV	n.d.
Cas40	C	<i>P. commune</i>	MV	n.d.
Cal3f	F	<i>P. commune</i>	SV	CPA
CalC	PP	<i>P. commune</i>	MV	CPA
X2	PP	<i>P. palitans</i>	NP	-
SP2	PP	<i>P. palitans</i>	NP	-
SP3	PP	<i>P. palitans</i>	MV	n.d.
XL	PP	<i>P. palitans</i>	SV	n.d.
XF	PP	<i>P. solitum</i>	HV	CPN, CPL, VIR, VOL
3B1	I	<i>P. discolor</i>	SV	CPN, CPL, VIR, VOL, ChA
3B4	I	<i>P. discolor</i>	MV	CPN, CPL, VIR, VOL, ChA
3B5	I	<i>P. discolor</i>	HV	CPN, CPL, VIR, VOL, ChA
3B6	I	<i>P. discolor</i>	HV	CPN, CPL, VIR, VOL, ChA
F3A	F	<i>P. discolor</i>	HV	CPN, CPL, ChA
Cas13	I	<i>P. discolor</i>	SV	ChA
Cas9	I	<i>P. discolor</i>	HV	CPN, CPL, VIR, VOL, ChA

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

469

470 Source: C: chestnut; F: flour; I: indoors; PP: processing phases. Virulence: NP: non-pathogenic, SV: slightly  
471 virulent; MV: moderately virulent; HV: highly virulent; -: not tested. Secondary metabolites: MPA:  
472 mycophenolic acid; MEL: meleagrins; AndA: andrastin A; RoqC: roquefortine C; PAT: patulin; CHA:  
473 chaetoglobosin A; CIN: cyclophenin; COL: cyclophenol; VIR: viridicatin; VOL: viridicatol; PenA: penitrem A;  
474 CPA: cyclopiiazonic 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,  $\beta$ -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	$\beta$ -tubulin accession number	Calmodulin accession number
<i>P. bialowiezense</i>	10	-	-	2	<b>12</b>	MG821357 (B1)	MF100873	MF100893
<i>P. brevicompactum</i>	-	1	-	1	<b>2</b>	MG821358 (Cas18)	MF100870	MF100890
<i>P. chrysogenum</i>	-	-	-	1	<b>1</b>	MG821359 (Cas26)	MF100859	MF100879
<i>P. citrinum</i>	-	1	-	-	<b>1</b>	MG821360 (CalA)	MF100868	MF100888
<i>P. commune</i>	2	1	1	-	<b>4</b>	MG821361 (CalC)	MF100877	MF100897
<i>P. corylophilum</i>	-	-	1	-	<b>1</b>	MG821362 (F1A)	MF100865	MF100885
<i>P. crustosum</i>	2	11	24	7	<b>44</b>	MG821363 (5A)	MF100874	MF100894
<i>P. discolor</i>	-	-	1	7	<b>8</b>	MG821364 (3B4)	MF100876	MF100896
<i>P. expansum</i>	4	1	1	1	<b>7</b>	MG821365 (PCAS)	MF100860	MF100880
<i>P. glabrum</i>	5	1	9	-	<b>15</b>	MG821366 (XD)	MF100875	MF100895
<i>P. glandicola</i>	-	2	-	2	<b>4</b>	MG821367 (3C)	MF100862	MF100882
<i>P. manginii</i>	1	-	-	-	<b>1</b>	MG821368 (YELL)	MF100867	MF100887
<i>P. nordicum</i>	-	-	1	-	<b>1</b>	MG821369 (F8)	MF100864	MF100884
<i>P. palitans</i>	-	4	-	-	<b>4</b>	MG821370 (SP2)	MF100871	MF100891
<i>P. pancosmium</i>	4	-	-	-	<b>4</b>	MG821371 (CP2)	MF100866	MF100886
<i>P. polonicum</i>	-	4	1	1	<b>6</b>	MG821372 (MO4)	MF100858	MF100878
<i>P. solitum</i>	-	1	-	-	<b>1</b>	MG821373 (XF)	MF100861	MF100881
<i>P. viridicatum</i>	-	4	-	-	<b>4</b>	MG821375 (XA)	MF100872	MF100892
<i>P. yezoense</i>	1	1	-	-	<b>2</b>	MG821376 (Cas33)	MF100869	MF100889
<i>P. verrucosum</i>	-	-	2	-	<b>2</b>	MG821374 (F1B)	MF100863	MF100883
<b>Total</b>	<b>29</b>	<b>32</b>	<b>41</b>	<b>22</b>	<b>124</b>			

477

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

	<b>Culture characteristics</b>	<b>YES*</b>	<b>MEA*</b>	<b>CYA #479</b>
<i>P. bialowiezense</i>	Velutinous	2.8 ±0.2	1.6 ±0.1	1.2 ±0.1
<i>P. brevicompactum</i>	Velutinous	1.9 ±0.1	2.1 ±0.1	1.3 ±0.1
<i>P. chrysogenum</i>	Floccose to velutinous	3.6 ±0.1	2.1 ±0.1	2.8 ±0.2
<i>P. citrinum</i>	Velutinous	2.3 ±0.1	1.2 ±0.1	1.8 ±0.1
<i>P. commune</i>	Velutinous to fasciculate	3.2 ±0.1	2.3 ±0.2	2.9 ±0.2
<i>P. corylophilum</i>	Velutinous	1.9 ±0.1	1.6 ±0.2	1.6 ±0.1
<i>P. crustosum</i>	Velutinous to crustose	3.7 ±0.2	3.1 ±0.1	3.0 ±0.1
<i>P. discolor</i>	Velutinous to fasciculate	3.5 ±0.3	3.0 ±0.2	2.8 ±0.1
<i>P. expansum</i>	Velutinous to fasciculate	3.6 ±0.3	2.8 ±0.1	3.3 ±0.3
<i>P. glabrum</i>	Velutinous	3.0 ±0.1	3.0 ±0.3	2.8 ±0.1
<i>P. glandicola</i>	Fasciculate	2.8 ±0.1	2.1 ±0.2	1.1 ±0.2
<i>P. manginii</i>	Velutinous to floccose	3.0 ±0.1	2.5 ±0.1	2.8 ±0.1
<i>P. nordicum</i>	Velutinous to floccose	2.5 ±0.4	2.0 ±0.2	2.0 ±0.1
<i>P. palitans</i>	Velutinous	3.2 ±0.2	2.4 ±0.1	1.4 ±0.1
<i>P. pancosmium</i>	Velutinous to floccose	2.9 ±0.1	2.7 ±0.2	2.3 ±0.3
<i>P. polonicum</i>	Velutinous	2.7 ±0.2	2.2 ±0.1	2.4 ±0.4
<i>P. solitum</i>	Velutinous	3.2 ±0.2	2.7 ±0.2	2.7 ±0.2
<i>P. viridicatum</i>	Velutinous to fasciculate	2.8 ±0.1	2.9 ±0.1	2.7 ±0.2
<i>P. yezoense</i>	Velutinous	3.7 ±0.3	3.4 ±0.1	3.4 ±0.5
<i>P. verrucosum</i>	Velutinous to floccose	2.7 ±0.1	1.4 ±0.1	1.4 ±0.2

480

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

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

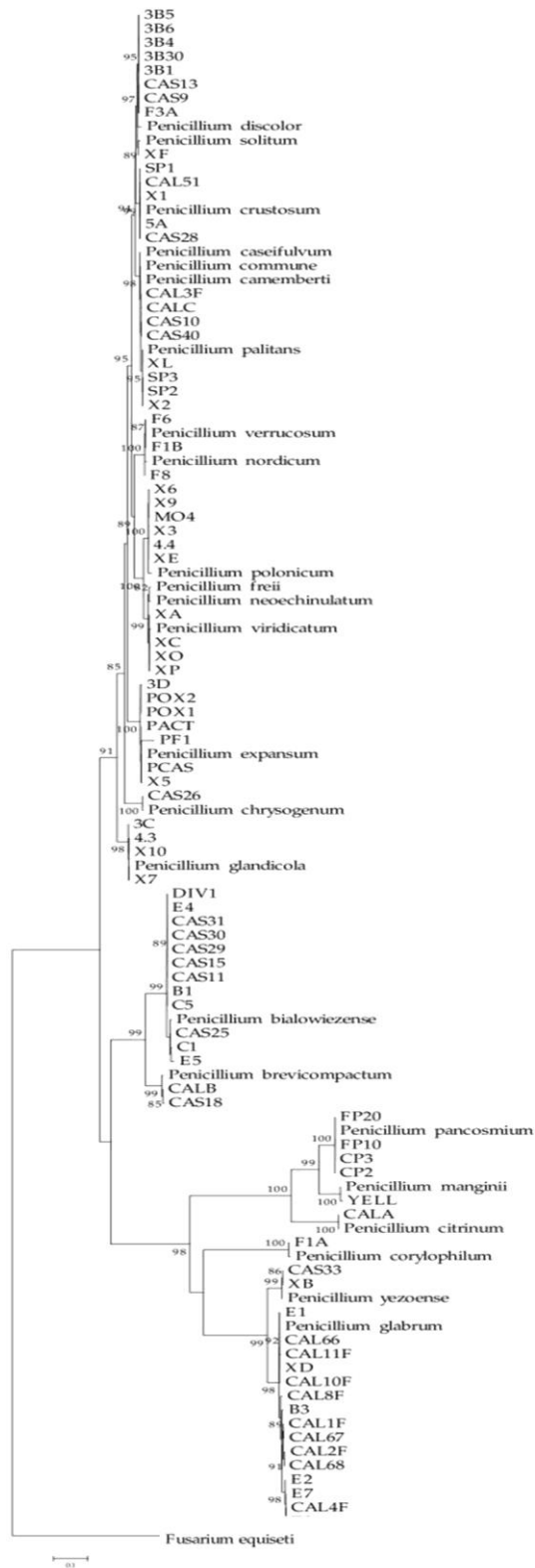
	<b>NP</b>	<b>SV</b>	<b>MV</b>	<b>HV</b>	<b>Number of isolates</b>
<i>P. bialowiezense</i>	5	-	4	3	<b>12</b>
<i>P. brevicompactum</i>	1	-	-	1	<b>2</b>
<i>P. chrysogenum</i>	-	-	-	1	<b>1</b>
<i>P. citrinum</i>	-	-	1	-	<b>1</b>
<i>P. commune</i>	-	1	2	1	<b>4</b>
<i>P. corylophilum</i>	1	-	-	-	<b>1</b>
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

483

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

486

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



491

492

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

