Eur J Plant Pathol (2023) 167:621–635
https://doi.org/10.1007/s10658-023-02700-4

A role for *Penicillium rubens* strain 212 xylanolytic system in biocontrol of Fusarium wilt disease in tomato plants

Elena Requena · María Carreras · Eduardo A. Espeso · Inmaculada Larena

Accepted: 30 May 2023 / Published online: 13 June 2023

Abstract *Penicillium rubens* strain 212 (PO212) is an effective inducer of resistance mechanisms in tomato plants against *Fusarium oxysporum* f.sp. *lycopersici*. During the interaction of PO212 with the plant, different classes of molecules may act as elicitors such as certain secreted endo-xylanases. The aim of this work was to elucidate the possible role of the xylanolytic system of PO212 in its biocontrol activity. We identified potential genes coding for xylanases (*xlnA*, *xlnE*, *xylP)*, β-xylosidase (*xlnD)* and their transcriptional regulators (*xlnR* and *araR*) in PO212, and evaluated their transcriptional patterns in response to tomato root extracts or synthetic medium containing xylan as main carbon source. For this work we compared data from biocontrol strain PO212 with those of two strains of *Penicillium, P. rubens* S27, and *P. chrysogenum* IPLA33001, lacking the biocontrol efficacy. Time-course experiments showed the effect of these carbon sources on the expression rates of xylanase genes. To achieve a strong reduction in expression of xylanolytic genes, we generated a null allele of XlnR, as the specific regulator of the xylanase pathway. Absence of XlnR function prevented growth of PO212 on media containing xylan as main carbon source and consequently, expression levels of xylanases were downregulated. The relationship of the *xlnR* gene regulated pathway to the efficacy of PO212 as a biological control agent was evaluated. Null *xlnR* strains did not reduce either disease severity or incidence as wild-type PO212 does. Thus, there is a relationship between a correct expression of xylanolytic system and the capability of these enzymes as effective elicitors to trigger of plant defense systems in tomato plants against *F. oxysporum* f.sp. *lycopersici*.

Keywords PO212 · Biological control agent · Endo-β-xylanases · Elicitor · Resistance induction

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10658-023-02700-4.

E. Requena · M. Carreras · I. Larena (✉)
Grupo Hongos Fitopatógenos, Departamento de Protección Vegetal, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Consejo Superior de Investigaciones Científicas (INIA-CSIC), Madrid, Spain
e-mail: ilarena@inia.csic.es

E. A. Espeso
Laboratorio de Biología Celular de Aspergillus, Departamento de Biología Celular y Molecular, Centro de Investigaciones Biológicas Margarita Salas, CSIC, (CIB-CSIC), Madrid, Spain

Introduction

Penicillium rubens strain 212 (PO212, ATCC201888), formerly *Penicillium oxalicum,* (Villarino et al., 2016) is an effective biocontrol agent (BCA) against several pathogens in horticultural crops (De Cal et al., 2008; Larena et al., 2003b; Martinez-Beringola et al., 2013) including *Fusarium oxysporum* f.sp. *lycopersici* (FOL)

(Sacc.) W. C. Snyder & H. N. Hans, causing the vascular wilt disease in tomato plants (De Cal et al., 1995). The application of PO212 conidia (Pascual et al., 2000) and conidial contact with roots (De Cal et al., 2000) make to PO212 effective as a BCA. PO212 is applied as a conidial suspension by watering seedlings in seedbeds seven days before transplanting (De Cal et al., 1999, 2000; Larena et al., 2003a). The main PO212 mode of action is the induction of resistance in tomato plants (De Cal et al., 1997, 1999, 2000). However, the mechanism by which PO212 induces plant resistance is still unknown. Investigations have been made to determine whether its biocontrol capacity is due to a genetic component. Thus, recent genomic comparative analysis between the PO212 and S27 genomes, the latter strain lacking biocontrol activity (BA), revealed a high conservation of their sequences, and therefore, the absence of a specific genetic basis for BA in PO212 (Requena et al., 2023).

The induction of resistance in plants occurs through a mechanism by which plants activate defense responses against pathogens. This activation occurs through the recognition receptors (PRRs) perceiving the presence of pathogen- and microbe-associated molecular patterns (PAMPs and MAMPs, respectively) (Jones & Dangl, 2006). Pathogen compounds or plant- and microorganism derived molecules acting as elicitors can trigger plant defense responses. In this context, and even though xylanases have been classified as enzymes with an essential role in the degradation of xylan, some studies identified endo-xylanases as active elicitors in plant defense systems (Dean et al., 1989). Enzymatic activity of xylanases is dedicated to xylan degradation. Xylan is the main component of hemicellulose, which is a heterogeneous polysaccharide consisting of a chain of β-1,4-units linked by arabinose, methyl glucuronic acid and acetate groups in its side chains (Lei et al., 2016). For xylan degradation, the presence of several endo-xylanases and β-D-xylosidases in microorganisms is essential (Sunna & Antranikian, 1997). Endo-β-xylanases cleave the main chain of xylan and liberate xylo-oligosaccharides with diverse degree of polymerization and then, β-D-xylosidases degraded to xylose (Tanaka et al., 2014). On the other hand, some studies have focused on the elicitor activity xylanases. An endo-xylanase from *Trichoderma viride*, referred to as the ethylene-inducing xylanases (EIX), has been used to study elicitor activity of endo-xylanases (Dean et al., 1989). A number of studies presented insights into the possible mechanisms for this endo-xylanases-inducing elicitor activity (Enkerli et al., 1999; Furman-Matarasso et al., 1999; Noda et al., 2010). Elicitor activity of *Trichoderma reesei* and *Botrytis cinerea* endo-xylanases remains active even when the enzymatic activity was inactivated (Frías et al., 2019). Frias and collaborators, showed that a short 25-residue peptide (named Xyn25) from the *B. cinerea* xylanase BcXyn11A was able to produce the elicitation as well as the whole BcXyn11A protein and, in addition, they found that two regions were essential to this process (Frías et al., 2019).

Xylanases have been extensively studied in *Trichoderma* and *Aspergillus* spp. In *Trichoderma* strain SY, a well-known xylanase producer isolated from soil, it was found that this strain produced 27-fold of xylanases, and mRNA was highly expressed when this fungus was grown on cellulose or xylan as a sole carbon source (Min et al., 2002). In *Aspergillus spp.,* endo-xylanases were found in *A. tubingensis* and *A. niger*, both saprophytic filamentous fungi. *A. tubingensis* genome encodes three endo-xylanases (XlnA, XlnB and XlnC) whereas *A. niger* present only two endo-1,4-β-xylanases (XlnB and XlnC) being the *xlnA* homologue of *A. tubingensis* absent in *A. niger* (de Graaff et al., 1994). *A. nidulans* secretes at least three xylanases of molecular masses 22 (XlnA), 24 (XlnB) and 34 kDa (XlnC) growing on xylan or xylose as carbon sources (Fernández-Espinar et al., 1992, 1993, 1994, 1996; Piñaga et al., 1994). For the complete hydrolysis of xylan in *A. niger*, xylosidase activity, encoded by *xlnD*, is also essential (van Peij et al., 1997). According to Tamayo et al. (2008), transcription of the *xlnA*, *xlnB* and *xlnD* genes are regulated by CreA and XlnR involved in a transcription factor cascade regulated by carbon catabolite repression. In Aspergilli, *xlnR* gene encodes a Zn2Cys6 transcription factor necessary for the synthesis of the main xylanolytic enzymes (Tamayo et al., 2008; van Peij et al., 1998a, b).

In *Penicillium* spp. multiple studies have focused on the study of xylanolytic enzyme production depending on the carbon source tested (Chávez et al., 2006). This work distributed the xylanolytic enzymes into three families of Glycosyl Hydrolases (GH): GH7, GH10 and GH11 and one xylosidase. Only one endo-xylanase gene from *P. chrysogenum (xylP*) belonging to family 10, has been studied in

greater detail (Haas et al., 1993). The work of Yang et al. (2018) revealed that strain P33 of *P. chrysogenum* secreted three xylanases, including two GH10 and one GH11 family xylanases, in presence of wheat bran plus microcrystalline.

In this work, we focus on the identification of the genes required for xylan catabolism in PO212 and S27 strains belonging to *P. rubens* and its possible role in BA. We verified the integrity of the xylanolytic system in the biocontrol strain PO212 and the non-biocontrol strain S27. The expected positive regulation of xylanases encoding genes by xylan or tomato root extracts, and the strong dependence in the activity of transcriptional regulator XlnR. By deleting *xlnR* in PO212, we explored the effects of downregulating xylanase expression in the BA of PO212.

Materials and methods

Fungal strains and growth conditions

Penicillium rubens strains used in this work are listed in Table 1. PO212 (ATCC 201888), PO212_18.2, and S27 are from strain collection at INIA-CSIC. Dr Mayo (Instituto de Productos Lácteos de Asturias-CSIC, Spain) provided the strain IPLA 33001. This strain was isolated from cheese and identified as *P. chrysogenum* (Flórez et al., 2007). Table 1 also contains the strains generated by transformation using PO212_18.2 as recipient strain. Conidia from these strains were long-term stored in 20% glycerol at -20 ºC except for PO212, which were stored at 4 ºC as dried conidia. For the maintenance of strains potato dextrose agar (PDA; Difco, Detroit, MI, USA) was used and cultures were stored at 4 ºC until further uses. Conidia were obtained growing mycelium on PDA in Petri dishes and incubated at 22–25 ºC for 7 days. Dried conidia of PO212 were produced in a solid-state fermentation system and dried as previously described by (Larena et al., 2003a). Minimal Medium (MM) (Espeso et al., 2019) was used for growth tests, using D-glucose 1% (w/v) and 5 mM ammonium tartrate as carbon and nitrogen sources, respectively (Villarino et al., 2018). When required D-glucose was substituted by 1% xylan as indicated in the text. Uracil and uridine (uu) were added to solid or liquid media to allow the growth of strains carrying *pyrG1* mutation.

The pathogenic isolate 1A of *F. oxysporum* f. sp. *lycopersici* (Sacc) Snyder and Hansen (FOL) was used to test BA of *P. rubens* strains. FOL strain was provided by Dr Cristina Moyano from the Laboratory for Assessment of Variety, Seed and Nursery Plants, INIA-CSIC (Madrid, Spain). FOL was stored at 4 ºC in tubes containing sterile sand. For mycelial production, conidia from FOL were germinated on Czapek Dox agar (CDA) (Difco Laboratories, Detroit, MI, USA) and cultivated in darkness at 25 ºC for seven days. Microconidial inoculum of FOL was produced in 250-ml flasks containing 150 ml of sterile Czapek Dox broth (Difco). Flasks were inoculated with three mycelial plugs (1 cm diameter) from the 7-day-old cultures on CDA 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 final conidial concentration in biocontrol assays was adjusted to 5×10^6 microconidia ml^{-1} (De Cal et al., 1995).

Table 1 *Penicillium* strains used in this work

Strain	Genotype	Reference
PO212[a]	WT[b]	De Cal et al. (1995)
S27[a]	WT[b]	Villarino et al. (2016)
IPLA 33001	WT[b]	Flórez et al. (2007) (1AM2)
PO212_18.2	*pyrG1*	Villarino et al. (2018)
1.1Δ*ku70*	Δ*ku70::pyrG*^{Af}, *pyrG1*	This work
T2.7	Δ*ku70*, *pyrG1*	This work
Δ*xlnR* T1.2	Δ*ku70*, *pyrG1*, Δ*xlnR::pyrG*^{Af}	This work
Δ*xlnR* T2.9	Δ*ku70*, *pyrG1*, Δ*xlnR::pyrG*^{Af}	This work

[a] Genomes published in (Requena et al., 2023). [b] *WT* Wild type

Database search and alignment

Target genes included three β-1,4-endo-xylanases (*xlnA, xlnE* and *xylP*), one β-xylosidase (*xlnD*) and two transcriptional regulators (*xlnR* and *araR*) (see Table 2). These genes were identified using homologues from *Aspergillus nidulans* (FungiDB) and *P. rubens* Wisconsin 54–1255 (abbreviated as PrWis) (NCBI) in local BlastP searches using PO212 and S27 proteomes (Requena et al., 2023). Nucleotide sequences were aligned with the Multiple Sequence Alignment tool of Clustal Omega (EMBL-EBI).

Expression assays

In order to study the expression of target genes, independent experiments in presence of lyophilized tomato roots or xylan as main carbon source were carried out with the strains under study. Using an inoculum of 5×10^6 conidia ml^{-1} (final concentration in each flasks), mycelium from each strain was growth in 2-L flasks containing 400 ml of MM with 1% glucose as carbon source. Mycelia were collected by filtration using Miracloth after incubation in a rotary shaker (GFL, Orbital Shaker 3020) at 150 rpm and 25 ºC for 24 h in darkness. Filtered mycelia were washed with sterile distilled water (SDW) and equally divided into four aliquots. Sample indicated as T0 was immediately frozen in liquid nitrogen and used as control. The other three samples designated as T30, T60 and T90 were used to inoculate fresh medium in 250 ml flasks containing 1% of lyophilized powdered roots (tomato roots were lyophilized in a Cryodos at -80 °C during 24 h (Telstar, S.A. Spain)) or 1% of xylan (Xylan from beechwood, Sigma-Aldrich). Mycelium from these samples in flasks was collected at 30 (T30), 60 (T60) and 90 (T90) minutes of incubation at 25 ºC, and was frozen using liquid nitrogen and stored at -80 ºC until processing.

RNA extraction and RT-qPCR

For total RNA extraction, mycelial samples, previously frozen in liquid nitrogen, were pulverized using a pestle and then, we followed the protocol described in Picazo et al. (2020). The quality and quantity of the RNA was checked using the NanoDrop 2000 (Thermo Scientific™). The integrity of RNA was visualized by 1.2% agarose gel electrophoresis. Total RNA samples were flash-frozen in liquid nitrogen and stored at 80 °C until use. cDNA was synthetized using the SuperScript™ First-Strand Synthesis System for RT-PCR kit (Invitrogen, Thermo Fisher Scientific Inc.), following the manufacturer's instructions. Samples were pre-treated with Deoxyribonuclease I, Amplification Grade (Invitrogen), according to the manufacturer's instructions. First-Strand was synthesized using oligo-dT primer. Dilutions of the cDNA were prepared with nuclease-free water (Promega Biotech Ibérica, S.L.) to a final concentration suitable for RT-qPCR. The samples were stored at -20 °C. RT-qPCRs were performed on the 7500 Fast real-time PCR System thermocycler (Applied Biosystems) with Master Mix SYBR Green (Promega). For each RT-qPCR specific primers were designed using Vector NTI™ Suite 8 (Supplementary Table S1). Three technical replicates were done per sample. RT-qPCR conditions were as follows: a 5 min pre-incubation cycle at 95 °C and 40 cycles of 10 s at 95 °C and 30 s at 65 °C. At the end of the program, a cycle consisted of lowering the temperature from 95 °C to 65 °C at a rate of 4.4 °C

Table 2 Genes encoding β-1,4-endo-xylanases, β-xylosidase and regulators, identified in PO212 and S27 genomes

A. nidulans	PrWis	PO212	S27
AN3613 (***xlnA***)	Pc12g01520	g7689.t1(***xlnA***)	S27g1384.t1
AN9365 (***xlnB***)	NF[a]	NF[a]	NF[a]
AN7401 (***xlnE***)	Pc22g00820	g7883.t1 (***xlnE***)	S27g4813.t1-00001
AN1818 (***xlnC***)	Pc20g07020 (***xylP***)	g2520.t1 (***xylP***)	S27g3708.t1
AN2359 (***xlnD***)	Pc21g23540	g9304.t1 (***xlnD***)	S27g5339.t1
AN7610 (***xlnR***)	Pc21g02790	g8768.t1 (***xlnR***)	S27g6043.t1
AN0388/AN10550 (***araR***)	Pc22g22560	g8278.t1 (***araR***)	S27g7769.t1

[a] *NF* not found

every 10 s was added. The expression levels were normalized using as housekeeping the tubulin (*benA*), from PO212 and was calculated following the $2^{-\Delta CT}$ or $2^{-\Delta\Delta CT}$ model specified for each case.

Construction of transformation cassettes

For the cassette construction, genomic fragments were obtained from PO212 strain and the selection marker *pyrG* of *Aspergillus fumigatus* (*pyrG*^{Af}) was obtained from plasmid pFNO3 (Villarino et al., 2018). Transformation cassettes were obtained using the fusion PCR protocol (Markina-Inarrairaegui et al., 2011; Nayak et al., 2006). The oligonucleotides used in generating fragments and transformation cassettes are listed in Supplementary Table S1. The proofreading enzyme PrimeStar (Takara) was used in PCR procedures following manufacturer's instructions. PCR conditions were, an initial denaturation cycle 98 ºC/2 min, 30 denaturation cycles 98 ˚C/10 s, oligonucleotide binding 55 ºC/5 s and extension 72 ºC/1 min/kb. Samples remained at 4 ºC. Fragments were purified using the PCR clean-Up kit (Macherey–Nagel), and following manufacturer's instructions.

Generation of mutant strains

To improve the yield of homologous recombination in transformation of PO212 derived strains we generated a strain lacking the *ku70* gene. The transformation procedure is described in Villarino et al. (2018). For this purpose, PO212_18.2 strain (Espeso et al., 2019; Villarino et al., 2018) which is an auxotrophic strain for *pyrG* (*pyrG1*) was used as a recipient strain (Step 1). This transformation was done by locus replacement, using the transformation cassette 1 (TC 1) consisting of the 5' UTR region of the *ku70* gene (1.5 kb), the CDS region of the selected marker *pyrG*^{Af} gene (1.9 kb) and the 3' UTR region of *ku70* gene (1.5 kb). The whole cassette had 5 kb. The strain obtained was genotyped to confirm the insertion of the cassette. Once confirmed as Δ*ku70*, this strain, named 1.1Δ*ku70,* was selected as recipient strain for the next transformation.

The next transformation (Step 2) was done with a transformation cassette 2 (TC 2) consisting of the 5' UTR fused to 3' UTR region of *ku70* gene (3.1 kb). For the transformation, it was used 5-fluororotic acid (5-FOA) in the medium, necessary to promote the recombination and expulsion of the marker (Villarino et al., 2016). An agarose gel electrophoresis confirmed that the amplified fragment matched to the expected insert size (3.1 kb), in contrast to the recipient strain (1.1Δ*ku70*), which had an amplicon size of 4.9 kb. The obtained strain named T2.7 was a strain *ku70* null and auxotrophic for *pyrG*.

Finally, from T2.7 strain used as recipient strain, we obtained two *xlnR* null strains named T1.2 and T2.9. It was achieved by the insertion of a transformation cassette (4.9 kb), consisting of the 5' UTR region of *xlnR* gene (1.5 kb), the marker *pyrG*^{Af} (1.9 kb) and the 3' UTR region of *xlnR* gene (1.5 kb). Genomic integration of this cassette complements the pyrimidine auxotrophy of recipient strain T2.7, allowing positive selection of transformants with this genetic marker.

These two strains were obtained using alternative strategies for gene deletion. The transformant Δ*xlnR* T1.2 was obtained using a complete transformation cassette of 4.9 kb (TC 3) integrated in the recipient strain (T2.7) by homologous recombination, favored by the absence of the *ku70* gene. The transformant Δ*xlnR* T2.9 strain was obtained through a co-transformation with two overlapping fragments. One fragment of 2.8 kb (TC 4) consisted in 5' UTR region of *xlnR* gene fused to a part of the *pyrG*^{Af} gene and the other fragment of 2.4 kb (TC 5) consisted in the other part of the *pyrG*^{Af} gene fused to 3' UTR *xlnR* region. Both fragments contained an overlapping *pyrG*^{Af} gene region. Once the strains were obtained, PCR genotyping and digestion enzymes validated them.

Biocontrol efficacy assays

Assays were performed on tomato plants in growth chambers in order to study the effect of the *xlnR* gene on PO212 BA against FOL such as described in (Larena & Melgarejo, 2009; Villarino et al., 2018). The previously described transformants and PO212 were used for these assays. Tomato seeds from cv "San Pedro", which is susceptible to races 1 and 2 of FOL, were used in the efficacy experiments. Tomato seeds were sown in sterile trays (27×42×7 cm) with autoclaved (1 h at pressure of 1 kg cm^{-2} and temperature of 121 ºC, during three consecutive days) vermiculite (Termita, Asfaltex, S.A., Barcelona, Spain), and watered with sterile Hoagland solution (Hoagland & Arnon, 1950) and tap water. The trays were maintained in a growth chamber at 24–26 ºC

with fluorescent light (100 µE m^{-2} s^{-1}, 16 h photoperiod) and 80–100% relative humidity during 2 weeks. Tomato seedlings (with at least two true leaves) were treated with an aqueous conidial suspension of each PO212 strain (6×10^6 conidia g^{-1} vermiculite) 7 days before be transplanted. Conidial suspensions of PO212 and derivates for treatments were prepared as follows. Dried conidia of PO212 were rehydrated in SDW for 2 h using a rotatory shaker at 150 rpm (CERTOMAT® RM). Conidia of the remaining strains were collected from colonies grown on PDA for 7 days in the dark at 25 °C. The day before treatment, conidial viability of all strains was checked by counting germinated conidia according to previously described (Larena et al., 2003a). Tomato seedlings were transplanted into 100-ml flasks containing 100 ml sterile Hoagland solution (Hoagland & Arnon, 1950) with roots in contact with the solution as described by De Cal et al. (1997). At this time, flasks were inoculated with an aqueous (SDW) suspension of conidia of FOL at 5×10^6 conidia ml^{-1}. Plants inoculated with FOL and non-treated with any strain of PO212, were used as the control. Each treatment consisted of five flasks with four plants per flask. Flasks were placed in growth chamber during 4 weeks under the conditions described earlier in this subsection. Disease severity was graded on days 7, 14, 21 and 28 days after transplanting. Disease severity followed a 1 to 5 index scale: 1, healthy plants (0 to 24%); 2, yellow lower leaves (25 to 49%); 3, dead lower leaves and some yellow upper leaves (50 to 74%); 4, dead lower leaves and wilted upper leaves (75 to 99%); and 5, dead plants (100%) (De Cal et al., 1995). At 28 days after transplant, roots were placed in humidity chambers at 25 ºC during 5 days, in order to determine the presence of the pathogen (incidence) in the crowns of the roots. The complete assay was repeated at least two times. If the replicate confirmed the previous results assay, only results from one assay was reported and discussed.

Data analysis

Data were analyzed by analysis of variance (ANOVA) with the Statgraphics program (XVII Centurion. v. 17.2.00). When variances were not homogeneous, data were transformed to $\log_{10}$ to improve the homogeneity before analysis. The Duncan Test was used for comparisons of media, when the F test was significant at $p \leq 0.05$ (Duncan, 1955). When homogeneity of variances was not obtained, data analysis was performed using the Kruskal–Wallis nonparametric test, with a confidence level of 5% (Kruskal & Wallis, 1952).

Results

Identification of genes encoding β-1,4-endo-xylanases in PO212 and S27 genomes

To study the role of xylanases in PO212 BA we searched for genes encoding β-1,4-endo-xylanases. Using amino acid sequences of *A. nidulans* and PrWis xylanases coding genes (*xlnA*, *xlnE* and *xylP*); we performed local BlastP searches in PO212 and S27 proteomes. Table 2 summarizes these searches. In these proteomes, we identified putative homologues for all PrWis xylanases but not for XlnB (AN9365) from *A. nidulans*. In addition, we searched for additional xylanolytic machinery finding a β-xylosidase coding gene, *xlnD*, and those genes coding for putative transcriptional regulators XlnR and AraR (a putative arabinolytic regulator). Supplementary Fig. S1 indicates the domains for each protein in PrWis. Hereafter, we designate genes in PO212 and S27 as in *Aspergillus* putative homologues except for *xylP* (Table 2).

Xylanolytic system in PO212, S27 and IPLA 33001

To understand the regulation of genes encoding β-1,4-endo-xylanases identified in PO212 and S27 genomes, during colonization of tomato roots, we analyzed the effect in their transcription of adding tomato lyophilized roots to culture media. RT-qPCR results showed that PO212 and S27 present a similar expression pattern of *xlnE*, *xylP*, *xlnR* and *araR* genes. Because PO212 and S27 strains were isolated from soil, we included in this test the IPLA 33001 strain isolated from cheese as a control. IPLA 33001 presented a basal expression for these genes in root-extract medium. The *xlnA* gene conserved a similar pattern in the three strains, and PO212 and IPLA 33001 strains showed a similar expression for *xlnD*

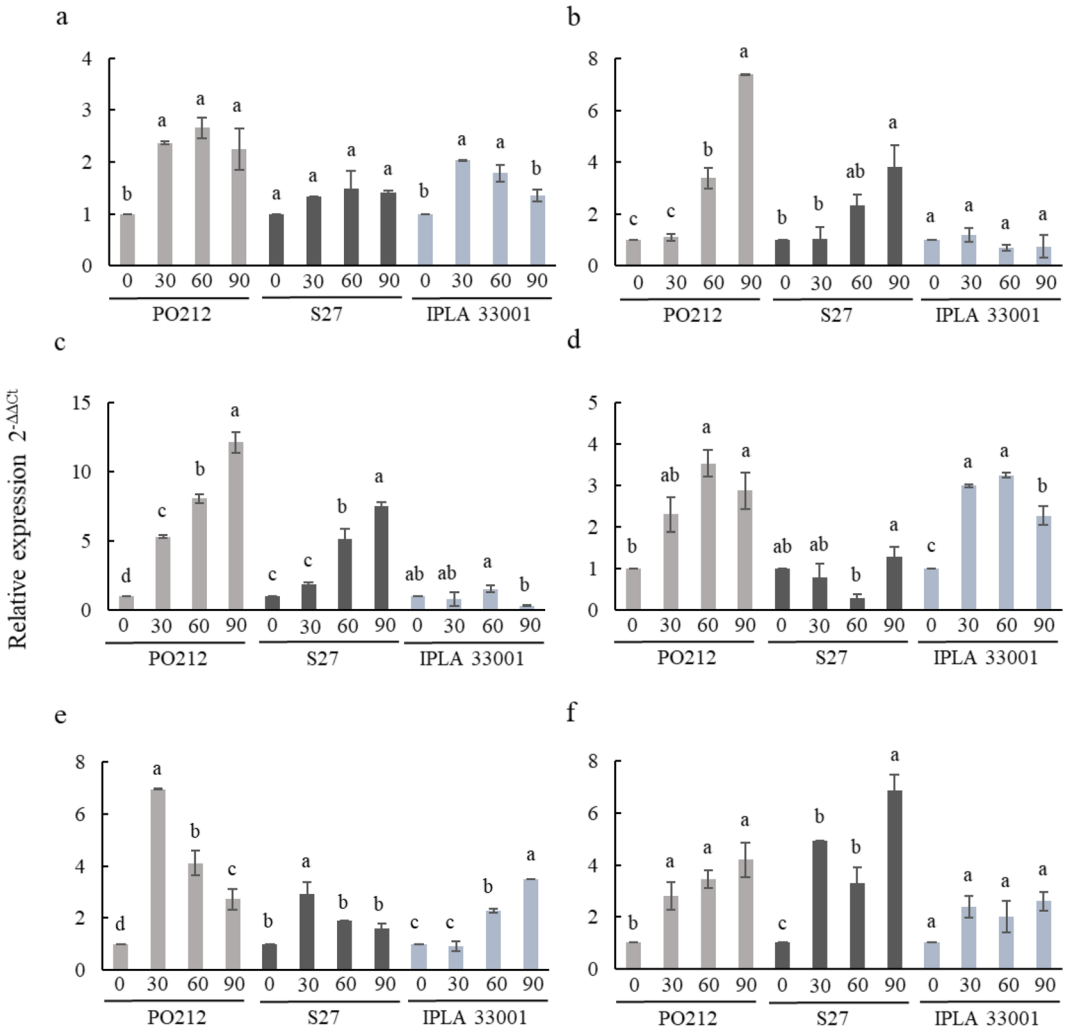

Fig. 1 Relative expression ($2^{-\Delta\Delta Ct}$) of endo-xylanases genes *xlnA* (**a**), *xlnE* (**b**) and *xylP* (**c**), β-xylosidase *xlnD* (**d**) and regulators *xlnR* (**e**) and *araR* (**f**) in the expression assays with PO212, S27 and IPLA 33001 strains in MM with lyophilized tomato roots as carbon source. Mycelial samples were collected at 30, 60 and 90 min. Time 0 indicates mycelium grown in glucose medium for 24 h prior to transfer to medium containing lyophilized tomato roots. Means with the same letter for each strain and for each gene are not significantly different from each other ($p \geq 0.05$) according to the Duncan test. Vertical bars represent the standard error of the mean of three biological replicates with three technical replicates

gene (Fig. 1d). Notably expression levels for most of the xylanolytic genes were higher in PO212 (Fig. 1).

Effect of xylan in the regulation of xylanolytic system of PO212 and S27

To determine the effect of xylan in the regulation of xylanolytic system of PO212 BCA and non-biocontrol strain S27, we proceed to culture mycelia from these strains in medium containing xylan as main carbon source. PO212 and S27 are closely related strains (Requena et al., 2023) and, as expected from that study, no differences in the nucleotide sequences of endo-xylanases were found (Supplementary Fig. S2). Presence of xylan in the media caused a variation in transcriptional levels of target genes between the

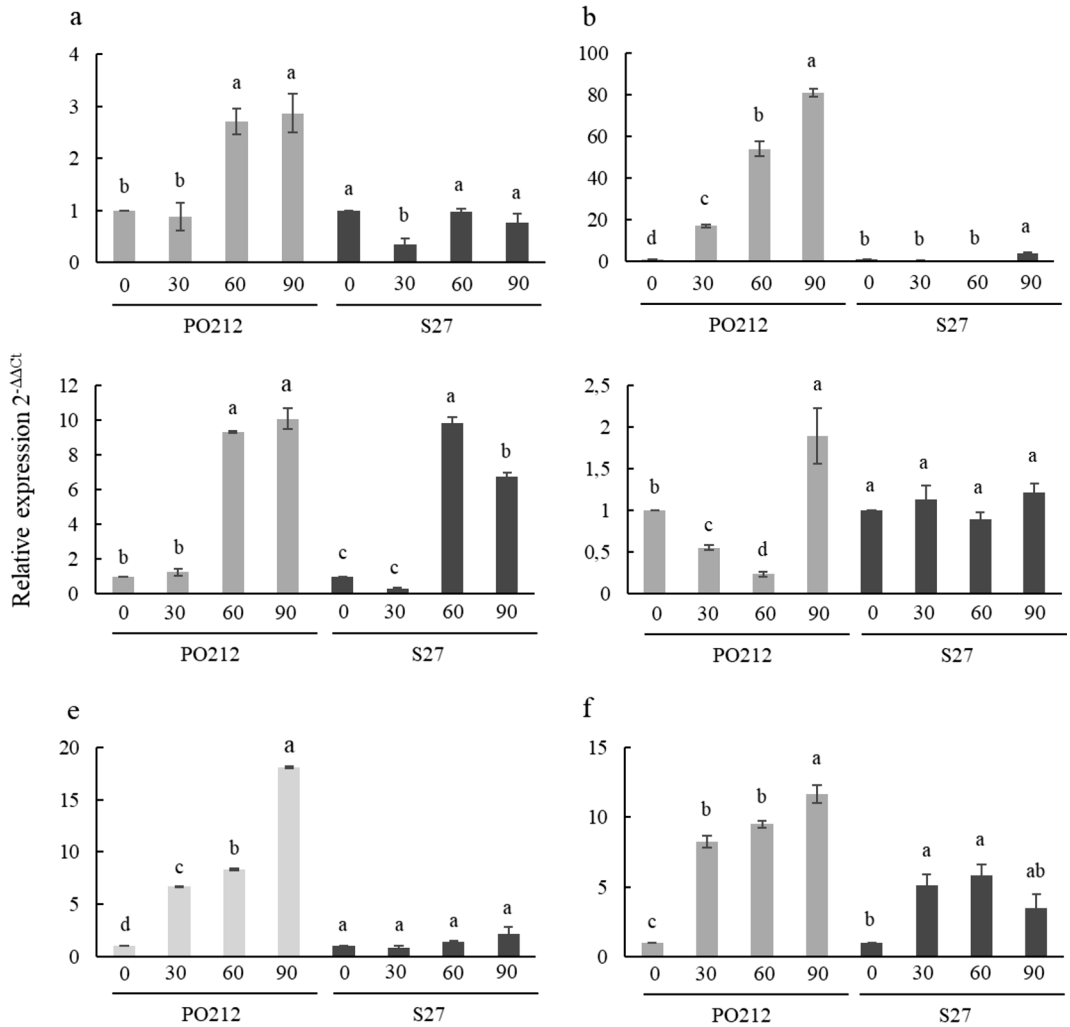

Fig. 2 Relative expression ($2^{-\Delta\Delta Ct}$) of endo-xylanases genes *xlnA* (**a**), *xlnE* (**b**) and *xylP* (**c**), β-xylosidase *xlnD* (**d**) and transcriptional regulators *xlnR* (**e**) and *araR* (**f**) in the expression assays with PO212 and S27 strains in MM with xylan as carbon source. Mycelial samples were collected at 30, 60 and 90 min. Time 0 indicates mycelium grown in glucose medium for 24 h prior to transfer to medium containing xylan. Data from *xlnD* (d) (PO212) were subject to $\log_{10}$ transformation to improve the homogeneity of variances before analysis. Means with the same letter for each strain and for each gene are not significantly different from each other ($p \geq 0.05$) according to the Duncan test. Vertical bars represent the standard error of the mean of two biological replicates with three technical replicates

two strains. *xlnA*, *xlnE* (Fig. 2a, b), *xlnR* and *araR* (Fig. 2e, f) showed higher levels in PO212 compared to S27. The expression of *xylP* was similar between both strains (Fig. 2c). The *xlnD* expression was variable in PO212 with a notable increase at 90 min, but in S27 *xlnD* levels remained constant in an intermediate state compared to PO212 (Fig. 2d). The observed differential expression between PO212 and S27 strains, prompted us to study how deletion of the transcriptional regulator XlnR in PO212 might alter this regulation and its BA.

Generation of a null allele of *xlnR* in PO212

Generation of genetic tools

To understand the implication of PO212 endo-xylanases in BA, we deleted the transcriptional

regulator *xlnR*. For this purpose, we generated a series of strains lacking the gene encoding the non-homologous joining helicase (Δ*ku70*) and able to accept the construct for deleting *xlnR*.

The first step towards obtaining a null *xlnR* strain was the transformation of recipient strain PO212_18.2 to generate Δ*ku70* allele. We replaced *ku70* coding sequence by the auxotrophic selectable marker *pyrG* from *A. fumigatus*. A linear DNA fragment of 4.9 kb was constructed by fusion PCR procedures (TC 1). Figure 3a step 1 describes the procedure for constructing the transformation cassette and the expected recombination events leading to the gene replacement of *ku70* CDS.

Further to obtaining primary transformants for Δ*ku70* allele, we selected strain 1.1Δ*ku70* and proceeded to reutilize the *pyrG1* mutation present in recipient strain PO212_18.2 by a second round of transformation but using a new linear fragment (TC 2, 3.1 kb) comprising the promoter and terminator fused together (Fig. 3a, step 2). Positive transformants were selected on regeneration medium containing 5-FOA. Among transformants, we selected mutant *pyrG1* strains T2.6 and T2.7 (Fig. 3b) and PCR analyses showed that the precise deletion of *ku70* CDS was carried out without remains of selectable marker (Fig. 3c). The recombinant strain T2.7 was selected as the recipient strain for future transformations.

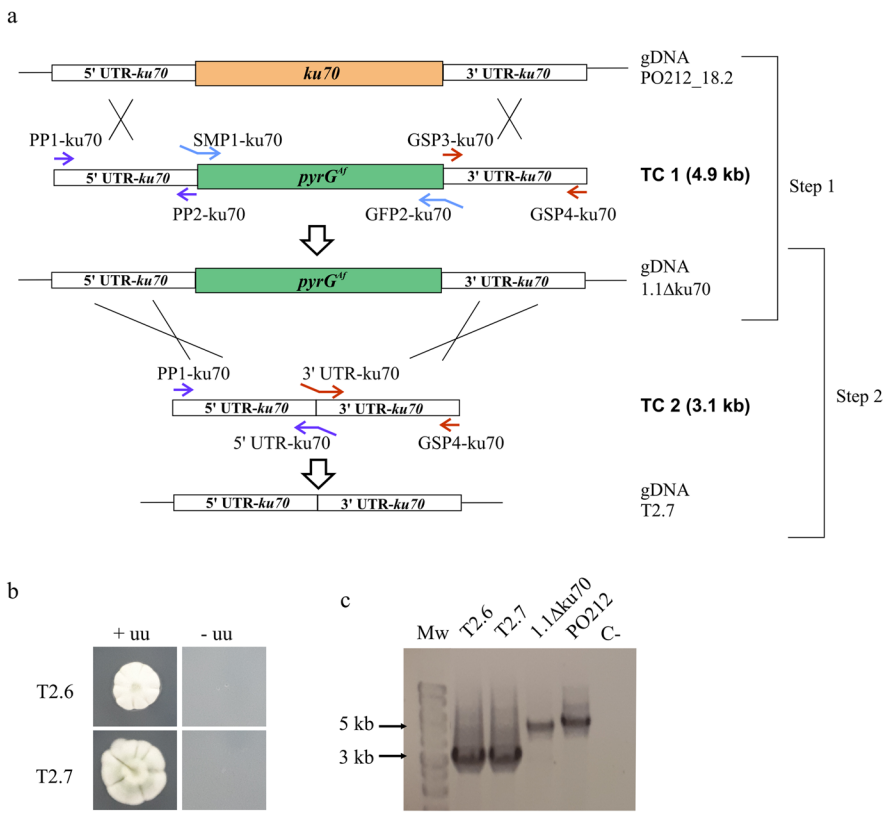

Fig. 3 Generation of null *ku70* strains. **a.** Scheme of transformation cassettes used in Step 1 and Step 2. In step 1, the locus of the recipient strain PO212_18.2, the transformation cassette 1 (TC 1) composed by 5' UTR-*ku70*, *pyrG*^{Af} gene and 3' UTR-*ku70* fused (4.9 kb) and the final product after the locus replacement, resulting in a new strain, 1.1Δ*ku70*. In step 2, the locus of the recipient strain 1.1Δ*ku70*, the TC 2 composed by 5' UTR and 3' UTR of *ku70* gene fused (3.1 kb) and the final product after locus replacement by adding 5-FOA. The new strain was T2.7. **b.** Growth test of T2.6 and T2.7 in MM in presence (+) or absence (-) of uracil and uridine (uu) as selected marker. The strains were grown for 5 days at 25 °C. **c.** PCR product. The order from left to right are: T2.6 and T2.7 are two transformants with the correct amplified fragment size (3.1 kb), 1.1Δ*ku70* is the recipient strain, with the size of the fragment of 4.9 kb, corresponding to 5' UTR fused to *pyrG*^{Af} and 3' UTR region and PO212, with wild type locus. C- Corresponds to the negative control of PCR. Mw: Molecular weight marker

Generation of ΔxlnR strains

The *xlnR* null allele was generated through precise locus replacement using mutant T2.7 strain as recipient. Two approaches were followed to increase the probability of obtaining the null *xlnR* allele. A single transformation cassette designated as TC 3 was constructed by fusion of fragments 5' UTR *xlnR*, *pyrG* gene of *A. fumigatus* and 3' UTR *xlnR* using PCR techniques. Alternatively, we constructed two fragments overlapping truncated versions of $pyrG^{Af}$ selectable marker, TC 4 and TC 5 (Fig. 4a). Both strategies should render similar results and we were able to obtain transformants in both approaches (Fig. 4b).

The correct insertion of these fragments was confirmed by PCR for most transformants (Fig. 4c). Strains lacking *xlnR* gene were unable to grow healthy on medium containing 1% xylan as carbon source but grew normally when the medium was supplemented with glucose. Only a residual growth was possible to appreciate due to there must be other enzymes able to degrade xylan (Fig. 4d).

Regulation of xylanolytic system in PO212

To determine the effect of the absence of XlnR function we measured the expression levels of three β-1,4-endo-xylanases (*xlnA*, *xlnE* and *xylP*), one β-xylosidase gene (*xlnD*) and the regulator gene (*araR*) coding genes in T1.2 and T2.9 strains and compared to PO212. Expression levels of *xlnR* in PO212 elevated along time in mycelium cultivated in medium containing 1% xylan (Fig. 5e). As expected *xlnR* transcript was not detected in samples from null *xlnR* T1.2 and T2.9 (Fig. 5e).

After confirmation of the absence of *xlnR* gene expression in null strains, we studied the gene expression of β-1,4-endo-xylanases *xlnA xlnE* and *xylP* (Fig. 5a-c), and β-xylosidase *xlnD* (Fig. 5d) under the same conditions described previously and were compared to PO212. The *xlnA* expression levels in PO212 increased in presence of xylan (T90); whereas in null strains, *xlnA* showed a basal expression either glucose or xylan during the studied times (Fig. 5a). Expression levels of *xlnE*, *xylP and xlnD* (Fig. 5b-d) genes showed similar profiles. These genes had higher expression in PO212 after 90 min in xylan compared to null strains, where there was hardly any increase in the level of these genes over time. When determining the expression levels of *araR* (Fig. 5f), this putative transcriptional regulator involved in the catabolism of arabinose branches from xylan, showed a significant higher expression in null *xlnR* strains compared to PO212.

Efficacy assays

To assess the effect of Δ*xlnR* allele on BA we performed biocontrol assays in which we compared T1.2 and T2.9 Δ*xlnR* strains to PO212. We also included in these experiments the intermediate strains 1.1Δ*ku70* and T2.7 as control of the possible effect of deleting *ku70* gene. Thus, 1.1Δ*ku70*, T2.7, T1.2 and T2.9 strains were used in these assays and compared to PO212 efficacy on reducing Fusarium wilt disease in tomato plants. Conidia suspensions from all strains had a germination rate higher than 95% before the treatment. Plants treated with conidia from PO212, 1.1Δ*ku70* and T2.7 strains showed a reduction of the disease severity, indicating that 1.1Δ*ku70* and T2.7 strains had not lost the efficacy of control against Fusarium wilt. However, the treatment with T1.2 and T2.9 conidia did not reduce either disease severity or incidence (Table 3).

Discussion

The ability of PO212 as an effective BCA against several pathogens in horticultural crops such as FOL in tomato plants has been widely demonstrated (De Cal et al., 1995, 2008; Larena et al., 2003a, b; Martinez-Beringola et al., 2013). It is documented that PO212 confers resistance against FOL in tomato plants via the host by triggering induced resistance (De Cal et al., 1997, 1999, 2000). However, the molecular basis of biocontrol phenotype displayed by PO212 is still poorly understood.

Since β-1,4-endo-xylanases have been previously described as possible elicitors of the plant defense response (Dean et al., 1989), we considered studying the effect of these xylanolytic enzymes on the BA of PO212. Based on the extended literature in *Aspergillus* spp. and the availability of protein sequences of endo-xylanases in *A. nidulans* and PrWis proteomes, we retrieved and defined the xylanolytic system in PO212 and S27 strains composed by

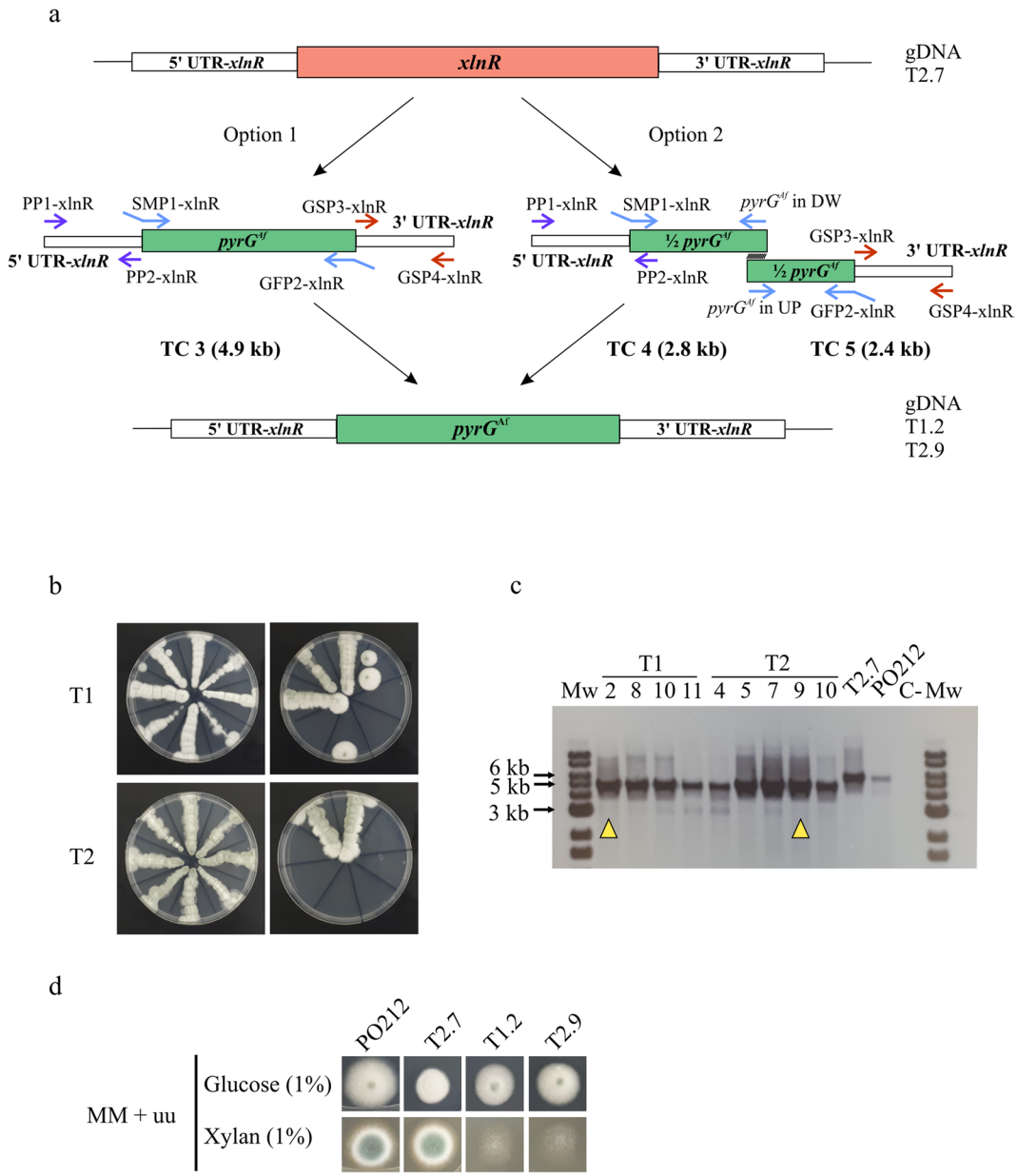

Fig. 4 Generation of null *xlnR* strains. **a.** Scheme of the transformation cassettes used for T1.2 and T2.9 strains generation. **b.** Cultures on selective medium allowing purification of homokaryotic *xlnR* null colonies. T1: transformation with the whole transformation cassette (TC 3). T2: co-transformation with the overlapping cassettes (TC 4 and TC 5). **c.** PCR product. The order from left to right are: different transformants obtained from different ways (T1 or T2). T2.7 is the recipient strain with a PCR product of 5.9 kb as well as PO212, as expected. C- Corresponds to the negative control of PCR. Mw: Molecular weight marker. Yellow triangles indicate the selected strains for the following assays. **d.** Growth test of *xlnR* null strains (T1.2 and T2.9) in comparison to the recipient strain T2.7 and PO212 in MM supplemented with glucose (1%) or xylan (1%) as carbon source. The strains were grown for 5 days at 25 °C. The addition of uracil and uridine (uu) in order to complement the auxotrophy of T2.7 caused by the lack of *pyrG* marker was necessary

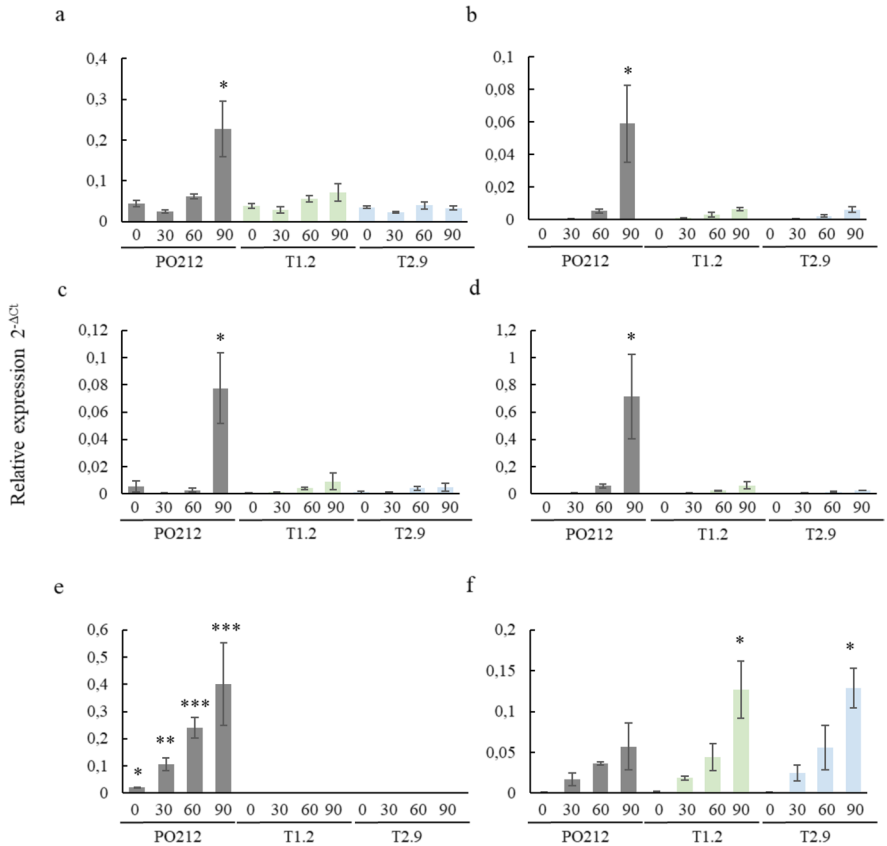

Fig. 5 Relative expression ($2^{-\Delta Ct}$) of endo-xylanases genes *xlnA* (**a**), *xlnE* (**b**) and *xylP* (**c**), β-xylosidase *xlnD* (**d**) and regulators *xlnR* (**e**) and *araR* (**f**) in the expression assays with PO212, T1.2 and T2.9 strains in MM with xylan as carbon source. Samples were collected at 30, 60 and 90 min. Time 0 indicates mycelium grown in glucose medium for 24 h prior to transfer to medium containing xylan. The Kruskal–Wallis test of independence was used for statistical analysis. Vertical bars represent the standard error of the mean of three biological replicates each one including three technical replicates

Table 3 Effect of PO212 and derive strains on the severity (%) and incidence (%) of the disease caused by *Fusarium oxysporum* f.sp. *lycopersici* in tomato plants cv. "San Pedro" at 28 days after inoculation in growth chamber

Treatment [a]	Severity (%)	Incidence (%)
Control [b]	34.3 a ± 1.7	85.0 a ± 6.1
PO212	16.6 b ± 4.2	30.0 c ± 3.3
1.1Δ*ku70*	20.1 b ± 2.1	50.0 bc ± 7.9
T2.7	22.6 b ± 1.8	65.0 ab ± 6.1
T1.2	32.8 a ± 3.8	85.0 a ± 10.0
T2.9	38.1 a ± 3.1	90.0 a ± 10.0
MSwithin [c]	44.69	354.17

Data are mean value of five replicates (flasks) per treatment and four plants per replicate. Letters in each parameter are significantly different from each other ($p \leq 0.05$) according to the Duncan Test. [a] The strains were PO212 (WT), 1.1Δ*ku70* (Δ*ku70*::*pyrG*^{Af}, *pyrG1*), T2.7 (Δ*ku70, pyrG1*), T1.2 (Δ*ku70, pyrG1*, Δ*xlnR*::*pyrG*^{Af}) and T2.9 (Δ*ku70, pyrG1*, Δ*xlnR*::*pyrG*^{Af}). Treatments of the strains were applied to seedlings seven days before transplanting by watering with a conidial suspension to final concentration of 6×10^6 conidia g^{-1}. [b] Control plants watered with sterilized distilled water (SDW) and inoculated with the pathogen. [c] MSwithin is mean squared error of analysis of variance.

three endo-xylanases (*xlnA*, *xlnE* and *xylP*), one β-xylosidase (*xlnD*) and two transcriptional regulators (*xlnR* and *araR*). The nucleotide sequence of each endo-xylanase coding genes was compared between PO212 and S27 strains, and no differences were observed between both strains. These results confirmed those published in Requena et al. (2023), since these genes were not within those genes that presented differences. In PO212 and S27 genomes, the *xlnB* coding gene was not found, unlike in *A. nidulans* and *P. purpurogenum.* In each of these fungi, two endo-xylanases were found (named XlnA and XlnB in *A. nidulans*) which were regulated by ambient pH via PacC, being differentially expressed in alkaline and acidic growth conditions (Belancic et al., 1995; MacCabe et al., 1998). The fact that PO212 present difficulties to grow well on alkaline growth conditions may explain the presence of a single gene encoding these β-1,4-endo-xylanases (Pascual et al., 1997).

To study the enzymatic regulation of endo-xylanases in PO212 BCA, *in vitro* assays with lyophilized tomato roots and xylan were carried out.

Understanding the regulation of PO212 endo-xylanases could give us an insight into their involvement in biocontrol activity. In presence of xylan, the increase of *xlnA*, *xlnE* and *xylP* genes in PO212 appears to be correlated with the increase of *xlnR*. This analysis revealed high differences in the expression patterns of endo-xylanases genes between PO212 and S27 strains, since S27 presented a lower expression of *xlnR* and therefore, a lower expression of *xlnA* and *xlnE* was observed. A similar regulation was observed in *A. niger* by van Peij et al. (1998a, b), regulating the expression of *xlnB*, *xlnC* and *xlnD* genes. The low expression of *xlnR* in presence of glucose was also observed confirming that *xlnR* promoter is repressed by glucose via CreA, and when the repression is abolished, the expression of *xlnA*, *xlnB* and *xlnD* stops being repressed as in *A. nidulans* (de Graaff et al., 1994; Tamayo et al., 2008).

The use of *xlnR* null strains is a powerful tool which has allowed us to evaluate the role of the transcriptional regulator on the BA of PO212. Moreover, we have developed a homologous transformation system in PO212 to delete any gene and be able to study its function on the biocontrol phenotype background. In this study, two different strains were obtained following two different strategies, and having the similar behavior. These two *xlnR* null strains were unable to grow on xylan as well as they did when MM was supplemented with glucose. A residual growth was possible to appreciate since, due to the xylan heterogeneity, a complex set of enzymes is required for its degradation (Biely, 1985).

In vitro assays with null *xlnR* strains and PO212 showed that in absence of *xlnR*, the expression level of *xlnA*, *xlnE*, *xylP* and *xlnD* are very low and did not experience a rise at time T90 as does PO212. This confirms what it was already seen in *A nidulans*, in which XlnR regulates the transcription of the endo-xylanases *xlnB* and *xlnC*, and the β-xylosidase *xlnD* coding genes (van Peij et al., 1998a, b). Moreover, an increase of *araR* gene expression in the Δ*xlnR* background was observed in presence of xylan as carbon source. This may be related to the catabolism of the arabinose residues present in the xylan side chains, as is the case with *A. niger*, responding to arabinose by AraR (Battaglia et al., 2011). The residual growth observed in XlnR null strains on media contained xylan as carbon source, could be due to a higher expression of this *araR* gene. In addition, efficacy assays showed that Δ*xlnR* strains lost their ability to reduce Fusarium wilt in tomato plants, demonstrating the involvement of this system in PO212 BA.

These results strongly supports a model in which the activity of the xylanolytic system driven by XlnR or the predicted extracellular presence of xylanases are key elements in mediating the biocontrol activity of PO212. This work showed that the search of key elements in biocontrol, which is a complex interaction, involves exploring pathways that may be differentially expressed, and supports the conclusions reached with the sequencing of the two strains used in this study, PO212 and S27, that show a differential phenotype in terms of their capacity as BCAs.

Author contributions Inmaculada Larena and Eduardo Antonio Espeso contributed to the study conception and design. Material preparation, data collection and analysis were performed by María Carreras and Elena Requena. The first draft of the manuscript was written by Elena Requena and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding Open Access funding provided thanks to the CRUE-CSIC agreement with Springer Nature. This study was supported by the RTA2013-00060-C05-01 (Plan Nacional de Ministerio de Economía y Competitividad), RTA2017-00019-C03-01 (Plan Nacional de I+D, MICIU, Spain) and PID2021-123594OR-C21 (MCIN/AEI/10.13039/501100011033/FEDER, UE). Elena Requena received a scholarship from the MICIU and María Carreras received a scholarship from SGIT-INIA (Ministerio de Economía y Competitividad).

Data Availability All data supporting the findings of this study are available within the paper and its Supplementary Information. Primer sequences are provided in Supplementary Table 1. Genomes of PO212 y S27 were published in Requena et al (2023). The datasets presented in that study can be found in on line repositories.The dataset of PO212 genome are deposited in the GenBank repository, accession number JAPDLE000000000. The dataset of S27 genome are deposited in the GenBank repository, accession number JAPDLD000000000.

Declarations

Conflicts of interest The authors declare no conflict of interest.

References

Battaglia, E., Visser, L., Nijssen, A., van Veluw, G. J., Wösten, H. A. B., & de Vries, R. P. (2011). Analysis of regulation of pentose utilization in *Aspergillus niger* reveals evolutionary adaptations in the Eurotiales. *Studies in Mycology, 69*, 31–38. https://doi.org/10.3114/sim.2011.69.03

Belancic, A., Scarpa, J., Peirano, A., Díaz, R., Steiner, J., & Eyzaguirre, J. (1995). *Penicillium purpurogenum* produces several xylanases: Purification and properties of two of the enzymes. *Journal of Biotechnology, 41*, 71–79. https://doi.org/10.1016/0168-1656(95)00057-w

Biely, P. (1985). Microbial xylanolytic systems. *Trends in Biotechnology, 3*, 286–290. https://doi.org/10.1016/0167-7799(85)90004-6

Chávez, R., Bull, P., & Eyzaguirre, J. (2006). The xylanolytic enzyme system from the genus *Penicillium*. *Journal of Biotechnology, 123*, 413–433. https://doi.org/10.1016/j.jbiotec.2005.12.036

De Cal, A., Pascual, S., Larena, I., & Melgarejo, P. (1995). Biological control of *Fusarium oxysporum* f. sp. *lycopersici*. *Plant Pathology, 44*, 909–917. https://doi.org/10.1111/j.1365-3059.1995.tb02750.x

De Cal, A., Pascual, S., & Melgarejo, P. (1997). Involvement of resistance induction by *Penicillum oxalicum* in the biocontrol of tomato wilt. *Plant Pathology, 46*, 72–79. https://doi.org/10.1046/j.1365-3059.1997.d01-204.x

De Cal, A., García-Lepe, R., Pascual, S., & Melgarejo, P. (1999). Effects of timing and method of application of *Penicillium oxalicum* on efficacy and duration of control of fusarium wilt of tomato. *Plant Pathology, 48*, 260–266. https://doi.org/10.1046/j.1365-3059.1999.00324.x

De Cal, A., Garcia-Lepe, R., & Melgarejo, P. (2000). Induced resistance by *Penicillium oxalicum* against *Fusarium oxysporum* f. sp. *lycopersici*: Histological studies of infected and induced tomato stems. *Phytopathology, 90*, 260–268. https://doi.org/10.1094/PHYTO.2000.90.3.260

De Cal, A., Redondo, C., Sztejnberg, A., & Melgarejo, P. (2008). Biocontrol of powdery mildew by *Penicillium oxalicum* in open-field nurseries of strawberries. *Biological Control, 47*, 103–107. https://doi.org/10.1016/j.biocontrol.2008.07.010

de Graaff, L. H., van den Broeck, H. C., van Ooijen, A. J. J., & Visser, J. (1994). Regulation of the xylanase-encoding xlnA gene of *Aspergillus tubigensis*. *Molecular Microbiol, 12*, 479–490. https://doi.org/10.1111/j.1365-2958.1994.tb01036.x

Dean, J. F. D., Gamble, H. R., & Anderson, J. D. (1989). The ethylene Biosynthesis-Inducing Xylanase: Its Induction in *Trichoderma viride* and Certain Plant Pathogens. *Phytopathology, 79*, 1071–1078. https://doi.org/10.1104/pp.95.1.316

Duncan, D. B. (1955). Multiple range and multiple F tests. *Biometrics, 11*, 1–42. https://doi.org/10.2307/3001478

Enkerli, J., Felix, G., & Boller, T. (1999). The enzymatic activity of fungal xylanase is not necessary for its elicitor activity. *Plant Physiology, 121*, 391–397. https://doi.org/10.1104/pp.121.2.391

Espeso, E. A., Villarino, M., Carreras, M., Alonso-Guirado, L., Alonso, J. M., Melgarejo, P., & Larena, I. (2019). Altered nitrogen metabolism in biocontrol strains of *Penicillium rubens*. *Fungal Genetics and Biology, 132*, 103263. https://doi.org/10.1016/j.fgb.2019.103263

Fernández-Espinar, M. T., Ramón, D., Piñaga, F., & Vallés, S. (1992). Xylanase production by *Aspergillus nidulans*. *FEMS Microbiology Letters, 91*, 91–96. https://doi.org/10.1016/0378-1097(92)90665-B

Fernández-Espinar, M. T., Piñaga, F., Sanz, P., Ramón, D., & Vallés, S. (1993). Purification and characterization of a neutral endoxylanase from *Aspergillus nidulans*. *FEMS Microbiology Letters, 113*, 223–228. https://doi.org/10.1111/j.1574-6968.1993.tb06518.x

Fernández-Espinar, M., Piñaga, F., de Graaff, L., Visser, J., Ramón, D., & Vallés, S. (1994). Purification, characterization and regulation of the synthesis of an *Aspergillus nidulans* acidic xylanase. *Applied Microbiology and Biotechnology, 42*, 555–562. https://doi.org/10.1007/BF00173920

Fernández-Espinar, M. T., Vallès, S., Piñaga, F., Pérez-González, J. A., & Ramón, D. (1996). Construction of an *Aspergillus nidulans* multicopy transformant for the x/nB gene and its use in purifying the minor X24 xylanase. *Applied Microbiology and Biotechnology, 45*, 338–341. https://doi.org/10.1007/s002530050693

Flórez, A. B., Álvarez-Martín, P., López-Díaz, T. M., & Mayo, B. (2007). Morphotypic and molecular identification of filamentous fungi from Spanish blue-veined Cabrales cheese, and typing of *Penicillium roqueforti* and *Geotrichum candidum* isolates. *International Dairy Journal, 17*, 350–357. https://doi.org/10.1016/j.idairyj.2006.04.002

Frías, M., González, M., González, C., & Brito, N. (2019). A 25-residue peptide from *Botrytis cinerea* xylanase bcxyn11a elicits plant defenses. *Frontiers in Plant Science, 10*, 1–10. https://doi.org/10.3389/fpls.2019.00474

Furman-Matarasso, N., Cohen, E., Du, Q., Chejanovsky, N., Hanania, U., & Avni, A. (1999). A point mutation in the ethylene-inducing xylanase elicitor inhibits the β-1-4-endoxylanase activity but not the elicitation activity. *Plant Physiology, 121*, 345–351. https://doi.org/10.1104/pp.121.2.345

Haas, H., Friedlin, E., Stöffler, G., & Redl, B. (1993). Cloning and structural organization of a xylanase-encoding gene from *Penicillium chrysogenum*. *Gene, 126*, 237–242. https://doi.org/10.1016/0378-1119(93)90372-A

Hoagland, D. R., & Arnon, D. I. (1950). Preparing the nutrient solution. *The Water-Culture Method for Growing Plants without Soil, 347*, 29–31.

Jones, J., & Dangl, J. (2006). The plant immune system. *Nature, 444*, 323–329. https://doi.org/10.1038/nature05286

Kruskal, W. H., & Wallis, W. A. (1952). Use of ranks in one-criterion variance analysis. *Journal of the American Statistical Association, 47*, 583–621.

Larena, I., & Melgarejo, P. (2009). Development of a method for detection of the biocontrol agent *Penicillium oxalicum* strain 212 by combining PCR and a selective medium. *Plant Disease, 93*, 919–928. https://doi.org/10.1094/PDIS-93-9-0919

Larena, I., Melgarejo, P., & De Cal, A. (2003a). Drying of conidia of *Penicillium oxalicum*, a biological control agent against Fusarium wilt of tomato. *Journal of Phytopathology, 151*, 600–606. https://doi.org/10.1046/j.0931-1785.2003.00772.x

Larena, I., Sabuquillo, P., Melgarejo, P., & De Cal, A. (2003b). Biocontrol of fusarium and verticillium wilt of tomato by *Penicillium oxalicum* under greenhouse and field conditions. *Journal of Phytopathology, 151*, 507–512. https://doi.org/10.1046/j.1439-0434.2003.00762.x

Lei, Z., Shao, Y., Yin, X., Yin, D., Guo, Y., & Yuan, J. (2016). Combination of Xylanase and debranching enzymes specific to wheat Arabinoxylan improve the growth performance and gut health of broilers. *Journal of Agricultural and Food Chemistry, 64*, 4932–4942. https://doi.org/10.1021/acs.jafc.6b01272

MacCabe, A. P., Orejas, M., Perez-Gonzalez, J. A., & Ramon, D. (1998). Opposite patterns of expression of two *Aspergillus nidulans* xylanase genes with respect to ambient pH. *Journal of Bacteriology, 180*, 1331–1333. https://doi.org/10.1128/jb.180.5.1331-1333.1998

Markina-Inarrairaegui, A., Etxebeste, O., Herrero-Garcia, E., Araujo-Bazan, L., Fernandez-Martinez, J., Flores, J. A., Osmani, S. A., & Espeso, E. A. (2011). Nuclear transporters in a multinucleated organism: Functional and localization analyses in *Aspergillus nidulans*. *Molecular Biology of the Cell, 22*, 3874–3886. https://doi.org/10.1091/mbc.E11-03-0262

Martinez-Beringola, M. L., Salto, T., Vázquez, G., Larena, I., Melgarejo, P., & De Cal, A. (2013). *Penicillium oxalicum* reduces the number of cysts and juveniles of potato cyst nematodes. *Journal of Applied Microbiology, 115*, 199–206. https://doi.org/10.1111/jam.12213

Min, S. Y., Kim, B. G., Lee, C., Hur, H. G., & Ahn, J. H. (2002). Purification, characterization, and cDNA cloning of xylanase from fungus *Trichoderma* strain SY. *Journal of Microbiology and Biotechnology, 12*, 890–894.

Nayak, T., Szewczyk, E., Oakley, C. E., Osmani, A., Ukil, L., Murray, S. L., Hynes, M. J., Osmani, S. A., & Oakley, B. R. (2006). A versatile and efficient gene-targeting system for *Aspergillus nidulans*. *Genetics, 172*, 1557–1566. https://doi.org/10.1534/genetics.105.052563

Noda, J., Brito, N., & González, C. (2010). The *Botrytis cinerea* xylanase Xyn11A contributes to virulence with its necrotizing activity, not with its catalytic activity. *BMC Plant Biology, 10*. https://doi.org/10.1186/1471-2229-10-38

Pascual, S., Rico, J. R., De Cal, A., & Melgarejo, P. (1997). Ecophysiological factors affecting growth, sporulation and survival of the biocontrol agent *Penicillium oxalicum*. *Mycopathology., 139*, 43–50. https://doi.org/10.1023/A:1006898724724

Pascual, S., De Cal, A., Magan, N., & Melgarejo, P. (2000). Surface hydrophobicity, viability and efficacy in biological control of *Penicillium oxalicum* spores produced in aerial and submerged culture. *Journal of Applied Microbiology, 89*, 847–853. https://doi.org/10.1046/j.1365-2672.2000.01189.x

Picazo, I., Etxebeste, O., Requena, E., Garzia, A., & Espeso, E. A. (2020). Defining the transcriptional responses of *Aspergillus nidulans* to cation/alkaline pH stress and the role of the transcription factor SltA. *Microbial Genomics, 6*, mgen000415. https://doi.org/10.1099/mgen.0.000415

Piñaga, F., Fernández-Espinar, M. T., Vallés, S., & Ramón, D. (1994). Xylanase production in *Aspergillus nidulans*: Induction and carbon catabolite repression. *FEMS Microbiology Letters, 115*, 319–323. https://doi.org/10.1111/j.1574-6968.1994.tb06657.x

Requena, E., Alonso-Guirado, L., Veloso, J., Villarino, M., Melgarejo, P., Espeso, E. A., & Larena, I. (2023). Comparative analysis of *Penicillium* genomes reveals the absence of a specific genetic basis for biocontrol in *Penicillium rubens* strain 212. *Frontiers in Microbiology, 13*, 1–15. https://doi.org/10.3389/fmicb.2022.1075327

Sunna, A., & Antranikian, G. (1997). Xylanolytic enzymes from fungi and bacteria. *Critical Reviews in Biotechnology, 17*, 39–67. https://doi.org/10.3109/07388559709146606

Tamayo, E. N., Villanueva, A., Hasper, A. A., de Graaff, L. H., Ramón, D., & Orejas, M. (2008). CreA mediates repression of the regulatory gene *xlnR* which controls the production of xylanolytic enzymes in *Aspergillus nidulans*. *Fungal Genetics and Biology, 45*, 984–993. https://doi.org/10.1016/j.fgb.2008.03.002

Tanaka, T., Hirata, Y., Nakano, M., Kawabata, H., & Kondo, A. (2014). Creation of cellobiose and xylooligosaccharides-coutilizing *Escherichia coli* displaying both β-glucosidase and β-xylosidase on its cell surface. *ACS Synthetic Biology, 3*, 446–453. https://doi.org/10.1021/sb400070q

van Peij, N. N., Brinkmann, J., Vrsanská, M., Visser, J., & de Graaff, L. H. (1997). beta-Xylosidase activity, encoded by xlnD, is essential for complete hydrolysis of xylan by *Aspergillus niger* but not for induction of the xylanolytic enzyme spectrum. *European Journal of Biochemistry, 245*, 164–173. https://doi.org/10.1111/j.1432-1033.1997.00164.x

van Peij, N. N., Gielkens, M. M., de Vries, R. P., Visser, J., & De Graaff, L. H. (1998a). The transcriptional activator XlnR regulates both xylanolytic and endoglucanase gene expression in *Aspergillus niger*. *Applied and Environmental Microbiology, 64*, 3615–3619. https://doi.org/10.1128/aem.64.10.3615-3619

van Peij, N. N., Visser, J., & de Graaff, L. H. (1998b). Isolation and analysis of *xlnR*, encoding a transcriptional activator co-ordinating xylanolytic expression in *Aspergillus niger*. *Molecular Microbiology, 27*, 131–142. https://doi.org/10.1046/j.1365-2958.1998.00666.x

Villarino, M., De Cal, A., Melgarejo, P., Larena, I., & Espeso, E. A. (2016). The development of genetic and molecular markers to register and commercialize *Penicillium rubens* (formerly *Penicillium oxalicum*) strain 212 as a biocontrol agent. *Microbial Biotechnology, 9*, 89–99. https://doi.org/10.1111/1751-7915.12325

Villarino, M., Espeso, E. A., Melgarejo, P., & Larena, I. (2018). Transformation of *Penicillium rubens* 212 and expression of GFP and DsRED coding genes for visualization of plant-biocontrol agent interaction. *Frontiers in Microbiology, 9*, 1–14. https://doi.org/10.3389/fmicb.2018.01653

Yang, Y., Yang, X., Dong, Y., & Qiu, D. (2018). The *Botrytis cinerea* xylanase BcXyl1 modulates plant immunity. *Frontiers in Microbiology, 9*, 1–13. https://doi.org/10.3389/fmicb.2018.02535