The development of genetic and molecular markers to register and commercialize *Penicillium rubens* (formerly *Penicillium oxalicum*) strain 212 as a biocontrol agent

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Summary

*Penicillium oxalicum* strain 212 (PO212) is an effective biocontrol agent (BCA) against a large number of economically important fungal plant pathogens. For successful registration as a BCA in Europe, PO212 must be accurately identified. In this report, we describe the use of classical genetic and molecular markers to characterize and identify PO212 in order to understand its ecological role in the environment or host. We successfully generated pyrimidine (*pyr*) auxotrophic mutants. In addition we also designed specific oligonucleotides for the *pyrF* gene at their untranslated regions for rapid and reliable identification and classification of strains of *P. oxalicum* and *P. rubens*, formerly *P. chrysogenum*. Using these DNA-based technologies, we found that PO212 is a strain of *P. rubens*, and is not a strain of *P. oxalicum*. This work presents PO212 as the unique *P. rubens* strain to be described as a BCA and the information contained here serves for its registration and commercialization in Europe.

Introduction

The soil-borne fungus, *Penicillium oxalicum* Currie and Thom strain 212 (PO212), is an effective biocontrol agent (BCA) against a large number of economically important fungal plant pathogens that infect different horticultural crops in growth chamber, glasshouse and open-field experiments (De Cal et al., 1995; 2009; Larena et al., 2003; Sabuquillo et al., 2005). PO212 is also effective against potato cyst nematodes under laboratory conditions (Martinez-Beringola et al., 2013).

For successful registration of a potential BCA, the microorganism in the BCA must be accurately identified (Strauch et al., 2011). Accurate identification of the microorganism in a potential BCA is not an inconsequential process because taxonomic classifications are continuously revised and incorrect synonymizations are frequent (Visagie et al., 2014). DNA-based technologies, such as DNA fingerprinting, and molecular markers, such as those that are based on the internal transcribed spacer (ITS) of ribosomal RNA genes, are now commonly used to detect and identify fungi (McCartney et al., 2003; Lievens and Thomma, 2005). According to Regulation (EC) 1107/2009 of the European Parliament and of the Council of 21 October 2009 (EU 2009), the ecological interactions of PO212 in the rhizosphere and in soil must be fully understood in order to register PO212 as a BCA in Europe. The ecological interactions between a BCA, the pathogen and the host involve a number of genes, compounds and molecular mechanisms. Accordingly, understanding these interactions necessitates developing means for detecting PO212 in the ecosystem, and our current knowledge of the cellular and molecular basis of these interactions for PO212 is lacking (Vinale et al., 2008). Hence, the main purpose of this investigation was to develop genetic and molecular markers for detecting PO212 in the ecosystem. To this end, we undertook a series of genetic and molecular studies to develop DNA-based technologies for identifying, characterizing and monitoring PO212 in the environment when it is used as a BCA.

In transformation experiments, antibiotic and/or antifungal-resistant genes or auxotrophic complementation are frequently used as selectable markers. Although dominant selection markers in fungi are often antifungal-resistant genes, the permanent expression of antifungal-resistant genes is of great concern for food-related organisms, such as *Penicillium camemberti* (Navarrete et al., 2009) and for BCAs, such as PO212. Consequently, metabolic selectable markers are preferred, and the pyrimidine biosynthetic pathway is a frequently used source of such markers. In *Saccharomyces cerevisiae*, the *URA3* gene encodes for orotidine-5’-monophosphate.
decarboxylase (OMPD) (Boeke et al., 1987) and URA3 gene homologues exist in filamentous fungi, such as the pyr-4 gene in Neurospora crassa and the pyrG gene in Aspergillus nidulans and other Penicillium spp. (Palmer and Cove, 1975; Perkins et al., 1982; Díez et al., 1987). ura3 or pyrG mutants can be easily obtained by selecting for resistance to the toxic antimetabolite, 5-fluoroorotic acid (5-FOA) (Díez et al., 1987). The OMPD enzyme catalyses the synthesis of uridine 5′-monophosphate (UMP) from orotidine 5′-monophosphate (OMP) (Wittmann et al., 2008). The six biochemical steps of the de novo pathway for pyrimidine biosynthesis, which comprises the pathway to UMP, the precursor for all pyrimidine nucleotides, are conserved in all known organisms (Aleksenko et al., 1999; Ralli et al., 2007). The biosynthesis of UMP in filamentous fungi proceeds from aspartate and carbamoyl phosphate through the intermediate, orotic acid to OMP (Díez et al., 1987). In animals and fungi, the first two steps of the pathway are performed by a multifunctional enzyme, pyrABCN, which comprises the activities of carbamoyl phosphate synthetase (CPSase) and aspartate transcarbamylase (ATCase) (Aleksenko et al., 1999). The last steps of the pathway are performed by the following enzymes: dihydroorotase (DHOase, pyrD, gene designation follows the A. nidulans nomenclature), dihydroorotate dehydrogenase (DHOdehase, pyrE), orotate phosphoribosyltransferase (OPRTase, pyrF) and orotidine 5′-monophosphate decarboxylase (OMPdecase, pyrG) (Fig. 1). Pyrimidine auxotrophic orotidine-5′-phosphate decarboxylase mutants of several fungi, such as S. cerevisiae (Boeke et al., 1984), Podospora anserina (Boeke et al., 1984; Razanamparany and Begueret, 1986), Penicillum chrysogenum (Díez et al., 1987), Aspergillus niger (Goosen et al., 1987) and Trichoderma reesei (Berges and Barreau, 1991), have been isolated by screening for resistance to 5-FOA (Fig. 1).

Although DNA-based technologies have been used to detect and identify Penicillium spp. in various commercial areas, such as the food industry (Navarrete et al., 2009), these technologies have not been developed for identifying PO212 when it is used as a BCA. Hence, the secondary objectives of the investigation were (i) to obtain pyr-mutants of PO212 in order to study the in vitro interactions between PO212, the pathogen and the host; (ii) to develop a molecular technique for accurately detecting and identifying PO212; and (iii) to identify different Penicillium spp. in field samples using a pyr gene as a molecular marker.

**Results**

**Isolation of 5-FOA-resistant PO212 mutants**

First, we analysed the sensitivity of PO212 to 5-FOA. PO212 did not grow on potato dextrose agar (PDA) or A. nidulans minimal medium (MMA) supplemented with 4 mg ml⁻¹ of 5-FOA after 4 days, but the growth of PO212 was restricted when supplemented with 1.5 and 2 mg ml⁻¹ of 5-FOA. Thus, we used the last conditions to isolate pyr- mutants. Twenty putative pyr- mutants of PO212 were obtained on MMA that was supplemented with 1.5–2 mg ml⁻¹ of 5-FOA, 1.22 mg ml⁻¹ of uridine and 0.56 mg ml⁻¹ of uracil after 7 days of incubation at 22–25°C. Twelve of the 20 5-FOA-resistant PO212 colonies were found to be uridine/uracil auxotrophs.

**Sequence analysis of the CDS of the pyrF and pyrG genes**

The most common mutations in the pyrimidine biosynthetic pathway that confer resistance to 5-FOA are

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**Fig. 1.** Pyrimidine biosynthetic pathway in Penicillium spp. The chart is depicting the genes and enzymes belonging to the pyrimidine biosynthetic pathway in Penicillium chrysogenum (Pc) and Penicillium oxalicum (Po/PDE). Gene designations are indicated on the left and enzyme names are on the right, and Aspergillus nidulans gene designation for each step. Each of the products in this pathway is indicated in the squares. Mutations allowing 5-FOA resistance involved the last two steps prior to UMP biosynthesis.

A. nidulans minimal medium (MMA) supplemented with 4 mg ml⁻¹ of 5-FOA after 4 days, but the growth of PO212 was restricted when supplemented with 1.5 and 2 mg ml⁻¹ of 5-FOA. Thus, we used the last conditions to isolate pyr- mutants. Twenty putative pyr- mutants of PO212 were obtained on MMA that was supplemented with 1.5–2 mg ml⁻¹ of 5-FOA, 1.22 mg ml⁻¹ of uridine and 0.56 mg ml⁻¹ of uracil after 7 days of incubation at 22–25°C. Twelve of the 20 5-FOA-resistant PO212 colonies were found to be uridine/uracil auxotrophs.
described in the pyrF and pyrG genes (Campuzano et al., 1993). Consequently, we proceeded to identify such mutations by sequence analysis of the polymerase chain reaction (PCR)-amplified fragments that corresponded to the CDS of the pyrF and pyrG genes of PO212. For this purpose, we relied on the nucleotide sequence of the pyrF homologue, PDE_03104, which was recently deposited in the genome database of P. oxalicum strain 114-2 (Liu et al., 2013), to generate specific oligonucleotides, complementary to sequences at untranslated regions (UTRs), for amplifying the complete CDS of the pyrF gene (Po-pyrF) (Fig. 1 and Table 2). We were unable to amplify any fragment by PCR using the Po-pyrF primers and genomic DNA (gDNA) from PO212. However, we were able to amplify a fragment using gDNA from strain PO6 (P. oxalicum strain DAOM213171; Table 1). Interestingly, the nucleotide sequence of the ITS1-ITS2 regions of the rDNA of PO212, which is deposited in GenBank database (entry EF103449), was similar to that of P. chrysogenum/rubens and other closely related species. Hence, we decided to design specific oligonucleotides from the reference genome of P. rubens Wisconsin strain 54–1255 (van den Berg et al., 2008) for amplifying a P. chrysogenum pyrF homologue (Pc-pyrF, Pc15g00490, Table 2) by PCR. To this end, a DNA fragment (860 bp) of gDNA of PO212 was first amplified using specific oligonucleotides that were complementary to the Pc-pyrF gene, and its nucleotide sequence was then compared with the genome of P. rubens Wisconsin strain 54–1255. We found that the sequence of this fragment was identical to that of the Pc15g00490 gene (738 nucleotides, Fig. S1) that encodes a protein of 245 amino acids with putative OPRTase activity.

Table 1. Culture collection number, source, origin and GenBank accession number of the 28 Penicillium strains used in the investigation.

| Isolate | Culture collection | Source | Origin | GenBank accession no. |
|---------|--------------------|--------|--------|-----------------------|
| PO212  | ATCC 201888        | Soil   | Spain  | EF103449              |
| PO1    | CBS 300.97         | Soil   | Slovenia | EF103450              |
| PO2    | UAMH 5148          | Poultry feed | Australia | EF103451              |
| PO3    | IMI 253788         | Air    | Spain  | EF103452              |
| PO4    | DAOM 192259        | Foam insulation | Canada | EF103453              |
| PO5    | DAOM 213268        | Stored seeds | Canada | EF103454              |
| PO6    | DAOM 213171        | Cucumber cankers | Canada | EF103455              |
| PO7    | DAOM 214729        | Old cucumber roots | Canada | EF103456              |
| PO8    | ATCC 16501         | Soil   | Mexico | EF103457              |
| PO9    | ATCC 22095         | Maize  | South Africa | EF103458              |
| PO10   | IMI 112755         | Rhizosphere of Vicia faba | Egypt | EF103459              |
| PO11   | IMI 093376         | Rhizosphere of Cicer | India | EF103460              |
| PO12   | CBS 838.96         | Purple Shiso  | Netherlands | EF103461              |
| PO13   | ...                | Stored tobacco | Spain | EF103462              |
| PO15   | ATCC 34885         | PDA contaminant | California, USA | KR233455              |
| PO16   | S53                | Soil   | Spain* | KR233456              |
| PO17   | S73                | Soil   | Spain* | KR233457              |
| PO18   | S62                | Soil   | Spain* | KR233458              |
| PO19   | S27                | Soil   | Spain* | KR233459              |
| PO20   | S56                | Soil   | Spain* | KR233460              |
| PO21   | S17                | Soil   | Spain* | KR233461              |
| PO22   | S49                | Soil   | Spain* | KR233462              |
| PO23   | S59                | Soil   | Spain* | KR233463              |
| PO24   | S60                | Soil   | Spain* | KR233464              |
| PO25   | S71                | Soil   | Spain* | KR233465              |
| PO26   | S63                | Soil   | Spain* | KR233466              |
| PO27   | S73                | Soil   | Spain* | KR233467              |
| PO28   | A1                 | Soil   | Spain* | KR233468              |

Table 2. List and nucleotide sequence of the primers used in this work.

| Primer code | Sequence (5′-3′) |
|-------------|------------------|
| Pc pyrD 1   | GCAAAAAGTGAAGATCGAC |
| Pc pyrD 2   | GTAAGAGGGATGTGCATGTG |
| Pc pyrE 1   | TAATCGCCGTTATAGGTCG |
| Pc pyrE 2   | CAGGATCTATCAAAGACCG |
| Pc pyrF 1   | GACTCTTTGACTCTTTGAC |
| Pc pyrF 2   | TCCATCCTGTTGTCTTTGC |
| Po114-2 PyrF 1 | ACCCTGCCACTTGTGTCGCC |
| Po114-2 PyrF 2 | AGTCGAGTCTTGGTCTTCCTG |
| Pc pyrG 1   | GCCATGTCCTCAAGTGCG |
| Pc pyrG 2   | CTCCTTATTGGCGACCCACGC |
| ITS5        | GGAATGAAAGTGCTAACAG |
| ITS4        | TCTCCGCTTATGTATGCT |
| ITS1        | TCCATCCTGTGTCCTTCG |
| ITS2        | GCTGCGTCTCCCTACCTAGCT |
| ITS3        | GATGCTGGAGAAGACAGCG |
| BOX-A1R     | CTACGGCAAGGCGACGCTAC |
| REP-1R      | IIICGIGCATCIIGG |
| REP-2R      | IICIGCTTATCGGCGCTAC |

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renders a null allele because translation of pyrF mRNA must be affected. Because all mutants are indistinguishable from each other with respect to pyr auxotrophy and they strictly require supplementation with pyrimidines for growth (Fig. 2), we concluded that these pyr- mutants of PO212 carry complete loss of function.

Sequence analysis of the ITS1-5.8S-ITS2 regions

Because the nucleotide sequences of the pyrF and pyrG genes of PO212 were identical to those of the P. rubens homologues, we questioned whether the previous morphological classifications of the P. oxalicum isolates were accurate. Consequently, we amplified and then sequenced the ITS1-5.8S-ITS2 regions of the rDNA gene from the 28 Penicillium strains (Table 2). The nucleotide sequences of the ITS1, 5.8S and ITS2 regions were then compared with that of PO212, and a dendrogram that was based on the pairwise comparison of the nucleotide sequences of the ITS1-5.8S-ITS2 regions of PO212 and the 27 Penicillium strains was constructed. Two distinct groups were identified with a mean level of similarity of 99% (Fig. 3). Most of the Spanish Penicillium isolates clustered in one clade with PO15 isolate from the United States. The other clade comprised the non-Spanish Penicillium isolates except PO3, with PO4 and PO10 being the most divergent isolates (Fig. 3).

Analysis of the BOX and repetitive extragenic palindromic (REP) DNA fingerprints

The DNA fingerprints of PO212 and the 27 Penicillium strains were obtained by amplifying the BOX elements and

Table 3. List of 5-fluoroorotic acid-resistant mutants of Penicillium strain PO212 isolated in this work.

| Mutant strain | Gene | DNA mutation | Change in protein |
|---------------|------|--------------|-------------------|
| PO212_1.5     | pyrF | G377A        | R126H             |
| PO212_3.1     | pyrG | T851G        | W266G             |
| PO212_6.1     | pyrG | C365T        | Q104stop          |
| PO212_18.2    | pyrG | T513G        | L153R             |
| PO212_20.1    | pyrG | G3A          | M11               |

The nucleotide sequence of the CDS of the pyrG gene from P. rubens Wisconsin strain 54–1255 is 831 bp long (GenBank accession number 211583497), and encodes OMPdecase (276 amino acids). Therefore, we amplified the nucleotide sequence of the CDS of the pyrG gene using gDNA of PO212 and specific oligonucleotides (Table 2). The nucleotide sequence of the CDS of the pyrG gene of PO212 was identical to that of the pyrG gene of P. rubens Wisconsin strain 54–1255 (Pc13g04420, Fig. S2). We used the same strategy to amplify, identify and characterize the nucleotide sequence of the CDS of the pyrD and pyrE genes of PO212. We then compared the nucleotide sequences of the CDS of these PO212 genes and the orthologues of P. rubens Wisconsin 54–1255 (Pc06g00380 and Pc22g21410, Fig. S3 and S4), and the results of this sequencing analysis revealed that PO212 is very closely related to P. rubens.

Next we sequenced the CDS of the pyrG gene of each of the five pyrimidine auxotrophic PO212 mutants. We detected changes in the nucleotide sequence of the CDS of the pyrG gene of three pyr- mutants. In the two remaining pyr- mutants in which pyrG sequence was unaltered, we then amplified and sequenced the CDS of their respective pyrF gene. Nucleotide changes were then found in the CDS of the pyrF gene in those two pyr- mutants. Mutations found and the derived amino acid substitutions of pyr- mutants of PO212 are shown in Table 3. Except for the PO212_18.2 mutant that carries a mutation in the pyrG gene that causes a truncation of PyrG protein at amino acid 104, the rest were missense mutations (Table 3). For mutant PO212_3.1, the mutation changes the initiation methionine codon of PyrF to a codon for isoleucine (Table 3), and this mutation probably
the REP sequences in their gDNA. The sizes of all DNA fingerprints were between 100 and 1200 bp. Dendrograms of the phylogenetic relationship between PO212 and the 27 Penicillium strains that were based on the BOX and REP DNA fingerprints were similar to those that were based on the ITS markers: the Spanish Penicillium strains were distinctly different from the non-Spanish Penicillium strains (PO1 from Slovenia, PO6 and PO7 from Canada and PO12 from the Netherlands).

PCR products were obtained from gDNA of the Spanish Penicillium isolates when using the Pc-pyrF oligonucleotides. We also obtained PCR products when we amplified the pyrF gene of PO26 using the primer pairs, Pc-pyrF or Po-pyrF. In contrast, PCR products were not obtained when using gDNA from PO3 and the primer pairs, Pc-pyrF or Po-pyrF. We attributed this failure to amplify the gDNA of PO3 to the low similarity of this Spanish Penicillium isolate with either the Spanish or non-Spanish Penicillium isolates. Furthermore, this finding of a low similarity is consistent with our previous finding that PO3 is closely related to *Penicillium corylophilum* (entry EF103452) in dendrograms that are based on the nucleotide sequences of the ITS1-5.8S-ITS2 regions.

The American and European Penicillium isolates, PO1, PO6, PO7 and PO12, were originally classified as *P. oxalicum* species according to the nucleotide sequence of their ITS1-5.8S-ITS2 regions. We were also able to obtain PCR products when we amplified the pyrF gene of PO1, PO6, PO7 and PO12 using specific Po-pyrF oligonucleotides. These results verify the use of Pc-pyrF and Po-pyrF as primers for amplifying the pyrF gene of *P. rubens* and *P. oxalicum* in order to reclassify Penicillium strains in laboratory collections.

Multiple sequence alignment of the CDS of the pyrF gene of PO212 and the 27 Penicillium strains revealed a high level of conservation of the nucleotide sequence of the CDS of the pyrF homologues (Fig. S1). Examination of the dendrogram that is based on the nucleotide sequence of the CDS region of the pyrF gene also revealed that PO212 is a strain of *P. oxalicum*. Examination of this dendrogram also revealed strong divergence between PO212 and *P. digitatum* and *P. glabrum*. In fact, the nucleotide sequence of the pyrF gene of *Aspergillus clavatus*, which is used to root the phylogenetic tree, revealed that *A. clavatus* is more closely related to the other *Penicillium* spp. than to *P. oxalicum* (Fig. S1). This strong conservation in the nucleotide sequences of the CDS of the pyrF gene, together with the presence of the macro- and micromorphological characteristic of the *Penicillium* strains, strongly suggests that the nucleotide sequences
of the CDS of the pyrF gene can be used to detect and accurately identify *Penicillium* isolates.

**Discussion**

The evaluation and authorization of plant protection products that contain microorganisms are regulated in the European Union (EU) by Regulation 546/2011 (EU 2011). According to these regulations, the identity of a microorganism should be clearly stated for placing of plant protection products on the market. Previous taxonomic classifications of the BCA PO212 and the soil-borne Spanish isolates identified these isolates as strains of *P. oxalicum*, and this identity was based on colour, size and shape of the colony and the various characteristics, such as conidial size and conidiophore morphology (Ramirez, 1982). Using these morphological and phenotypic characteristics to identify *P. oxalicum* strains can be...
challenging, and contradictory findings and grouping of more than one species often occur (Taylor et al., 2000). Species in the *Penicillium* genus exhibit only few distinguishing features, which additionally may vary depending on the growth conditions and the culture medium (Henk et al., 2011). Taxonomic classification in the genus *Penicillium* is challenging because it is being continually reviewed. This ongoing review stems from the genetic diversity of this genus that has been unmasked by random amplified polymorphic DNA markers and nucleotide sequencing and restriction fragment length polymorphism markers of the ITS1-5.8S-ITS2 regions of their rDNA (Dupont et al., 1999; Tiwari et al., 2011). For example, the reference fungus of the genus *Penicillium*, *P. chrysogenum*, has recently been reclassified (Houbraken et al., 2011) following a phylogenetic analysis of numerous *P. chrysogenum* isolates. The results of this analysis revealed the presence of two highly supported clades that represent two species: *P. chrysogenum* and *P. rubens*. Even Fleming’s original penicillin-producing strain and the full genome-sequenced strain of the filamentous fungus, *P. chrysogenum* Wisconsin 54–1255 (NRRL 1951) (van den Berg et al., 2008), have been re-identified as *P. rubens*. Such reclassifications show that no strain of *P. chrysogenum sensu stricto* has had its entire genome sequenced (Houbraken et al., 2011). In this study, we found that well-defined clusters in the dendrograms were based on the ITS1-5.8S-ITS2 regions and the BOX and REP DNA fingerprints of PO212 and the 27 *Penicillium* isolates. These phylogenetic analyses also revealed that all the Spanish *Penicillium* isolates are grouped together, suggesting that they belong to the same *Penicillium* species. Thus, the identification of a strain needs to be based on the newest available methodologies and knowledge about the species and its genus. Progress and advances in molecular and *in silico* genetics have resulted in the invention of novel genetic and molecular tools for rapid identification and accurate taxonomic classification of fungal species (Dupont et al., 1999).

Here we show that distinguishing between strains of *P. rubens* and *P. oxalicum* can be achieved by the use of the *pyrF* gene. Given that the pyrimidine biosynthetic pathway is a highly conserved pathway in the fungal kingdom (see below), sequence analysis of those genes that encode the enzymes in this pathway can be used to accurately classify fungal isolates. Because the size of the CDS of the *pyrF* gene of PO212 is only 738 nucleotides, it is adequate for a standardized process of PCR amplification and sequencing. Conservation of the nucleotide sequence of the CDS of the *pyrF* gene homologues is high, even at the third codon position, and this high level of conservation is most likely a consequence of the reduced size of the *pyrF* gene homologues (Fig. S1). Thus, when changes in nucleotide sequence are found, as in the case of PO212-*pyrF* and *P. oxalicum* 114-2 *pyrF* genes, these can be taken as evidence of variability among *Penicillium* species. Additionally, despite a high level of conservation of the nucleotide sequence of the CDS of the *pyrF* gene homologues, the level of conservation of the nucleotide sequence of the flanking UTRs is low. We found that this feature was very useful for designing the specific primers that we used for amplifying the CDS of the *pyrF* gene by PCR from either *P. rubens* or *P. oxalicum*. We consider that these *pyrF*-specific primers would serve, as those previously designed for other phylogenetic markers (e.g. β-tubulin, calmodulin, RNA polymerase), to achieve with accuracy the identification of these two fungal species at any global location.

Furthermore, identification of *pyrG* and *pyrF* gene homologues of PO212 prompted us to revise the taxonomic classification of PO212 and these other strains in our laboratory collection. As a result of this examination, we concluded that many of naturally occurring isolates from diverse Spanish locations, which were initially classified as *P. oxalicum* using standard taxonomic clues of the genus *Penicillium*, were actually classified as *P. rubens*. Interestingly, the Center for Agricultural Bioscience International has also classified PO212 as a strain of *P. rubens/chrysogenum* using classical phylogenetic markers. This new finding that PO212 is a strain of *P. rubens/chrysogenum* is intriguing because a biocontrol activity has been shown for strains of *P. rubens*. Curiously, it has only been reported to date that dry mycelium preparations of *P. chrysogenum* (*P. rubens*), which is a waste product of the pharmaceutical industry and sometimes used as an organic fertilizer in commercial agriculture, does protect plants against fungal pathogens, such as *Fusarium oxysporum* f. sp. *vasinfectum* and *Verticillium dahliae* Kleb (Chen et al., 2006), and infestations of the nematode, *Meloidogyne javanica* (Gotlieb et al., 2003).

Finally, our work demonstrates that traditional genetic techniques, focused on the isolation of auxotrophic mutants, can be used with a field sample. We successfully isolated uridine- and uracil-requiring mutants of PO212 by selecting 5-FOA-resistant mutants. Loss of function mutations in *pyr* genes have been extensively used in combination with molecular tools to understand numerous biological and biochemical processes in fungi (Ballance and Turner, 1985; Woloshuk et al., 1989). The introduction of such DNA-based technologies will improve our current understanding of the cellular and molecular mechanisms of the interactions between PO212, phytopathogenic fungi and plants. Similar strategies to study plant–fungal interactions have been already used in other fungi (Lagopodi et al., 2002; Olivain et al., 2006). In *Penicillium* (Diez et al., 1987) and other fungi, mutations
resulting in both pyrimidine auxotrophy and resistance to 5-FOA locate in two independent loci (Boeke et al., 1984; Razanamparany and Begueret, 1986; Akileswaran et al., 1993; Takeno et al., 2004). Mutations that modify the activity of OMP-pyrophosphorylase, encoded by the pyrF gene, and the activity of OMP decarboxylase, encoded by the pyrG gene, can be isolated and the resultant mutants exhibit an identical and non-additive pyrimidine-requiring phenotype. Because we were able to characterize these mutations in PO212, we were also able to demonstrate universality of this selection procedure that can be applied to other Penicillium.

In conclusion, we have generated auxotrophic mutants of PO212 in order to improve our current understanding of PO212 as a BCA. We have designed specific oligonucleotides of the pyrF gene to be used in a standard PCR that can be used to rapidly and reliably identify two species of the genus Penicillium, P. rubens and P. oxalicum, in field samples. Furthermore, nucleotide sequencing of the CDS of the pyrG and pyrF genes revealed that PO212 is a strain of P. rubens, and is not a strain of P. oxalicum. The availability of phylogenetic markers and nucleotide sequencing of the genome of fungi has created exciting opportunities for comparative genomics between fungal species. Additionally, their availability and genome sequencing will enable reliable and definitive classification of fungal strains at the species level within a genus. Furthermore, these technologies can be used to characterize the mode of action, the ecology and the fitness of PO212, which is also a required information for their registration and commercialization as a BCA in Europe. In the future, we plan to fully sequence the genome of PO212 and then compare its genome to full-genome-sequenced strains of Penicillium species. Results of all these studies will improve the efficacy and practical application of PO212 when it is used as a BCA for plant disease control.

Experimental procedures

Penicillium strains, growth media and culture conditions

The investigation comprised 28 different strains of Penicillium that were obtained from different geographical regions, habitats and culture collections (Table 1). The Spanish strains were mainly soil-borne fungi and the non-Spanish strains came from various hosts in other global regions. The identity of each Penicillium strain was confirmed using the macro- and micromorphological characteristics of Ramirez (Ramirez, 1982).

The 28 Penicillium strains were stored at −80°C in 20% glycerol (long-term storage) and at 4°C on PDA (Difco, Detroit, MI, USA) slants in the dark (short-term storage). The 28 Penicillium strains were propagated at 22–25°C on PDA, A. nidulans complete medium or MMA, which contained 5 mM of ammonium tartrate as a nitrogen source and D-glucose 1% (p/v) as a carbon source (Cove, 1966). Uridine (1.22 mg ml$^{-1}$) or uracil (0.56 mg ml$^{-1}$) was added to the growth medium when required.

Isolation of nucleic acids

PO212 (ATCC number 201888) was used as the source of gDNA for genome sequencing and for obtaining pyr mutants in mutagenesis experiments. Conidia from wild-type (wt) PO212, the 27 Penicillium strains and the pyrimidine auxotrophic mutants of PO212 were used as the starting material for obtaining mycelia, which were then used for extracting gDNA. These conidial cultures were grown on MMA or PDA at 25°C, and the mycelia were collected by filtration. gDNA was extracted from the mycelia using our previously published protocols (Etxebeste et al., 2009; Larena and Melgarejo, 2009), and the gDNA samples were stored at −20°C until required.

Strategies for obtaining 5-FOA-resistant mutants of PO212

The isolation of 5-FOA-resistant mutants of PO212 was carried out on PDA or MMA plates that were supplemented with 1–4 mg ml$^{-1}$ of 5-FOA (Apollo Scientific, Stockport, UK), 1.22 mg ml$^{-1}$ of uridine and 0.56 mg ml$^{-1}$ of uracil, and incubated at 20–25°C for 5 days. Clones that were resistant to 5-FOA would lack either OPRTase, which is encoded by the pyrF gene, or OMPdecase, which is encoded by the pyrG gene.

The 5-FOA-resistant mutants were isolated, purified through two selective passes on MMA plates that were supplemented with 1–4 mg ml$^{-1}$ of 5-FOA and then tested for uridine auxotrophy on PDA plates with and without uridine and uracil. The pyr mutants do not grow on MMA without uridine and uracil (Fig. 2).

Amplification and sequence analysis of the coding region of the pyr genes

For amplification of the pyr genes, specific primers (Table 2) were designed using the genomic information of P. oxalicum strain 114-2 (formerly classified as P. decumbens; Liu et al., 2013) and P. rubens (formerly P. chrysogenum) (strain ATCC 28089/DSM 1075/Wisconsin 54–1255; van den Berg et al., 2008) in the National Center for Biotechnology Information’s database (http://www.ncbi.nlm.nih.gov/blast). The oligonucleotide pair, Pc-pyrF and Po-pyrF, was routinely used in the taxonomical analyses of all Spanish isolates and a selected number of non-Spanish strains (PO1 from Slovenia, PO6 and PO7 from Canada and PO12 from Netherlands).

The PCRs were performed in a 50 μl reaction mixture that contained standard Taq-polymerase under the following conditions: an initial denaturation step of 2 min at 94°C, followed by 25 cycles of 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C, and a final extension of 3 min at 72°C. The PCR products were purified using the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions, and then sequenced in an automated DNA sequencer (version 3.1; BigDye® Terminator) at the sequencing services of Secugen (http://www.secugen.es; Madrid,
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Spain). Multiple alignments of the orthologous pyrF genes were carried out using ClustalW2 online facility at EBI (http://www.ebi.ac.uk/Tools/msa/clustalw2/) (Fig. S1).

Amplification and sequence analysis of the ITS1-5.8S-ITS2 regions

The ITS1-5.8S-ITS2 regions of the rDNA of each isolate were amplified by PCR with the universal primers, ITS4 and ITS5 (White et al., 1990), whose sequences are shown in Table 2. The PCR conditions for these amplifications were identical to those that are described in Larena and Melgaréjo (2009). The PCR products were purified using the Wizard® SV Gel and PCR Clean-up System (Promega, Madison, WI, USA), and the purified products were then sequenced using the universal primers, ITS1, ITS2, ITS3 and ITS4 (White et al., 1990) (Table 2), in an automated DNA sequencer (ABI 3730, Applied Biosystems, Foster City, CA, USA) at the sequencing services of CiSA-INIA, Madrid, Spain, and the DNA Sequencing Services of Stab Vida España, Madrid, Spain. The nucleotide sequences of ITS1-5.8S-ITS2 regions of the rDNA of each isolate were aligned and compared using BioEdit Sequence Alignment Editor 5.0.6 (Hall, 1999) and ClustalW Multiple Sequence Alignment program (version 1.82; http://www.clustal.org/) (Thompson et al., 1994). The GenBank accession numbers of the nucleotide sequences from each isolate are presented in Table 2.

The nucleotide sequences of ITS1-5.8S-ITS2 regions of the rDNA of the 28 Penicillium strains were assigned to species using the basic local alignment search tool (BLAST; http://www.ncbi.nlm.nih.gov/blast). A dendrogram that was based on the nucleotide sequences of the ITS1-5.8S-ITS2 regions (Fig. 3) was constructed by MEGA (version 5.2; http://www.megasoftware.net/) (Kumar et al., 2004) using the neighbour-joining method (Saitou and Nei, 1987) and the Jukes–Cantor model (Jukes and Cantor, 1969). The reliability of the clusters was assessed by bootstrap analysis with 1000 replicates (Felsenstein, 1985).

Amplification of the BOX element and the REP sequence

BOX-PCRs using the BOX-A1R primer and REP-PCRs using the REP-1R and REP-2R primers (Table 2) (Loulos et al., 1999) were used to generate DNA fingerprints of PO212 and the 27 Penicillium strains. The PCR conditions were identical to those that are described in Redondo et al. (2009). The DNA fingerprints were then visualized in 2% agarose gels under ultraviolet light after their staining with ethidium bromide. Each PCR was carried out at least twice to verify the reproducibility of the bands and reliability of the reaction.

Determination of the phylogenetic relationships between PO212 and other Penicillium strains

In order to determine the phylogenetic relationship between PO212 and the 27 Penicillium strains, their DNA fingerprints were binarized (0 = absent, 1 = present), and Dice coefficients of similarity (Sneath and Sokal, 1973) for pairs of isolates were calculated using the NTSYS-pc software package (version 2.10b; Exeter Software, Setauket, NY, USA) (Rohlf, 2002). The resultant similarity matrices were converted into dendrograms using the unweighted pair group method with arithmetic average method and the sequential, agglomerative, hierarchical and nested clustering program of NTSYS-pc software package (Figs 4 and 5).

Acknowledgements

This study was supported by funds from RTA2007-00067, RTA2010-00093 (Plan Nacional de I + D, Ministerio de Ciencia e Innovación, Spain) and RTA2013-00060-C05-01 (Plan Nacional de I + D, Ministry of Economy and Competitiveness, Spain), and grant BFU2012-33142 from the Ministry of Economy and Competitiveness, Spain. M. Villarino held postdoctoral contracts that were associated to grants RTA2010-00093-00-00 and BFU2012-33142 from the Ministry of Economy and Competitiveness, Spain. The authors wish to thank Y. Herranz for his support and collaboration. The authors would also like to acknowledge Dr. Arieh Bomzon, ConsulWrite (http://www.consulwrite.com) for his editorial assistance in preparing this manuscript.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. Multiple alignment of nucleotide sequences of the coding region of the pyrF genes of Penicillium strain PO212, Penicillium chrysogenum Wisconsin 54–1255 (Pc_w, XM_002560230.1), P. chrysogenum v.1.0 (Pc, fgenesh1_pm.11_#_35, JGI code), P. digitatum (Pd, PDIG_06100m.01, JGI code), P. glabrum (Pg, CE13497_6539, JGI code), Aspergillus clavatus (Ac, XM_001275418.1) and P. oxalicum (Po, EPS28158).

Fig. S2. Multiple alignment of nucleotide sequences of the pyrG genes of Penicillium chrysogenum (rubens) Wisconsin 54–1255 (Pc_w, Pc13g04420), Penicillium strain PO212 (PO212) and P. oxalicum (Po, PDE_04166).

Fig. S3. Multiple alignment of nucleotide sequences of the pyrD genes of Penicillium chrysogenum (rubens) Wisconsin 54–1255 (Pc_w, Pte13g04420), Penicillium strain PO212 (PO212) and P. oxalicum (Po, PDE_04166).

Fig. S4. Multiple alignment of nucleotide sequences of the pyrE genes of Penicillium chrysogenum (rubens) Wisconsin 54–1255 (Pc_w, Pte22g21410), Penicillium strain PO212 (PO212) and P. oxalicum (Po, PDE_03073).