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## **PCR diagnosis of *P. brevicompactum*, a grape contaminant and mycophenolic acid producer.**

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**PCR diagnosis of *P. brevicompactum*, a grape contaminant and mycophenolic acid producer.**

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2 **PCR identification of *Penicillium brevicompactum*, a grape**  
3 **contaminant and mycophenolic acid producer.**

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1

2 **Abstract:**

3 *Penicillium brevicompactum* is a ubiquitous fungal species that contaminates  
4 diverse substrates and commodities and produces an array of metabolites toxic to  
5 human and animals. In this work, we have obtained evidence, by LC-ion trap-mass  
6 spectrometry, of the ability of *P. brevicompactum* strains isolated from grapes to  
7 produce mycophenolic acid, a potent immunosuppressor. In order to facilitate early  
8 diagnosis of this species on commodities for human and animal consumption, we  
9 have developed a rapid, sensitive and specific PCR assay for *P. brevicompactum*.  
10 The specific primers were designed on the basis of ITS1-5.8S-ITS2ITS (Internal  
11 transcribed Spacers of rRNA genes) multicopy region. This method provides a  
12 useful aid to detect the presence of this fungal species in grapes and other  
13 commodities in order to prevent the toxins produced entering the food chain.

14

15 **Key words:** *Penicillium brevicompactum*, Mycophenolic acid, PCR-diagnosis,  
16 grapes, LC-ion trap-mass spectrometry.

17 **Running title:** Detection of *P. brevicompactum* a mycophenolic acid producer

18

## 1 Introduction

2 *Penicillium brevicompactum* Dierckx is a ubiquitous fungus which has been  
3 isolated from air (Wu *et al.*, 2003), apple production, cheese (Kure *et al.*, 2004;  
4 Serra *et al.*, 2003) cashew (Freire *et al.*, 1999), pepper and nuts (Freire *et al.*,  
5 2000) and grapes (Barkai-Golan, 1980; Abrunhosa *et al.*, 2001; Serra *et al.*, 2003;  
6 Serra *et al.*, 2005). Some *P. brevicompactum* strains from diverse commodities  
7 have been described either as mycophenolic acid, brevianamide A, (Paterson *et*  
8 *al.*, 1990; Rundberget *et al.*, 2004) or patulin producers (Paterson *et al.*, 2003).  
9 Mycophenolic acid-producing strains of *P. brevicompactum* have been isolated  
10 from different origins (Vinokurova *et al.*, 2005) such as ginger (Overy & Frisvad,  
11 2005), but no reports of mycophenolic acid-producing strains from grapes have  
12 been published so far. The mycophenolic acid has been found to affect many  
13 physiological processes. It shows antibiotic, anti-tumour, anti-psoriasis and  
14 immunosuppressive features, and it is currently used in Medicine (Bentely, 2000).  
15 Additionally, it might be relevant in secondary mycotoxicosis (bacterial infection  
16 caused by intake of immunosuppressive mycotoxins). Since *P. brevicompactum*  
17 produces diverse toxic compounds such as mycophenolic acid, brevianamide A  
18 and patulin and it is an ubiquitous fungus, it should be considered as a threat to  
19 commodity system (Paterson *et al.*, 2004).

20  
21 *Penicillium brevicompactum* Dierckx (Section Coronata) is included in the  
22 important group of terverticillate penicillia which also includes the Sections  
23 *Roqueforti*, *Chrysogena*, *Penicillium*, *Digitata* and *Viridicata*. The classification of  
24 this group has been in a state of change during the history of the genus.

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3 1 Taxonomic keys to identify *Penicillium* spp. are based primarily on morphological  
4  
5 2 criteria, such as micromorphology, macromorphology and colours produced in the  
6  
7 3 mycelium or diffused into the medium (Raper & Thom, 1949; Ramírez, 1982; Pitt,  
8  
9 4 1991; Samson & Frisvad, 2004). However many authors have shown that some of  
10  
11 5 these characteristics (i.e. colony texture) are highly variable and therefore do not  
12  
13 6 always allow unambiguous classification (Frisvad & Filtenborg, 1983). As an  
14  
15 7 alternative to secondary metabolites profiling (Frisvad & Filtenborg, 1983),  
16  
17 8 detection of specific enzyme activity and isoenzyme electrophoretic patterns  
18  
19 9 (Frisvad, 1981; Paterson *et al.*, 1989), have been used. More recently genotypic  
20  
21 10 characterization (RFLPs, rRNA genes sequence, RAPDs) has proven its utility  
22  
23 11 (Dupont *et al.*, 1999; Pianzola *et al.*, 2004). However, although phylogenetic  
24  
25 12 studies of *P. brevicompactum* and related species have been recently developed  
26  
27 13 (Peterson, 2000; Peterson *et al.* 2004) a specific method to detect *P.*  
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29 14 *brevicompactum* based on PCR has not been reported.  
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15  
16 The objectives of this work were to analyse the ability to produce mycophenolic  
17 acid by *P. brevicompactum* strains isolated from grapes and to design specific  
18 primers, based on ITS region, and the corresponding PCR assay to detect this  
19 species.

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## 2 **Materials and Methods**

### 3 *Fungal Isolates and culture conditions*

4 All the isolates used in this study, along with their sources, are given in Table I.  
5 The isolates were obtained in our laboratory from grapes collected from Spanish  
6 vineyards or from culture collections: the Spanish Type Culture Collection (Spain),  
7 the Agroindustrial fungi & yeasts collection (MUCL, The Belgian Co-ordinated  
8 Collections of Micro-organisms) and the ARS culture collection (NRRL, United  
9 States Department of Agriculture).

10

11 [Insert table I about here]

12

13 Cultures were maintained on potato dextrose-agar (PDA) medium (Scharlau  
14 Chemie, Barcelona, Spain) at 4° C and stored as spore suspension in 15 %  
15 glycerol at –80 °C. The isolates were cultured in 100 mL Erlenmeyer flasks  
16 containing 20 mL Sabouraud liquid medium (Scharlau Chemie, Barcelona, Spain).  
17 Cultures were inoculated with mycelial disks cut from the plates and incubated at  
18 25 °C, 150 rpm. Mycelia from 2-days-old cultures were harvested by filtration  
19 through Whatman paper n° 1 and kept at –80 °C for DNA isolation.

20

### 21 *Fungal Identification*

22 The strains A and U isolated from Spanish grapes (*Tempranillo* variety) were  
23 identified by the Spanish Type Culture Collection following the protocol described

1  
2  
3 1 by Pitt (1999). The strains were inoculated in Czapek yeast extract agar (CYA),  
4  
5 2 incubated for 7 days at 5 °C, 26 °C and 37 °C, in Malt extract agar (MEA) at 26 °C  
6  
7  
8 3 and in 25% glycerol nitrate agar (G25N) at 26 °C. All identifications were confirmed  
9  
10 4 by amplification of 28S rRNA gene (598 bp) and subsequent sequencing. The  
11  
12 5 sequences showed 100% similarity to *P. brevicompactum* strains from the NCBI  
13  
14 6 and MycoBank (CBS) data bases.  
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#### 19 8 *DNA extraction and PCR amplification.*

20 9 Genomic DNA of the strains listed in Table I was obtained using either the genomic  
21  
22 10 DNA Extraction Kit (Genomix, Talent, Trieste, Italy), according to the  
23  
24  
25 11 manufacturer's instructions, or the protocol described by Querol *et al.* (1992).  
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31 13 The ITS1-5.8S-ITS2 region of the nuclear ribosomal DNA from several *Penicillium*  
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33 14 strains, including the *P. brevicompactum* strains isolated from grapes, was  
34  
35 15 amplified using primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5'  
36  
37 16 TCCTCCGCTTATTGATATG 3') described elsewhere (White *et al.*, 1990). The  
38  
39 17 PCR reaction was performed in an Eppendorf Mastercycler Gradient (Eppendorf,  
40  
41 18 Hamburg, Germany) using 10 pg-10 ng of genomic DNA. The amplification  
42  
43 19 program used was described by Henry *et al.*(2000) and it is as follows: 1 cycle of 4  
44  
45 20 min 30 s at 95 °C, 40 cycles of 30 s at 95 °C (denaturalization), 30 s at 50 °C  
46  
47 21 (annealing), 60 s at 72 °C (extension) and finally 1 cycle of 3 min at 72 °C. The  
48  
49 22 PCR products generated were purified using the High Pure PCR Product  
50  
51 23 Purification Kit (Roche, Mannheim, Germany) and cloned into the cloning vector  
52  
53 24 pCR4-TOPO with the TOPO TA Cloning Kit for Sequencing (Invitrogen, U.K.).  
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3 1 Plasmids were sequenced in both directions using the ABI PRISM DNA Sequencer  
4  
5 2 (Applied Biosystems, Foster City, USA) according to the manufacturer's  
6  
7 3 instructions in the Genomic Unit of the University Complutense of Madrid (Spain).  
8  
9 4 The ITS1-5.8S-ITS2 sequences of the *P. brevicompactum* strains U, A, and CECT  
10  
11 5 2361 were deposited in the EMBL database under accession numbers AJ877044,  
12  
13 6 AJ877045, and AJ877046, respectively.  
14  
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### 20 8 *Development and evaluation of species-specific PCR primers.*

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22 9 The ITS1-5.8S-ITS2 DNA sequences of *P. brevicompactum* and related species  
23  
24 10 obtained in our laboratory and retrieved from data bases (GenBank and EMBL)  
25  
26 11 were analysed and aligned by Clustal method using Dnastar (Lasergene, Madison,  
27  
28 12 Wis., USA). Primers were designed on the basis of these DNA alignments. The  
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30 13 primers obtained were named BRE1 (5'GCTGCCGGGGGACATCTGTCC 3') and  
31  
32 14 BRE2 (5'GGCGCCGGCCAGTCCT ACAAG 3'). The PCR amplification protocol  
33  
34 15 used was as follows: 1 cycle of 4 min 30 s at 95 °C, 25 cycles of 30 s at 95 °C, 20 s  
35  
36 16 at 69.5 °C, 30 s at 72 °C and finally 1 cycle of 5 min at 72 °C. All amplification  
37  
38 17 reactions were carried out in volumes of 25 µL containing 3 µL (10 pg-10 ng) of  
39  
40 18 template DNA, 1.50 µL of each primer (20 µM), 2.5 µL of 10 x PCR buffer, 1 µL of  
41  
42 19 MgCl<sub>2</sub> (50 mM), 0.25 µL of dNTPs (100 mM) and 0.2 µL of Taq DNA polymerase (5  
43  
44 20 U/µL) supplied by the manufacturer (Ecogen, Barcelona, Spain). PCR products  
45  
46 21 were detected in 2% agarose ethidium bromide gels in TAE 1 X buffer (Tris-acetate  
47  
48 22 40 mM and EDTA 1.0 mM). The DNA ladder "Real escala n°2" (Durviz, Valencia,  
49  
50 23 Spain) was used as molecular size marker.  
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3 1 Genomic DNA from the strains indicated in Table I was used for PCR amplification  
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5 2 using the primers BRE1/BRE2 in the conditions described above. The genomic  
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8 3 DNA from all the strains was tested for PCR amplification using primers ITS1 and  
9  
10 4 ITS4 according to Henry *et al.* (2000) using the same master mix used for specific  
11  
12 5 PCR amplification except primers.  
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#### 17 *Reagents and standards for mycotoxin assays*

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20 8 Mycophenolic acid standards were purchased from Sigma (Sigma-Aldrich,  
21  
22 9 Alcobendas, Spain). Acetonitrile, chloroform, acetic acid, methanol (all LC grade)  
23  
24 10 and phosphoric acid (85%, A.R.) were from J.T. Baker (Deventer, Holland). Pure  
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26 11 water was obtained from a Milli-Q Plus apparatus (Millipore, Billerica, Ma., USA)  
27  
28 12 and was used when water was required. Yeast extract and sucrose were  
29  
30 13 purchased from Panreac (Montcada i Reixac, Barcelona, Spain)  
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#### 36 15 *Mycotoxin production*

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38 16 One *Penicillium brevicompactum* strain (CECT 2316) and two isolates of this  
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40 17 species (A and U) from Spanish grapes (Table I) were tested for mycophenolic  
41  
42 18 acid production using Yeast Extract Sucrose (YES) broth (Singh *et al.*, 1991; Overy  
43  
44 19 & Frisvad, 2005) and grape must:water (20:80 v/v). Grape must:water (20:80 v/v)  
45  
46 20 was used to find out whether this substrate had any effect on the fungal  
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48 21 development and/or production of the studied mycotoxins, as two isolates were  
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50 22 from grapes grown in Spain.  
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3 1 Liquid medium (50 mL) was poured into 100 mL Erlenmeyer flasks. The flasks  
4  
5 2 were plugged with cotton, covered with aluminium foil and autoclaved at 115 °C for  
6  
7  
8 3 30 min. Sterilised liquid media were inoculated with 1 mL of a spore suspension  
9  
10 4 made in aqueous Tween 80 (0.5%) having  $10^5$  spores/mL and then they were  
11  
12 5 incubated at 25 °C for 2 weeks. Three flasks per isolate were assayed for  
13  
14 6 mycotoxin accumulation. Controls consisted of non-inoculated sterilized liquid  
15  
16 7 media incubated under the same conditions.  
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8

### 9 *Mycotoxin extraction, clean-up and analysis*

10 Extraction of mycophenolic acid and HPLC analysis was carried out according to  
11 the method described by Medina *et al.* (2004) with slight modifications.  
12

13 Five ml of culture medium were mixed with 500 µL of a 0.1 M solution of  
14 phosphoric acid, extracted with 2 x 5 mL chloroform and, after evaporation of the  
15 organic phase, the residue was dissolved in 0.5 mL of mobile phase  
16 (acetonitrile:water:acetic acid 47:52:1 v/v/v).  
17

### 18 *Chromatographic equipment*

19 The LC system consisted of a Waters 600 pump equipped with a Waters 717  
20 automatic injector and a Waters 474 scanning fluorescence detector (FLD)  
21 (Waters). A Waters 996 photodiode array detector (DAD) was usually set after the  
22 fluorescence detector. Millennium 32® software ver. 4.0 (Waters) was used to  
23 control the chromatograph and to process the signals. Separations were performed  
24 on a stainless steel LiChrospher 100 C<sub>18</sub> reversed-phase column (250 x 4 mm, 5

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3 1  $\mu\text{m}$  particle size) (Agilent Technologies, Waldbronn, Germany) connected to a  
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6 2 guard column (4 x 4 mm, 5  $\mu\text{m}$  particle size) filled with the same phase. The  
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8 3 column was kept at 40 °C. Alternatively, separation was performed on a  
9  
10 4 Phenomenex Gemini<sup>®</sup> C<sub>18</sub> column (150 x 4.6 mm, 5  $\mu\text{m}$  particle size)  
11  
12 5 (Phenomenex, Torrance, Ca., USA) connected to a Phenomenex Gemini<sup>®</sup> C<sub>18</sub>  
13  
14 6 SecurityGuard cartridge (4 x 3 mm, 5  $\mu\text{m}$  particle size). The column was kept at 35  
15  
16 7 °C. The flow rate was 1 mL/min and the mobile phase was acetonitrile:water:acetic  
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18 8 acid (47:52:1 v/v/v). Mycophenolic acid was quantified by DAD at 254 nm.  
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#### 25 10 *Toxin confirmation by LC-MS*

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27 11 LC-ion trap-mass spectrometry was used to unambiguously confirm the possible  
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29 12 presence of mycophenolic acid. The analysis was carried out on an Agilent 1100  
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31 13 liquid chromatograph (Agilent Technologies), equipped with a Zorbax SB-C<sub>18</sub>  
32  
33 14 column (150 x 4.6 mm, 5  $\mu\text{m}$  particle size) (Agilent Technologies) and coupled to a  
34  
35 15 Bruker Esquire 3000 Plus ion trap mass spectrometer (Bruker Instruments,  
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37 16 Billerica, Ma., USA).  
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44 18 For the analysis of mycophenolic acid the mobile phase was  
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46 19 acetonitrile:water:acetic acid (50:49:1, v/v/v). The ionization method was  
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48 20 electrospray ionization (ESI) in positive mode by using the following ionization  
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50 21 source parameters: N<sub>2</sub> nebulizer gas at 10 psi, dry gas at 5 L/min, dry temperature  
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52 22 at 250 °C, and capillary voltage at 4.5 kV.  
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## 1 Results

2 Two specific primers to detect *P. brevicompactum* were designed on the basis of  
3 the alignment of ITS1-5.8S-ITS2 regions performed with a representative sample  
4 of *Penicillium* species available in our laboratory, including A and U isolates from  
5 grapes and closely related species: *P. oslonii* and *P. biourgeianum* (Peterson *et al.*,  
6 2004), the latter considered a synonym of *P. bialowiezense* according to Frisvad &  
7 Samson (2004). Additionally, 26 sequences of *P. brevicompactum* from the  
8 GenBank were also used. Figure 1 shows ITS1-5.8S-ITS2 sequence of *P.*  
9 *brevicompactum* isolate A ([AJ877045](#)) indicating the position of primers BRE-1 and  
10 BRE-2. Primer BRE1 was located within the ITS1, at the position +77, and the  
11 primer BRE2 at the position +445 (within the ITS2).

12  
13 [insert figure 1 about here]

14  
15 All the *Penicillium* strains listed in Table I were tested for amplification using primer  
16 pair BRE1/BRE2. A single fragment of about 390 bp was amplified only when  
17 genomic DNA from *P. brevicompactum* strains was used. No product was  
18 observed with genomic DNA from other *Penicillium* isolates, nor in the case of  
19 species belonging to other *genera* (Figure 2). Control amplifications of the genomic  
20 DNA with primers ITS1 and ITS4 were positive for all the strains analysed.

21  
22 [Insert figure 2 about here]

23

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3 1 The ability of *P. brevicompactum* strains A, U and CECT 2361 to produce  
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5 2 mycophenolic acid was tested by chromatographic analysis. The chromatographic  
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7 3 analysis of culture media using the Phenomenex Gemini<sup>®</sup> C<sub>18</sub> column showed the  
8  
9 4 presence of a peak with a retention time of 5.4 min that was tentatively identified as  
10  
11 5 mycophenolic acid. The identity of mycophenolic acid was confirmed by the UV  
12  
13 6 spectrum that showed absorption maxima at 226.2 nm, 250.9 nm and 305.2 nm,  
14  
15 7 which matched to the absorption maxima shown by the standard. The  
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17 8 mycophenolic acid peak was found in the cultures of the three *P. brevicompactum*  
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19 9 strains (Figure 3).  
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27 11 [Insert figure 3 about here]  
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32 13 The presence of mycophenolic acid in cultures was confirmed by LC-MS based on  
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34 14 the protonated molecule  $[M+H]^+$  and the most abundant product ion  $[(M+H)^+ +$   
35  
36 15  $H_2O]$  whose  $m/z$  ratios were 321 and 338, respectively. The confirmation was also  
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38 16 made using the second fragmentation of ion 321 (LC-MS-MS), obtaining in both  
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40 17 standard and sample an ion with  $m/z$  303 corresponding to the fragmentation  
41  
42 18  $[(M+H)^+ - H_2O]$ .  
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48 20 Production of mycophenolic acid was compared in two different media, YES and  
49  
50 21 grape must:water (20:80 v/v). The results obtained did not show significant  
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52 22 differences between both media (Table II).  
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55 23 [insert table II about here]  
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## 1 Discussion

2 We have developed the first PCR detection assay specific for *P. brevicompactum*.

3 The specificity of this assay has been successfully tested in a wide and diverse  
4 sample of *P. brevicompactum* strains associated to different substrates, other  
5 *Penicillium* species and other fungal species often associated to grapes and  
6 related commodities. This PCR method represents a good alternative to  
7 conventional identification methods, especially difficult in the case of terverticillate  
8 penicillia (Paterson *et al.*, 2004), and does not require highly trained specialists or  
9 expensive equipment (Paterson *et al.*, 2004). The PCR assay described in this  
10 work also represents an advantage in terms of time of analysis and specificity in  
11 comparison with the conventional methods and will facilitate detection of this  
12 species in future surveys of agro-products.

13  
14 The assay developed in this study targeted the multicopy ITS1-5.8S-ITS2 region in  
15 order to improve the sensitivity of the detection. The detection limit of ITS  
16 amplification product, defined as the clearly visible product on agarose gels  
17 containing ethidium bromide, has been estimated between 1 to 10 pg of DNA  
18 template in *Fusarium* (Bluhm *et al.*, 2002). The sensitivity of our PCR assay based  
19 on ITS sequences would be in the same order of magnitude and, therefore, more  
20 sensitive than primers based on single copy gene, estimated between 0.1 and 1 ng  
21 of DNA template per reaction (Bluhm *et al.*, 2002).

22  
23 Those features are very important since *P. brevicompactum* is a ubiquitous and  
24 cosmopolitan fungi that has been isolated from widespread substrates.

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2  
3 1 Furthermore, most *P. brevicompactum* strains isolated from commodities other  
4  
5 2 than grapes were capable to produce mycophenolic acid. Some authors have  
6  
7 3 described production of this metabolite by all the *P. brevicompactum* isolates  
8  
9 4 tested (Overy & Frisvad, 2005) while Vinokurova *et al.* (2005), studying isolates  
10  
11 5 from different origins, pointed out that 60 % of the strains tested were able to  
12  
13 6 produce mycophenolic acid. In the present work, we also report production of  
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15 7 mycophenolic acid by *P. brevicompactum* isolated from grapes for the first time.  
16  
17 8 The high incidence of *P. brevicompactum* strains capable to produce mycophenolic  
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19 9 acid stresses the relevance of this species in terms of food safety.  
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27 11 The levels produced by our isolates grown under the conditions used in this work  
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29 12 were relatively low if compared with levels reported by other authors who found *in*  
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31 13 *vitro* production of this compound around 300 mg/L (Vinokurova *et al.*, 2005). In  
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33 14 their work, however, these authors suggested a strong influence of culture media  
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35 15 on the mycophenolic acid production. Therefore, more studies are necessary to  
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37 16 propose an optimised culture medium to evaluate this metabolite.  
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43 18 The cultivation of *P. brevicompactum* collected from grapes on a grape-like media  
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45 19 (grape must: water, 20:80 v/v) did not result in enhanced levels of mycophenolic  
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47 20 acid production in comparison with YES medium. However, more studies are  
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49 21 needed to confirm that production of mycophenolic acid by *P. brevicompactum*  
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51 22 isolates would not be positively influenced by grape substrate.  
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3 1 The occurrence of *P. brevicompactum* strains in grapes and their ability to produce  
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5 2 mycophenolic acid reported here increases the number and importance of the  
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8 3 dietary foods and derivatives susceptible to be contaminated by this metabolite and,  
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10 4 therefore, we suggest that its inclusion in future survey programs should be  
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12 5 considered by food safety organisations as well as more exhaustive studies on the  
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14 6 effects of mycophenolic acid on the immune response in humans (Overy & Frisvad,  
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16 7 2005).  
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Figure 1. Sequence of ITS1-5.8S-ITS2 of *Penicillium brevicompactum* isolate A (AJ877045). Specific Primers BRE1/2 are underlined. The genes 18S, 5.8S and 28S are shadowed.

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22 CCACCCGTGTTTATTTTACCTTGTGCTTCGGCGAGCCTGCCTTTTGGCTGCCGGGGGAC 120  
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24 ATCTGTC<sup>u</sup>CCCGGGTCCGCGCTCGCCGAAGACACCTTAGAACTCTGTCTGAAGATTGTAGT 180  
25  
26 CTGAGATTAAATATAAAATTATTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCG 240  
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28 ATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAATTCAGTGAATCATCGA 300  
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30 GTCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTCCGAGCGTCAT 360  
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32 TGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCTCCGTCCTCCTCCGGGGGACGGGCCCG 420  
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34 AAAGGCAGCGGGCACCCGCTCCGGTCCTCAAGCGTATGGGGCTTTGTCACCCG<sup>u</sup>CTTTG 480  
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36 TAGGACTGGCCGGCGCCTGCCGATCAACCAAAC<sup>u</sup>TTTTCCAGGTGACCTCGATCAGGAGGG 540  
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6 Figure 2. PCR amplification using primers BRE1/2 and DNA from *P.*  
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8 *brevicompactum* CECT 2316, MUCL 29153, MUCL 38823, MUCL 39543, A and  
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10 U lanes (2-7) respectively, *Penicillium chrysogenum* CECT 2306 (lane 8), *P.*  
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12 *expansum* CECT 2280 (lane 9), *P. simplicisimum* CECT 2747 (lane 10), *P.*  
13  
14 *aurantogriseum* CECT 2320 (lane 11), *P. commune* MUCL 31780 (lane 12), *P.*  
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16 *oslonii* NRRL 13634 (lane 13) and *P. biourgeianum* NRRL 865 (lane 14). Lane  
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1: Non-template control, M: DNA marker

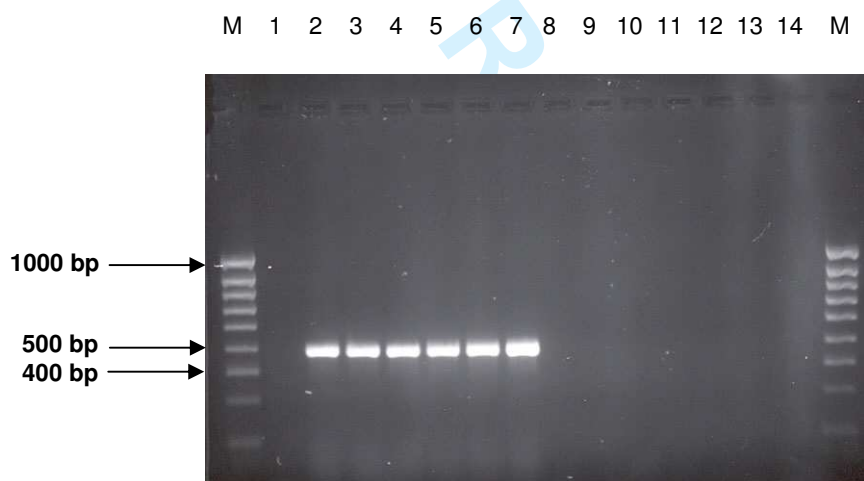
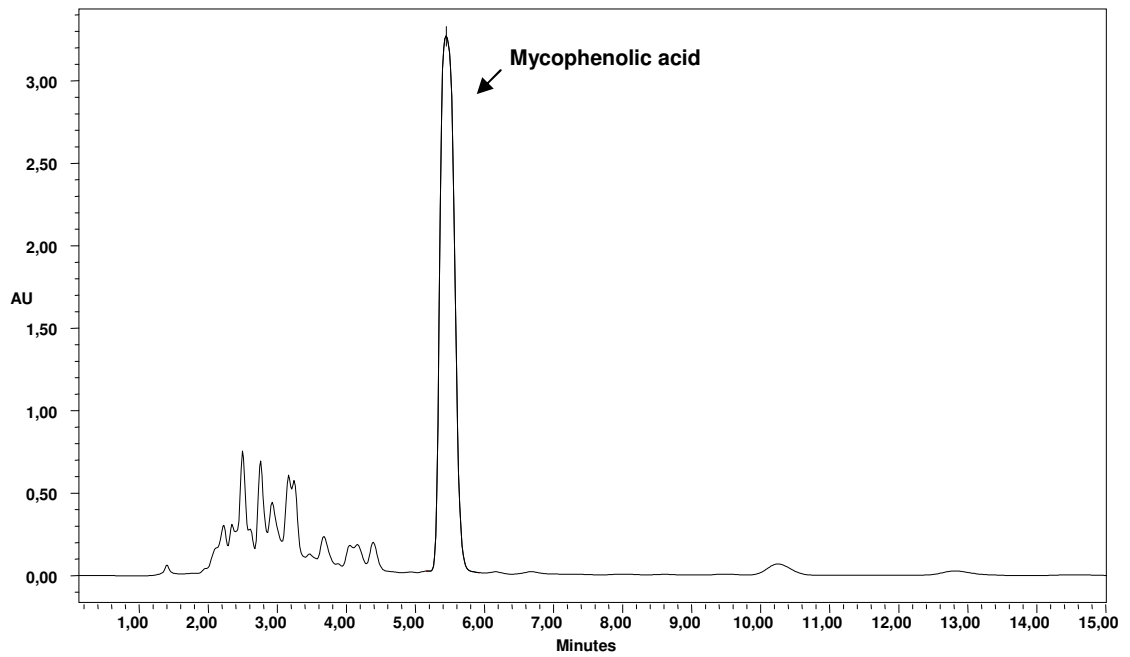


Figure 3. LC-PDA (254nm) chromatogram of water:grape must (80:20 v/v) culture of *P. brevicompactum* (isolate A).



Review Only

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<b>Mycophenolic acid (<math>\mu\text{g/ml}</math>)</b>				
Isolate	YES medium	RSD %	Grape must:water (20:80 v/v)	RSD %
2316	148.878	7.30	90.910	11.37
A	130.528	7.38	115.309	7.18
U	183.256	1.70	134.350	4.81

Table II: Mycophenolic acid production by strains of *P. brevicompactum* isolated from grapes grown in Spain. RSD: relative standard deviation.

Strains	Species	BRE1-2
A (a)	<i>P. brevicompactum</i>	+
U (a)	<i>P. brevicompactum</i>	+
CECT 2316	<i>P. brevicompactum</i>	+
MUCL 29153	<i>P. brevicompactum</i>	+
MUCL 38823	<i>P. brevicompactum</i>	+
MUCL 39543	<i>P. brevicompactum</i>	+
NRRL 2011	<i>P. brevicompactum</i>	+
NRRL 28120	<i>P. brevicompactum</i>	+
NRRL 865	<i>P. biourgeianum</i>	-
NRRL 28149	<i>P. biourgeianum</i>	-
NRRL 13634	<i>P. oslonii</i>	-
NRRL 31467	<i>P. oslonii</i>	-
CECT 2306	<i>P. chrysogenum</i>	-
CECT 2264	<i>P. chrysogenum</i>	-
CECT 2784	<i>P. chrysogenum</i>	-
CECT 2668	<i>P. chrysogenum</i>	-
CECT 2280	<i>P. expansum</i>	-
CECT 2275	<i>P. expansum</i>	-
CECT 2747	<i>P. simplicissimum</i>	-
CECT 2746	<i>P. simplicissimum</i>	-
CECT 2918	<i>P. aurantiogriseum</i>	-
CECT 2320	<i>P. aurantiogriseum</i>	-
CECT 2270	<i>P. corylophium</i>	-
CECT 2273	<i>P. corylophium</i>	-
CECT 2735	<i>P. raistrickii</i>	-
CECT 2737	<i>P. raistrickii</i>	-
CECT 2736	<i>P. raistrickii</i>	-

MUCL 28653	<i>P. commune</i>	-
MUCL 31780	<i>P. commune</i>	-
CECT 2906	<i>P. verrucosum</i>	-
C.AL.P.1 (a)	<i>P. sclerotiorum</i>	-
L (a)	<i>P. polonicum</i>	-
R.Te.P.4 (a)	<i>P. chrysogenum</i>	-
B.Me.P.13 (a)	<i>P. expansum</i>	-
CL.1 (a)	<i>Cladosporium sp</i>	-
UCO.1 (a)	<i>Alternaria consortiale</i>	-
T.MV.F.1 (a)	<i>Fusarium sp.</i>	-
BO.1 (a)	<i>Botrytis sp</i>	-
CECT 2086	<i>Aspergillus carbonarius</i>	-
Z.M.A.29 (a)	<i>Aspergillus niger</i>	-
T.TT.A5 (a)	<i>Aspergillus tubingensis</i>	-
T.MV.A .21 (a)	<i>Aspergillus fumigatus</i>	-
U-2003 (a)	<i>Aspergillus ochraceus</i>	-

Table I: Strains analysed indicating species and the occurrence of PCR amplification product with the pair of primers BRE1-2. (a): Strains isolated from different grape varieties