Book Chapter

Comparative Analysis of *Penicillium* Genomes Reveals the Absence of a Specific Genetic Basis for Biocontrol in *Penicillium Rubens* Strain 212

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Abstract

Penicillium rubens strain 212 (PO212) is a filamentous fungus belonging to the division Ascomycete. PO212 acts as an effective biocontrol agent against several pathogens in a variety of horticultural crops including *Fusarium oxysporum* f.sp. *lycopersici*, causing vascular wilt disease in tomato plants. We assembled draft genomes of two *P. rubens* strains, the biocontrol agent PO212 and the soil isolate S27, which lacks biocontrol activity. PO212 genome size is 2,982 Mb, which is currently organized into 65 scaffolds and a total of 10,164 predicted Open Reading Frames (ORFs). The comparative analysis between PO212 and S27 genomes showed high sequence conservation and a low number of variations mainly located in ORF regions. These differences found in coding regions between PO212 and S27 genomes can explain neither the biocontrol activity of PO212 nor the absence of such activity in S27, opening a possible avenue toward transcriptomic and epigenetic studies that may shed light on this mechanism for fighting plant diseases caused by fungal pathogens. The genome sequences described in this study provide a useful novel resource for future research into the biology, ecology, and evolution of biological control agents.

Keywords

PO212; Biocontrol Agent; *Penicillium rubens*; Genome; Comparative Genomics

Introduction

Penicillium rubens strain 212 (PO212, ATCC201888), formerly known *Penicillium oxalicum* [1], is a filamentous fungus belonging to the division Ascomycete. PO212 is a strain that mainly attracts agricultural and biotechnological interest because it is an effective biocontrol agent (BCA) against several pathogens in a variety of horticultural crops [2-5]. Among the fungal pathogens, PO212 acts against *Fusarium oxysporum* f.sp.

lycopersici (Sacc.) W. C. Snyder and H. N. Hans, (FOL), causing the vascular wilt disease in tomato plants [6]. The effective control of tomato wilt is based on the application of PO212 conidia [7] and conidial contact with roots [8]. Plant-fungus contact is achieved by watering seedlings 7 days before transplanting, in seedbeds with a conidial suspension of PO212, at a final conidial density in the seedbed substrate and rhizosphere between 10^6 and 10^7 conidia per gram [2,8,9]. PO212 acts against Fusarium wilt primarily through a mechanism of induced resistance in tomato plants [8-10]. Nevertheless, PO212 strain showed a competition for space and nutrients [11] and promotion of plant growth [6].

Genome sequencing is currently on the rise due to new and faster procedures and reduced sequencing costs. This allows for expanded comparative studies of the genomes of organisms with key roles in health, biotechnology or agriculture. Among these interesting organisms are species of the genus *Penicillium*, especially *P. rubens.* Genome sequences of several *P. chrysogenum* and/or *P. rubens* strains have been reported, as *P. chrysogenum*: P2niaD18 [12] and *P. rubens Wisconsin* 54-1255 (PrWis) [13]. These two strains, P2niaD18 and PrWis, were interesting from a sanitary point of view because of their high penicillin-producing capacity. Notably, these two strains are descendants from the natural isolate NRRL 1951 which was classified as *P. chrysogenum* [14]. Both strains have undergone various mutagenesis procedures and screening methods to improve their respective penicillin yields. This implies that the presence of sequence modifications and reorganizations in the genomes of these strains is highly expected.

PO212 strain was initially misclassified as *Penicillium oxalicum* based on morphological characteristics, such as the color of spore layer on the colony surface, the size and shape of the colony, conidial size, and conidiophore morphology [15]. Subsequent sequencing of internal transcribed spacer (ITS) regions and the isolation and identification of 5-fluoroorotic acid (5-FOA)-resistant mutants showed that PO212 is a strain of *P. rubens* [1]. The first study to understand the genetic basis of PO212 biocontrol activity (BA) focused on the analysis of genes involved in nitrate assimilation since PO212, and other *P. rubens*

strains showing a BA against Fusarium wilt lacked the ability to use nitrate as the main nitrogen source [16]. This nitrate assimilation-deficient phenotype was due to the presence of mutations in the NirA regulator or the nitrate transporter CrnA. However, the complementation of these mutations did not help to understand the BA of PO212 [16].

Genome-wide analyses proved to be a good method to determine the genetic basis behind the biocontrol process in several organisms [17-19]. Moreover, comparative genomics allowed the identification of pathways or mutations between the genomes of different organisms that may be specific to organisms with BA [20]. Comparative analyses of *Trichoderma* spp. genomes revealed notable differences in contrast to the genomes of other multicellular ascomycetes in comparison to publicly available genomes [21]. These analyses of *Trichoderma* spp. represent an useful new resource for the further development of improved and research-driven strategies to select and improve *Trichoderma* species as BCA [21]. Major efforts in technology and bioinformatics tools have significantly increased our knowledge of BCA and their properties [20].

In this study, we conducted a comparative genomic approach to understand the genetic basis of BA in *P. rubens*. We assembled draft genomes of two *P. rubens* strains, the BCA PO212 and the soil isolate S27, which lacks BA. These analyses revealed a significant conservation of genomic sequences among the two strains compared, and evidenced the absence of any specific genes related to biocontrol in PO212.

Materials and Methods

Strains and Growth Conditions

Penicillium rubens strain 212 [6] and S27 [1], were isolated from diverse agricultural soils and plant samples in Spain. Conidia from these strains were stored in 20% glycerol for a long term at -20ºC except for PO212, which was stored at 4ºC as dried conidia. Dried conidia of PO212 were produced in a solid-state fermentation system and dried as previously described by Larena et al. [22]. S27 strain was grown on potato dextrose agar (PDA) or minimal medium (MM; Espeso et al., [16]) with 5 mM ammonium tartrate and D-glucose 1% (w/v) as nitrogen and

carbon source, respectively, and incubated at 25ºC for 5 days. For short-term storage, strain was kept at 4°C on PDA.

The biocontrol activity of *P. rubens* strains was tested using the pathogenic isolate 1A of FOL, provided by Dr. Cristina Moyano from the Laboratory for Assessment of Variety, Seed and Nursery Plants, INIA-CSIC (Madrid, Spain). FOL was stored at 4ºC in tubes containing sterile sand. For mycelial production, conidia from FOL stored in sterile sand were germinated on Czapek Dox agar (CDA; Difco Laboratories, Detroit, MI, United States) and cultivated in darkness at 25ºC for seven days. Microconidial inoculum of FOL was produced in 250-ml flasks containing 150 ml of sterile Czapek Dox broth (Difco). Each flask was inoculated with three mycelial plugs (1 cm diameter) from the 7-day-old cultures on CDA [6] and incubated for 5 days at 25°C in a rotary shaker (model 3527; Lab-Line Instruments, Inc.) at 150 rpm. Microconidia were separated from mycelial mass by filtration through glass wool. The conidial concentration was determined using a hematocytometer and adjusted to 10^6microconidia/ml.

Efficacy Assays

At least two growth chambers experiments were carried out on tomato plants to evaluate the biocontrol efficacy of S27 against FOL such as described by Villarino et al. [23]. Seeds of tomato cultivar "San Pedro", which is susceptible to races 1 and 2 of FOL, were used in all experiments. Tomato seeds were sown in sterile trays (27 cm × 42 cm × 7 cm) that contained an autoclaved (for 1 h at pressure of 1 kg cm^{-2} and temperature of 121ºC, during 3 consecutive days) mixture of vermiculite (Termita; Asfaltex, S.A., Barcelona, Spain) and peat (Gebr. BRILL substrate GmbH & Co. KG; 1:1, *v*:*v*). The trays were maintained in a growth chamber at 25ºC with fluorescent lighting (100 $\mu Em^{-2}s^{-1}$, 16-h photoperiod) and 80-100% relative humidity for 3-4 weeks. Tomato seedlings (with at least two true leaves) were treated 7 days before transplanting with an aqueous conidial suspension (6×10^6 conidia per gram of substrate) of S27 or PO212. Conidial suspensions of PO212 and S27 were prepared as follows: Dried conidia of PO212 were rehydrated in sterile distilled water (SDW) using a rotatory shaker at 150 rpm

for 2 h (CERTOMAT® RM). Conidia of S27 were harvested from colonies grown on PDA and incubated in the dark at 25°C for 7 days. The day before treatment, the viability of PO212 and S27 conidia were estimated by measuring their germination as previously described [22]. For each replicate (three by sample type), the germination of 50 randomly selected conidia was counted and viability was calculated and expressed as a percentage [24]. Seven days after treatment, tomato seedlings were transplanted from seedbeds into 100-ml flasks containing 100 ml of Hoagland solution [25] so that the roots were in contact with the solution, as described by De Cal et al. [26]. An aliquot of an aqueous (SDW) conidial suspension of FOL was added to the flasks just before transplanting so that the final conidial concentration in the flasks was 1×10^5 conidia/ml. Plants that had been inoculated with the pathogen but not treated with any strain of *P. rubens*, were used as the control. Five replicate flasks, each containing four plants, were used per treatment. The flasks were placed in a randomized complete block design in a growth chamber for 4 weeks under the conditions described earlier in this subsection. The complete experiment was done two times. Disease severity was graded on days 7, 14, 21 and 28 after the transplant. Disease severity followed a 1-5 index scale: 1, healthy plants (0-24%); 2, yellow lower leaves (25-49%); 3, dead lower leaves and some yellow upper leaves (50-74%); 4, dead lower leaves and wilted upper leaves (75-99%); and 5, dead plants (100%) [6]. All plant roots were placed in humidity chambers at the end of each experiment, and the presence or absence of the pathogen in the crown after 5 days of incubation at 25°C was recorded.

Data of disease severity and incidence were analyzed by ANOVA with the STATGRAPHICS program (XVII Centurion. v. 17.2.00). When the *F* test was significant at a value of p of < 0.05, means were compared using Student-Newman-Keul's multiple range test [27].

Genomic DNA Extraction, Sequencing, and PCR

Genomic DNA (gDNA) was extracted from the mycelia of *P. rubens* strains (PO212 and S27) grown in liquid MM at 25ºC for 2 days. Mycelia were harvested by filtration using Miracloth

(Calbiochem, Merck-Millipore, Darmstadt, Germany). Samples were lyophilized for at least 6 hours. For each sample, mycelium was pulverized using a ceramic bead in a FastPrep-24 homogenizer (MP Biomedicals™), one pulse for 20 s at minimum speed. A sample of 100 mg of powdered mycelium was mixed with 1 ml of DNA extraction solution (25 mM Tris-HCl pH 8.0, 250 mM sucrose, and 20 mM EDTA pH 8.0). Before incubation for 15 min at 65ºC, 100 µl of 10% SDS were added to each sample. Next, proteins and cellular debris were removed by adding 1 ml of Phenol/Chloroform/Isoamyl alcohol mixture per sample and further mixing on a rotary shaker (Rotator Multi Bio RS-24) during 15 min at room temperature (RT). Organic and aqueous phases were separated by centrifugation on a benchtop centrifuge at maximum speed for 5 min. gDNA was precipitated from aqueous phase by the addition of 1/10 vol of sodium acetate pH 6 and 0.6 vol of 2-propanol, followed by incubation for 15 min at RT and centrifugation for 5 min at RT. To wash the gDNA pellet, 1 ml ethanol (80%) was added, followed by centrifugation at max. speed for 5 min at RT. After drying the ethanol in samples, pellets were dissolved in 500 µl sterile water, and samples were treated with DNase-free RNase A (5mg/ml; 37°C, 60 min). gDNA was newly precipitated and washed as described before. Finally, gDNA was dissolved in 200 µl nuclease-free water and stored at -20ºC until use.

Sequencing of PO212 gDNA was performed on an Illumina MiSeq 500 cycles at the "FPCM, Fundación Parque Científico de Madrid" sequencing facility using 150 bp and 250 bp paired-end sequencing reads. For S27 gDNA, Stab Vida (Portugal) performed the construction and sequencing of DNA libraries. DNA libraries were sequenced on the lllumina Hiseq 4000 platform, using 150 bp paired-end sequencing reads.

Standard PCR protocols were used: initial denaturation at 95ºC for 2 min, 30 cycles of denaturation at 95ºC for 10 s, annealing at 55ºC for 30 s, and extension at 72ºC for 1 min/kb. After 30 cycles, an extension at 72ºC for 10 min and storage at 4ºC. The polymerase Takara (TaKaRa Taq ™) was used for amplification. Oligonucleotides were designed using Vector NTI™ Suite 8 and are listed in Table 1. The oligonucleotides for the amplification

of MAT1-1 and MAT1-2 were previously described in Espeso et al. [16]. PCR products were analysed in 0.8% agarose-TAE electrophoresis and when required, they were purified using the PCR clean-Up kit (Macherey-Nagel), following the manufacturer's instructions. Sequencing of DNA fragments was done by Stab Vida's (Portugal) sequencing service.

Table 1: List and nucleotide sequence of the oligonucleotides used in this work.

Primer code	5'-3' Sequence
g196 F	GGACAGTACGGCATTGGATATTACGGACACC
g196 R	CCAGATTGTGTCCCATAGACGTTGTCCG
g1339 F	CCACACCGTCAGACTTTGGAATCCTATACC
g1339 R	CGACTTGCACGACAAGATGAGTTGGTTTCC
g3160 F	GCTCCGCTGGGAATGTATTATACACCTACG
g3160 R	CTCGCAATTCCTCTTGAGATGGAAGCTCG
g3741 F	GGATCGAACACGAGGGAAGATTCCTTGCC
g3741 R	CCAACACTGTTACAGAAAGCCTCGATGG
g3975 F	GCCAAAGCTCAACCAATACCCAGAGTACC
g3975 R	CGACTATGTCGCTAATTCGCAGGGTCGTGTC
g4471 F	CCAAGATCACCTCAACTTGTCTGCCTCACC
g4471 R	CGTATGGAGGAGCAACGATGAAAGAGGATCG
MAT1-1_fw	TGCAGCTCAAGTTCTACG
MAT1-1_rv[a]	AGGAGTACATCTCATCAACC
MAT1-2_fw	ATGGTGAAGTCTTCCTGCC
MAT1-2_rv[a]	AGAGAGTGGCTCGACACC
nirA 1	CACTAGGCATGCGAAGAGG
nirA 2	TACATCGCTGCTGATCTCGC

[a] These sequences correct the sequences previously published in Espeso et al. (2019).

Assemblies and Comparative Analyses of Genomes

For sequencing of the PO212 genome, two libraries of 150 and 250bp paired-end fragments were produced and raw sequencing reads were subjected to quality control using FastQC program (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). A Q>28 along the read length and the *k*-mers and nucleotide distribution were homogeneous in both libraries. An A5-miseq pipeline was used [28] to assemble PO212 genome. The nuclear reads were extended using FLASH [29] and were assembled using the A5-miseq pipeline, until the output, named final.scaffolds.fasta, was obtained. The previously aligned

mitochondrial reads were assembled to obtain a single mitochondrial contig of ~ 28kbp.

De novo assembly of the S27 genome was performed using the A5-miseq pipeline [28] and further revised with Gapcloser from the Soapdenovo assembler [30]. The aligned S27 mitochondrial reads were assembled apart from the genome in 13 contigs of ~ 34kbp.

Completeness of the genome assemblies was assessed by the Benchmarking Universal Single-Copy Orthologs (BUSCO) v.2.0.1 software tool [31]. The assembled genomes were annotated using the MAKER (v.2.31.9) pipeline [32]. Before annotation, a species-specific repeat library was constructed using RepeatModeler (v.1.0.8) to mask repeats [33]. Gene models were predicted with AUGUSTUS (http://bioinf.uni-greifswald.de/augustus/) [34] using two strategies, *ab initio* gene predictors and training with *Aspergillus nidulans* as a model genome.

Gene models of the PO212 and S27 genomes were manually curated using the P2niaD18 (GCA_000710275) and PrWis (GCA_000226395) strain proteins available in the EnsemblFungi and the National Center of Biotechnology Information (NCBI) database as evidence for gene prediction using Apollo [35]. Predicted proteins were functionally annotated using BLASTp [36] against the non-redundant database of the NCBI and classified using InterProScan and Pfam analysis [37]. The comparison of PO212 and S27 genomes was performed through the Quast bioinformatics tool [38].

MUMmer4 software package [39] was used for pairwise alignment of PO212 and S27 assemblies by setting PO212 as the reference; the minimum length of a matched group to 20 bp and the distance an alignment extension will attempt to extend to poor scoring regions before yielding to 100 bp. Comparative analysis was performed using Circos tools [40]. Moreover, CLC Genomics Workbench 12 (QIAGEN Bioinformatics; https://digitalinsights.qiagen.com/products-overview/discovery-insights portfolio/analysis-and-visualization/ qiagen-clc-

genomics-workbench/) was used to align the reads of S27 to the assembled genome of PO212. The CLC alignment workflow was used to map the raw reads to a reference genome and to detect variants. Default values were used, except for the minimum coverage and minimum count, which were set to 10 and 2, respectively, to avoid loss of information. Of all the variations found, homozygous variations were selected. Only variations with 100% of frequency were taken into account for manual verification by PCR amplification using specific primers and sequencing. To visualize amino acid changes in genes between PO212 and S27 strains, we used Integrative Genome Viewer (IGV version 2.9.4; Robinson et al., [41]).

Results

Genome Sequencing of PO212, Assembly, and General Characteristics

The genome of PO212 was sequenced *via* paired-end Illumina MiSeq technology, providing two DNA-seq libraries: one paired-end small fragment library and a second obtained from long-range DNA fragments. The resulting assembly was performed *de novo* and evaluated in terms of N50 and L50 using Quast. Automated assembly and manual sequence verification yielded an estimated PO212 genome size of 29.82 Mb at ~ 200X coverage. PO212 genome was organized into 65 scaffolds with an N50 scaffold length of 1.88 Mb, comprising the first six longest scaffolds and reaching an N90 scaffold length of 0.38 Mb, corresponding to the first 18 scaffolds (Table 2). PO212 genome had a GC content of 49.07%, similar to that of other *Penicillium* genomes. After the manual curation of automated ORF predictions by AUGUSTUS, we determined at least 10,164 ORFs in the PO212 genome.

The data presented in this study are deposited in the GenBank repository, accession number JAPDLE000000000.

Table 2: Assembly and gene prediction summary of PO212 and S27 genomes.

Strain	Scaffolds	Assembly Size (Mb)	Largest Scaffold (Mb)	N50 (Mb)	BUSCO completeness (%)	Predicted genes
PO212	65	29.82	3.49	1.88	98.5	10,164
S27	414	29.89	1.65	0.42	98.3	10,164

S27 Lacks Efficacy against Fusarium Wilt

The S27 strain was isolated from the soil and was primarily classified as a non-BA strain [1]. Notably, S27 displayed similar colonial morphology to PO212 (Figure 1). Sequencing of ITS1-5.8S-ITS2 regions classified S27 as a *P. rubens* strain, and a dendrogram based on BOX and repetitive extragenic palindromic (REP) DNA fingerprints placed S27 close to the isolate PO212 [1].

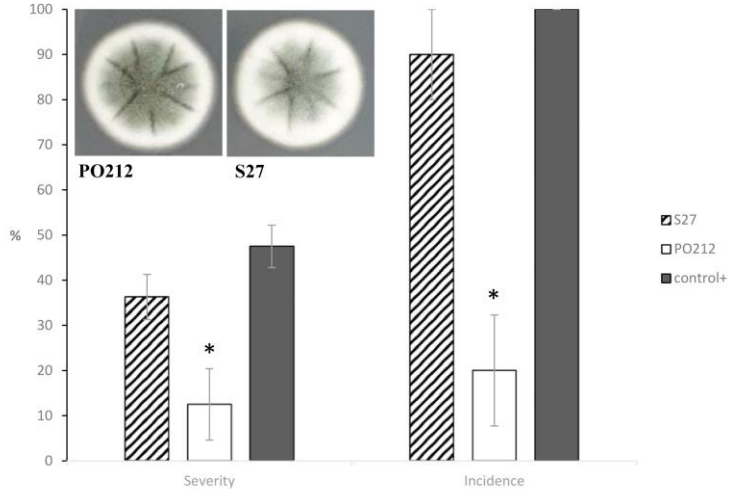

Figure 1: Cultures of 7-day-old *Penicillium rubens* strains 212 and S27 growing on potato dextrose agar (PDA) at 25ºC (front). The graph shows the effect of PO212- and S27- treatment on percentage of disease severity and incidence caused by *Fusarium oxysporum* f.sp. *lycopersici* (FOL) in tomato plants cv. "San Pedro" at 28 days after FOL-inoculation under controlled growth chamber conditions. PO212 and S27 treatments were applied to seedlings 7 days before transplanting by watering with a conidial suspension to a final concentration of $6x10^6$ conidia g^{-1}. Control+, untreated and FOL-inoculated plants. Data are the mean value of five replicates (flasks) per treatment and four plants per replicate. Asterisks in each parameter are significantly different from each other ($p< 0.05$) according to the Student–Newman–Keuls multiple range test. Vertical bars represent the standard error of the mean of five replicates. MSE is the mean squared error of ANOVA. MSE 182.292 (Severity) and 416.667 (Incidence).

The efficacy of S27 against Fusarium wilt in tomato plants was recently determined. In contrast to PO212, strain S27 did not

significantly ($p \leq 0.05$) reduce either the disease severity or incidence caused by FOL in tomato plants. FOL-inoculated and S27-treated plants showed similar symptoms to FOL-inoculated and untreated control plants (control +, Figure 1). Thus, we chose S27 for genomic sequencing as the best tool for a comparative genomic analysis with PO212 to target genes potentially related to PO212 BA.

Comparative of PO212 and S27 Genomes

The genome of the S27 strain was sequenced using the lllumina Hiseq 4000 platform by 150 bp paired-end sequencing reads that generated 2,345 Mbp (15,532,752 sequence reads). These reads showed a coverage of ~ 82.3x over PO212 assembly. De novo assembly of the S27 genome was performed and evaluated in terms of N50 and L50 using Quast. Automated assembly and manual sequence verification allowed estimating for the S27 strain a genome size of 29.89 Mb. S27 genomic assembly was organized into 414 scaffolds with an N50 scaffold length of 0.42 Mb. At least 10,164 coding sequences were estimated after manual curation of ORF prediction by AUGUSTUS. Table 2 shows a summary of the two genomes. The data presented in the study are deposited in the GenBank repository, accession number JAPDLD000000000.

Genome wide alignment showed the strong conservation of PO212 and S27 genomic sequences (Figure 2). MUMmer plot showed that 99.95% of the PO212 genome assembly matched that of the S27 strain with identity equal to or greater than 75%. Only 0.05% of the genomic sequence seemed to be unique to PO212 but did not contain any predicted gene model.

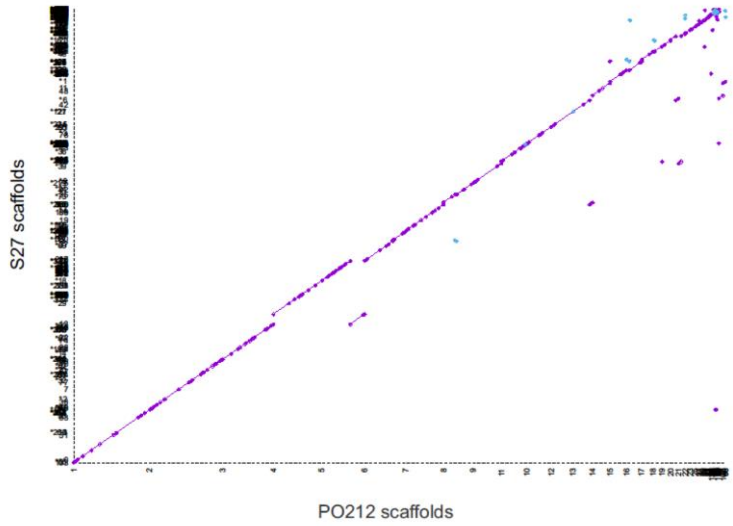

Figure 2: Whole-genome dot-plot showing regions of forward alignment (in purple), translocations (displacements from the diagonal), inversions (in blue), and duplications (parallel diagonal lines) between *Penicillium rubens* strains 212 (PO212) and S27. The PO212 assembly (*X* axis) was used as a reference.

Quast analysis also revealed the high similarity between PO212 and S27 genomes. Comparisons of the S27 and PO212 assemblies showed that up to 99.81% of the S27 genome was represented in PO212 and a duplication ratio of 1.002. In this comparison 1,890 mismatches were found, a rate of 6.34 changes per 100 kbp of genomic sequence, which was significantly lower than previous genomic comparison. The phylogenetic trees using either multilocus or genomic analysis also confirmed the proximity of the two strains PO212 and S27 and other *P. rubens* strains by joining them in a clade [42].

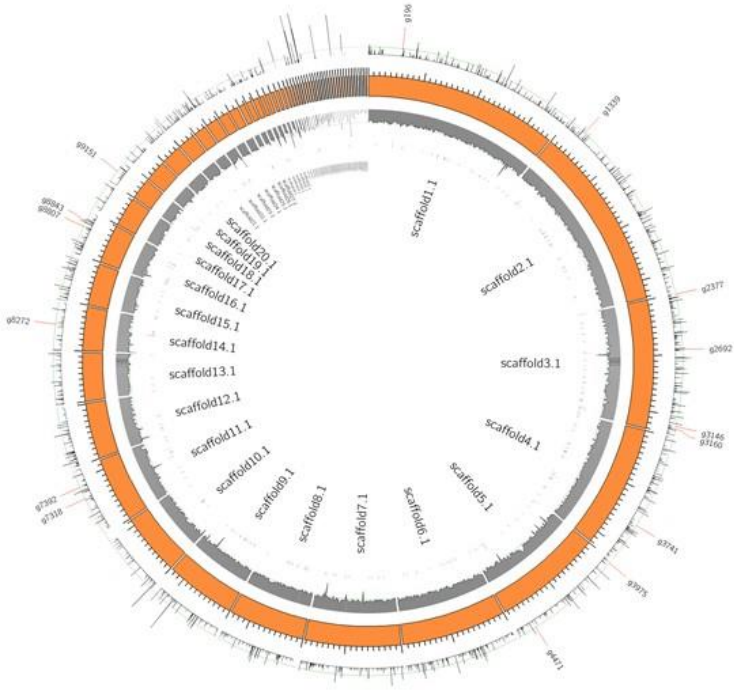

Figure 3: Genome wide SNVs of PO212 genome against S27. Orange boxes present scaffolds of the PO212 genome. The first inner track, the gray histogram, shows the coverage of the S27 reads aligned against the PO212 genome. The data show full coverage of the PO212 genome. The outermost track represents the SNV density of the PO212 genome against the aligned S27 reads. The innermost track, black ticks, represents genes containing SNVs in their coding region. 15 genes are highlighted with longer ticks in the innermost track and labeled in the outermost track. These 15 genes showed 100% allelic variation on the SNV loci of PO212 when compared with S27.

Figure 3 shows a circos ideogram representing a comparison between the assembly of PO212 and S27 raw reads. S27 sequencing data present full coverage of the PO212 genome. Figure 4A shows that 97.86% of S27 reads mapped over the 65 scaffolds PO212 genomic assembly. The remaining 2.14% of S27 reads mapped to the mitochondrial genome. With the aid of Circos analysis and CLC software, we searched for those Single Nucleotide Variants (SNVs) displaying 100% allelic variation between S27 and PO212 genome. We detected at least 104

variations between both genomic sequences. Most of this low number of variations was classified as SNVs (Figures 3, 4B). In addition to 71 nucleotide changes found in coding or intergenic regions, we found seven Multi-Nucleotide Variants (MNVs), 14 insertions, nine deletions, and three replacements (Figure 4B). Only 15 of these variations were found in coding regions (Figures 3, 4C). However, manual curation still reduced the number of variations to six with amino acid change (see Figure 5 and Table 3) and two silent mutations.

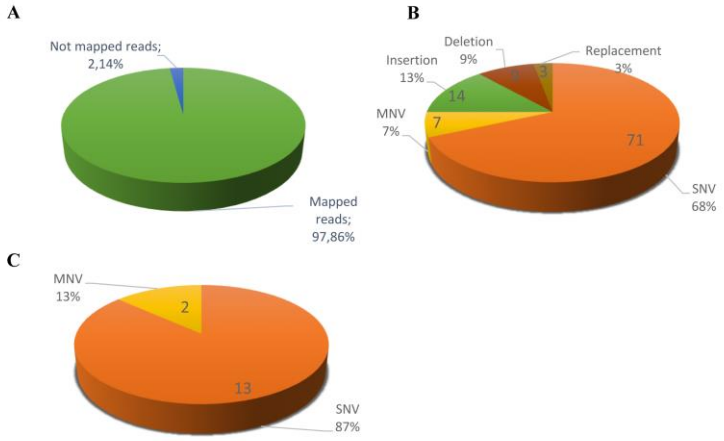

Figure 4: Variations between *Penicillium rubens* strains 212 (PO212) and S27 genomes. (**A)** S27 mapped reads vs. the un-mapped reads using PO212 as a reference genome. **(B)** Differences between two strains classified according the type of mutation: 71 SNVs, 7 MNVs, 14 Insertions, 9 Deletions, and 3 Replacements. **(C)** Differences between the two strains in the coding regions with 100% of frequency: 13 SNVs and 2 MNVs. After a review with preliminary RNA-Seq data support, we discarded seven variations, considered them as mistakes, and classified two as silent variations, since they did not cause an amino acid change.

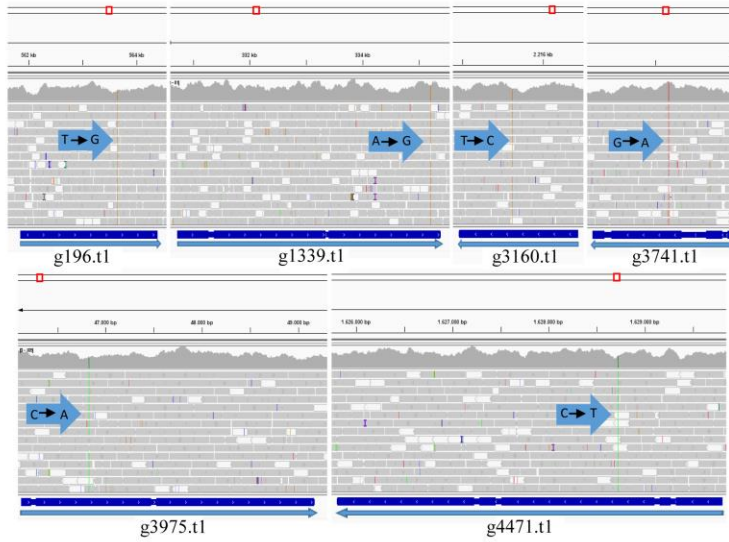

Figure 5: Graph mapping the S27 reads against the PO212 reference genome in the genes containing the variation (PO212 genes): g196.t1 (Dimethylglycine oxidase), g1339.t1 (Vegetative incompatibility protein HET-E-1), g3160.t1 (Putative mitochondrial chaperone), g3741.t1 (Hypothetical protein), g3975.t1 (RNA polymerase I specific TF), and g4471.t1 (Hypothetical protein). Arrows indicate the point of variation and nucleotide change (Visualized with IGV).

We focused on six genes that carried an SNV causing an amino acid change. Only one SNV caused an early stop in the coding sequence. In g4471.t1 gene (orthologue of Pc16g08360 in PrWis), the CGA codon for arginine 327 was changed to a TGA stop codon. This SNV caused the truncation of the hypothetical protein S27g3229.t1 at amino acid 326, missing 3/4 of the ORF compared to the predicted PO212 protein (g4471.t1; Table 3). The remaining SNVs described in Table 3 caused punctual amino acid substitutions (Figure 5). Notably, these SNVs were found only in S27. The orthologues of PO212 showed the same nucleotide sequences as in the PrWis and P2niaD18 reference genomes.

Table 3: List of the genes whose sequences present variations between PO212 and S27 strains and the affected amino acid.

Coding region change PO212/S27	**Scaffold in PO212 genome**	**Triplet PO212/S27 (5'→ 3')**	**Amino acid change**	**Protein**	***P. rubens* Wisconsin 54-1255**
g196.t1/ S27g134.t1	1.1	TGC/GGC	Cys605Gly	Dimethylglycine oxidase	Pc13g04270
g1339.t1/ S27g7851.t1	2.1	GAT/GGT	Asp1447Gly	Vegetative incompatibility protein HET-E-1	Pc12g06410
g3160.t1/ S27g8511.t1	3.1	TCC/CCC	Ser271Pro	Putative mitochondrial chaperone	Pc21g18720
g3741.t1/ S27g3030.t1	4.1	GGT/AGT	Gly135Ser	Hypothetical protein	Pc06g00910
g3975.t1/ S27g4439.t1	5.1	CCT/CAT	Pro219His	RNA polymerase I specific TF	E8E15_002124
g4471.t1/ S27g3229.t1	5.1	CGA/TGA	Arg327*	Hypothetical protein	Pc16g08360

Table 4: Nucleotide variations and alleles in loci of *Penicillium rubens* strains from INIA, CSIC collection.

Strain	**BCA[a]**	**MAT alleles**	***nirA* alleles**	**g196.t1**	**g1339.t1**	**g3160.t1**	**g3741.t1**	**g3975.t1**	**g4471.t1**
PO212	+	MAT1-1	*nirA1*	T	A	T	G	C	C
S27	-	MAT1-1	*nirA1*	G	G	C	A	A	T
S17	-	MAT1-1	*nirA1*	T	A	T	A	A	T
S71	-	MAT1-1	*nirA1*	T	A	T	A	A	T
S73	+	MAT1-1	*nirA1*	T	A	T	A	A	T
CH2	-	MAT1-2	WT	T	A	T	G	C	C
CH5	-	MAT1-2	WT	T	A	T	G	C	C
CH6	+	MAT1-1	*nirA1*	T	A	T	G	C	C
CH8*	+	MAT1-1	WT	T	A	T	G	C	C
CH16	+	MAT1-1	*nirA1*	T	A	T	G	C	C

BCA[a]: Biological Control Agent. The symbol + indicates biocontrol activity. The symbol – indicates no biocontrol activity. *CH8 carries the *crnA1* mutation. The name of the genes corresponds to PO212.

We then confirmed the presence of these variations between S27 and PO212 genomes by PCR amplification of these regions and subsequent sequencing (Table 4). To investigate whether any of these variations were specific to the biocontrol phenotype, we chose eight strains from our collection of *P. rubens* strains classified accordingly to their BA. We sequenced the regions where those SNVs were mapped. We found that SNVs present in g196.t1, g1339.t1, and g3169.t1 genes (nomenclature as in PO212 genome) were specific of S27 (Table 4). For the remaining genes, it was feasible to establish two well-differentiated groups based on specific changes in g3741.t1, g3975.t1, and g4471.t1 (Table 4).

Given the absence of specific mutations or genes that could explain the BA of PO212, we performed a search for putative homologs of 13 genes involved in biocontrol in *Trichoderma* species (Table 5; Sharma et al., [43]). Twelve were found in the genomes of PO212 and S27 without any difference in nucleotide sequences**.**

Table 5: List of genes described in *Trichoderma* spp. as genes related to biocontrol (Sharma et al., 2011).

Genbank number	Gene	Function	PrWis code	PO212 code	S27 code
AM050097	Squalene epoxidase (*erg1* gene)	Silencing of the *erg1* gene enhances resistance to terbinafine that shows antifungal activity	Pc22g15550	g7259.t1	S27g7219.t1
EU124654	Putative acetyltransferase and monooxygenase	Antagonist activity against *S. sclerotiorum*, *S. minor*, and *S. cepivorum*	Pc21g05060	g8955.t1	S27g5480.t1
EU311400	Heat shock protein 70 kDa (*hsp70* gene)	Increases fungal resistance to heat and abiotic stresses	Pc22g11240	g9809.t1	S27g8859.t1
AJ605116	mRNA for endochitinase (*ech42* gene).	Antifungal activity in transgenic tobacco	Pc13g09520	g648.t1	S27g7976.t1
EF407410	Carotenoid cleavage dioxygenase 1 (*ccd*1 gene)	Helps in hyphal growth, conidiospore development and carotenoid pigment production	Pc12g09530	g1595.t1	S27g829.t1
EU551672	Transcription factor CTF1 (*ctf1* gene)	Antifungal activity against *R. solani*, *Fusarium oxysporum*, and *B. cinerea* and production of 6-pentyl-2H-pyran-2	Pc21g11250	g5805.t1	S27g1152.t1
DQ910533	Protease gene SL41	Biocontrol activity against pathogens	AAG44693	g5366.t1	S27g8455.t1
AM421521	Endopolygalacturonase (*pg1* gene) exons 1–5	Secretion of plant cell wall degrading enzymes against *R. solani* and *P. ultimum*	Pc22g20290	g9752.t1	S27g1693.t1
EU399786	Hypothetical kelch domain containing protein (*Thkel1*)	Expression of this gene in *A. thaliana* modulates glucosidase activity, and enhances tolerance to salt and osmotic stresses	Pc13g04170	g188.t1	S27g126.t1
AY156910	Xylanase (*xyl* gene)	Helps in breakdown of hemicellulose	Pc12g01520	g7689.t1	S27g1384.t1
Accession number not available	*tmkA* gene	Induction of plant systemic resistance and biocontrol activity against *R. solani*. (Tested in green house condition)	Pc22g01670	g7951.t1	S27g4744.t1
Accession number not available	*qid74* gene	Involved in cell protection and adherence to hydrophobic surfaces that helps in antagonism against *R. solani*	not located	not located	not located
Accession number not available	*Sm1* gene	A small cysteine-rich protein that induces defense responses in dicot and monocot plants and in protecting crop diseases	Pc20g15140	g6346.t1	S27g2607.t1

The GenBank number, the gene name, and the function are detailed. In addition, the code in PrWis, PO212, and S27 if the gene is present in these strains.

Discussion

PO212 is a BCA not only capable of reducing the vascular wilt of tomatoes caused by FOL but also other diseases in a variety of horticultural crops [3-5,22]. The main mode of action for the control of Fusarium wilt by PO212 is the induction of resistance in tomato plants [7,9,10], although plant growth promotion and competition for space and nutrients are also described [6,11]. Despite these numerous studies, the molecular basis of PO212 BA remains unexplored. Therefore, the main objective of this study was to understand the genetic components governing the biocontrol mechanism. For this purpose, we sequenced and assembled the PO212 genome.

A *de novo* assembly of the PO212 genome was performed, yielding a genomic sequence organized into 65 scaffolds. The estimated size of the PO212 genome (29.82 Mb) is consistent with the predicted genome size from other strains such as KF-25 (29.91 Mb; Peng et al., [44]) and Biourge 1923 (30.45 Mb; Pathak et al., [45]). However, other *Penicillium* genomes have a larger genome, as it is the case of P2niaD18 and NCPC10086 with 32.5 and 32.3 Mb, respectively [12,46].

We considered the sequencing and comparison of a local strain similar and geographically close to strain PO212 but lacking BA, so we selected the isolate S27 [1].

Similar studies comparing biocontrol microorganisms with non-biocontrol microorganisms of the same species [47] and different species [21] have found notable differences and thus provide a genetic basis for understanding the biocontrol process. Comparative analysis of the PO212 and S27 genomes showed strong conservation of their genomic sequences. Only six variants, causing an amino acid change, were found in the coding regions between PO212 and S27. Sequencing of these ORFs in eight other strains from our stock collection, previously classified according to their BA, showed the presence of specific variations in soil isolates and in those taken from plants, but no correlation was found with their BAs. We also took advantage of these strains to determine whether the presence of known mutations or variations were the cause of BA. First, we

determined the presence of MAT1-1, which is believed to play an important role in several biotechnological traits [48] or MAT1-2. This analysis yielded no correlation between these loci and BA; in fact, both PO212 and S27 carry the MAT1-1 locus. Second, we had previously studied the complementation of the *nirA1* mutation causing a nitrate-assimilation deficient phenotype in PO212 [16]. Although complementation of *nirA1* mutation in PO212 transformants caused a reduction in their biocontrol phenotype, the S27 and other non-biocontrol strains carried the same *nirA1* mutation, indicating that the loss of NirA activity has no role in BA. In conclusion, PO212 and S27 are two strains very similar in sequence, with one important difference, the biocontrol capacity of PO212. However, current data do not suggest any correlation between the presence of the SNVs found and the biocontrol capacity of PO212 nor the absence or presence of extra genes specifically related to this phenotype.

Genome comparisons of *Postia placenta* strains evidenced high similarity between their genomes while showing important differences in phenotypes [49]. Hence, the high conservation of PO212 and S27 genomic sequences points to the presence of specific variants located in non-coding regions as candidates for a role in biocontrol. When located in putative promoter sequences, these variations could cause changes in gene expression patterns. Transcriptomic analyses are a convenient approach to studying the expression patterns of candidate genes with a potential role in biocontrol as previous works have shown [50,51]. Future lines of research will find the basis of the biocontrol phenotype in *Penicillium* focusing on epigenetics or the presence of RNA-based mycoviruses.

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