academicJournals

Vol. 10(32), pp. 1292-1305, 28 August, 2016
DOI: 10.5897/AJMR2016.8185
Article Number: A492DF860281
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Cloning and characterization of an endo-β-1,4-xylanase gene from *Colletotrichum lindemuthianum* and phylogenetic analysis of similar genes from phytopathogenic fungus

Ulises Conejo-Saucedo[1], Horacio Cano-Camacho[1], Everardo López-Romero[2], Maria G. Villa Rivera[1], Alicia Lara-Márquez[1] and María G. Zavala-Páramo[1]*

[1]Centro Multidisciplinario de Estudios en Biotecnología, FMVZ, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, México.
[2]Departamento de Biología, División de Ciencias Naturales y Exactas, Universidad de Guanajuato, Gto., México.

Received 24 June, 2016; Accepted 4 August, 2016

Colletotrichum lindemuthianum **is the etiological agent of anthracnose, one of the main diseases of bean (*Phaseolus vulgaris*). In this study, the complete cDNAs of two endo-β-1,4-xylanase genes (*xyl1*) from non-pathogenic (0) and pathogenic (1472) races of *C. lindemuthianum* were isolated and characterized. To get an insight into the role of endo-β-1,4-xylanases in their different lifestyles, *xyl1* gene expression and enzyme activity in mycelia of both races grown in the presence of xylan or *P. vulgaris* cell walls were investigated. The *xyl1* sequence analysis and Clustal alignment revealed the characteristic elements of genes coding for endo-β-1,4-xylanases of the GH11 family. The growth of the two races with glucose as the sole carbon source showed both basal transcription levels of *xyl1* and endoxylanase activity. When glucose was substituted with xylan or plant cell walls, *xyl1* transcription, and enzyme activity significantly increased in race 1472 as compared to race 0. The pathogenic race degraded xylan faster and grew better than the non-pathogenic counterpart. Seemingly, the regulation of xylanolytic gene expression, enzyme production and the nature of the assimilatory carbon substrates processed by these organisms play a determinant role in their lifestyle. Phylogenetic analyses of XYL1 and endo-β-1,4-xylanases from other fungi revealed a diversification process and separation of proteins from the same fungal species into different lineages.**

Key words: *Colletotrichum lindemuthianum*, *Phaseolus vulgaris*, endo-β-1,4-xylanase, gene expression, phylogeny.

INTRODUCTION

Colletotrichum lindemuthianum is an economically important phytopathogen and together with its host *Phaseolus vulgaris*, represents a convenient model for studying the physiological and molecular basis of plant-pathogen interactions (Dean et al., 2012; Perfect et al., 1999). This species encompasses different strains or special forms known as races, physiological races or pathotypes identified through the interaction with a group of 12 different cultivars of *P. vulgaris*, a system used worldwide (Rodríguez-Guerra et al., 2006). A non-

pathogenic race and more than 100 pathotypes with different virulence levels have been reported around the world. AFLP analyses of 10 out of the 54 *C. lindemuthianum* pathotypes identified in México have shown high genetic diversity with several lineages (Gonzalez et al., 1998; Sánchez-García et al., 2009). *C. lindemuthianum* is an intracellular hemibiotrophic whose physiological races invade the plant in a manner consistent with the gene-for-gene model interactions (Flor, 1971; Oblessuc et al., 2012). Monogenic dominant resistance in common bean cultivars leads to the appearance of localized necrotic spots that are typical of the hypersensitive response (HR) (O'Connell and Bailey, 1988). After penetrating a host epidermal cell in a susceptible cultivar, pathogenic races of the fungus develop an infection vesicle and extend into adjacent cells by means of large primary hyphae, which invaginate without penetrating the cell membrane and thus persisting as a biotrophic interaction (Münch et al., 2008; O'Connell and Bailey, 1988). Once a large area of the plant tissue has been colonized, necrotrophic hyphae develop. This step closely correlates with the production of a number of host cell wall degrading enzymes that are characteristic of phytopathogenic fungi (Dodds et al., 2009; King et al., 2011; Wijesundera et al., 1989). Currently, race 0 is one strain of *C. lindemuthianum* unable to infect 12 different cultivars of *P. vulgaris*, which contrasts with race 1472, one virulent isolated in México (Rodríguez-Guerra et al., 2006). This difference makes the comparison of the two races a convenient approach to investigate the role played by host cell wall degrading enzymes in the pathogenicity of *C. lindemuthianum*.

Analysis of genomic sequences from plant saprophytic and pathogenic fungi has led to the identification of putative genes encoding for carbohydrate-active enzymes (CAZymes) involved in the degradation of plant cell wall. Comparison of these genes has contributed to our understanding of their lifestyle and helped to create infection models (Zhao et al., 2013). For example, biotrophic fungi tend to have fewer CAZymes than necrotrophic and hemibiotrophic fungi. Saprophytic fungi have fewer CAZymes than plant pathogenic fungi, and dicot pathogens often contain more pectinases than monocot pathogens (Zhao et al., 2013). Nevertheless, there have been few studies examining the genetic expression and enzymatic activity of these CAZymes as compared to the vast diversity of substrates presented by hosts.

Endoxylanases are CAZymes produced by some saprophytic and pathogenic fungi (Polizeli et al., 2005; Sunna and Antranikian, 1997) and are responsible for the depolymerization of xylan in plant cell wall (Collins et al., 2005; van den Brink and de Vries, 2011). There are currently more than 100 families of glycoside hydrolases (GHs) in the CAZymes database (Cantarel et al., 2009; Lombard et al., 2014) (http://www.cazy.org/). Endoxylanases are distributed in families GH10 and GH11, which correspond to the F and G families, respectively (Ahmed et al., 2009; Biely et al., 1997; Gilkes et al., 1991). The endo-β-1,4-xylanases (EC 3.2.1.8) belonging to family GH11 hydrolyze the β-1, 4 bond of xylan generating xylooligosaccharides, which are further hydrolyzed by β-xylosidase to xylose units (EC 3.2.1.37) (Biely, 1985; Pollet et al., 2010). These endoxylanases fold into β jelly roll sheets that define their secondary structure (Paës et al., 2012). Phylogenetic analysis of endoxylanases of plant saprophytes and pathogens can contribute to the understanding of the evolutionary process in relation to host types and different invasion/nutritional strategies (biotrophic, necrotrophic, or hemibiotrophic).

On this background, here, for the first time, the isolation and characterization of *xyl1* cDNA, which encodes an extracellular endo-β-1,4-xylanase in non-pathogenic (0) and pathogenic (1472) races of *C. lindemuthianum* was reported. Moreover, to understand the role of β-1,4-xylanase in the different fungal lifestyles, *xyl1* gene expression and endoxylanase activity in mycelia of both races grown in the presence of xylan or *P. vulgaris* cell walls were investigated.

Finally, the results of Clustal alignment and phylogenetic analyses of XYL1 from *C. lindemuthianum* and similar enzymes reported in other species of fungi are also presented.

MATERIALS AND METHODS

Strains and culture conditions

C. lindemuthianum races 1472 and 0 were kindly provided by Dr. June Simpson (CINVESTAV-IPN, Unidad Irapuato, México), which were reported and characterized by interaction with differential varieties of bean (*Phaseolus vulgaris*) and molecular strategies (RAPD and AFLP) as the pathotypes 1472 and 0 by González et al. (1998) and subsequently analyzed by Rodríguez-Guerra et al. (2006). *C. lindemuthianum* was maintained on potato dextrose agar (PDA) (Difco, México) at 20°C. For expression analysis, 1.6 mg (approximately 5 cm^2) of mycelia from both races was inoculated into 250 mL-Erlenmeyer flasks containing 50 mL of PD medium and shaken (150 rpm) at 20°C. After 9 days, mycelia was collected by filtration, washed with water and transferred to 125 mL-Erlenmeyer flasks containing 50 mL of modified Mathur's medium (Acosta-Rodriguez et al., 2005), supplemented with 2.5% of glucose, xylan (from beechwood; Sigma-Aldrich, St. Louis, MO, USA) or cell walls from *P. vulgaris* (cv. Flor de Mayo). Flasks were shaken (150 rpm) at 20°C and after various periods of time, mycelia were collected by filtration, washed with water and stored at -80°C until use.

For enzyme analysis, 125 mL-Erlenmeyer flasks containing 50 mL of modified Mathur's medium (Acosta-Rodriguez et al., 2005)

*Corresponding author. E-mail: gzavalap@umich.mx or gzavpar@hotmail.com. Tel/Fax: +52 443-2958029.

Table 1. Sequences of endoxylanases used for the analyses.

Fungi	GenBank access	In this study	References
Didymella pisi	CAA93120.1	*Didymella_pisi*	(Lübeck et al., 1997a)
Botrytis cinérea	AAZ03776	*B.cinerea_xyn11A*	(Brito et al., 2006)
Cochliobolus carbonum	L13596	*Cochliobolus_carbonum-xyl1*	(Apel-Birkhold and Walton, 1996)
Cochliobolus carbonum	U58916	*C.carbonum-xyl3*	(Apel-Birkhold and Walton, 1996)
Cochliobolus sativus	CAA06151.1	*C. sativus-xyl2*	(Emami and Hack, 2001)
Claviceps purpurea	CAA76570	*Claviceps_purpurea-xyl1*	(Giesbert et al., 1998)
Fusarium oxysporum f. sp. lycopersici.	AAK27975.1	*Fusarium_oxysporum-xyl4*	(Gomez-Gomez et al., 2002)
Fusarium oxysporum f. sp. lycopersici.	AF246830_1	*F.oxysporum-xyl5*	(Gomez-Gomez et al., 2002)
Helminthosporium turcicum	CAB52417.1	*Helminthosporium_turcicum-htxyl1*	(Degefu et al., 2004)
Helminthosporium turcicum	CAD70174.1	*H. turcicum-htxyl2*	(Degefu et al., 2004)
Magnaporthe grisea	L37530	*Magnaporthe_grisea*-Xyn22	(Wu et al., 1995)
Lentinula edodes	AAL04152.1	*Lentinula_edodes*	(Lee et al., 2005)

supplemented with one of the carbon sources described above, were inoculated with 1.6 mg dry weight (approximately 5 cm^2) of a 9-day-old colony grown on PDA and incubated at 20°C with continuous shaking (150 rpm). After different periods of time, cultures were centrifuged at low speed and the mycelia and supernatants were saved. Fungal growth was measured as mg of wet mycelia except in those experiments where plant cell walls were used as carbon sources. In these cases, growth was measured as the amount of mycelial protein, as residual undegraded cell walls interfered with weight quantification. A 3-mL aliquot of the cell-free supernatant was filtered through a column (1.5 x 6 cm) of Bio-Gel P-6 (Bio-Rad, Hercules, CA, USA), equilibrated and eluted with 50 mM sodium acetate buffer, pH 5.0 (buffer A), at 4°C to prepare the enzymatic fraction. Fractions corresponding to the void volume (V_o) were pooled and the pool, labeled as the filtered extracellular medium (FEM), was used to determine protein and enzyme activity.

Preparation of plant cell walls

P. vulgaris seedlings (cv. Flor de Mayo) were grown for seven days, and cell walls were extracted and purified from hypocotyls by washing in organic solvents as described elsewhere (Fry, 2006).

RNA isolation

Total RNA was purified from mycelia using the Sokolovsky method (Sokolovsky et al., 1990). RNA samples were treated with DNAse I according to the manufacturer's instructions (Invitrogen, Grand Island, NY, USA) to eliminate DNA. The quality and concentration of total RNA were assessed using a Biophotometer Plus system (Eppendorf, Barkhausenweg, Hamburg, Germany).

cDNA isolation, sequencing and analysis

A cDNA fragment (223 bp) of the endo-β-1,4-xylanase gene (*xyl1*) from race 1472 of *C. lindemuthianum* was amplified using the reported primers XYNG2-F [5´-GA(A/G)TA(T/C)TA(T/C)AT(T/C/A)GT(A/T/G/C)GA(A/G)(A/T)(G/C)(A/C/G/T)TA-3´] and XYNG2-R [5´-GCCCA(A/C/G/T)GC(A/G)TT(A/G)AA(A/G)TG(A/G) TT-3´] according to Kimura (Kimura et al., 2000). Total RNA was isolated from mycelia induced with xylan for 24 h. This fragment was sequenced (data not shown), the specific primers Xyl-D110 (5'-GCGTGAACCAGCCCAGCATC-3') and Xyl-GSP1 (5'-CAGCGTTGAAGTGGTTG-3´) were designed. The cDNA of *xyl1* was amplified by 3´and 5´RACE as specified by the manufacturer using the SuperScript III First-Strand Synthesis System (Invitrogen, Grand Island, NY, USA) using total RNA isolated from mycelia of race 1472 induced with xylan for 24 h. Finally, the complete cDNAs of *xyl1* were amplified with the specific primers designed on the 5´ UTR, ClxiI1-F (5´-ACTTATCATCGTCCGCTTCAACCA-3´) and 3´ UTR ClxiI1-R (5´-GCAATCCTCGGAGTTCCAATCTGA-3´), using total RNA of mycelia from both races induced with xylan for 24 h. The PCR incubation mixture was heated at 96°C for 3 min in a Thermocycler (Eppendorf Master Cycler Gradient, Brinkmann, Westbury, NY), followed by 30 cycles of denaturation for 30 s at 96°C, annealing for 35 s at 60°C, extension for 1 min at 72°C and then by a final extension for 10 min at 72°C. All PCR products obtained from both races were ligated into the pCR 2.1 vector (Invitrogen).

The sequences of both strands of cDNA were determined by automatized sequencing using the dideoxy-chain termination method by the commercial service of Macrogen USA. Nucleotide sequences were analyzed using DNAsis (Hitachi), Mega6 (Tamura et al., 2013) and 4Peaks v 1.7.2 software (Griekspoor, 2012). The sequence of the N-terminal secretion signal was identified with SignalP 4.1 Server (Bendtsen et al., 2004). The protein molecular masses, pI values and *N*-glycosylation sites were calculated using ExPASy Proteomics Server (Wilkins et al., 1999). The nucleotide sequences of 12 endo-β-1,4-xylanases from fungal species were obtained from the NCBI GenBank (Table 1) and were numbered when more than one gene was present in a genome. Multiple sequence alignments were performed with Clustal X software (Larkin et al., 2007) using the default parameters. The signal peptide sequences and N-and C-terminal extensions were excluded.

Expression analysis of *xyl1*

Relative quantification of gene expression (RT-qPCR) was performed using the comparative Ct method (DDCt) on a Step One Plus Real-Time PCR System (Applied Biosystems Carlsbad, CA, USA) according to the manufacturer's instructions. Reactions were carried out with an SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). A fragment of cDNA (208 bp) was amplified with the designed specific primers TRxil2-F (5´TGTCGGAGGCAAGGGCTGGAATC 3´) and TRxil2-R (5´

ATAAGTGCCGCCGTCGCTGGTGA 3´) using total RNA obtained from mycelia induced with 2.5% glucose for 8 h, or xylan or CW from *P. vulgaris* for 0, 2, 4, 6, 12, 24 and 48 h and 3, 4, 5, 7 and 9 days. Fragments of cDNA (372 and 339 bp for races 1472 and 0, respectively) from the *C. lindemuthianum* β-tubulin gene (*btub*) were amplified with the primers B36F and B12R (5´ CACCCACTCCCTCGGTGGTG 3´ and 5´CATGAAGAAGTGAAGACGCGGGAA 3´, respectively) (Thon and Royse, 1999) using total RNA obtained from mycelia grown with glucose. The β-tubulin fragments were sequenced and deposited in GenBank (Accessions: KF487130 and KM587706). The specific primers TR-βtub2-D and TR-βtub2-R (5´ GAATTCCCCGACC GTATGATG 3´ and 5´ CGGAGAGGGTGGCGTTGTA 3´, respectively) were designed and used as an internal control (endogenous gene).

Data were obtained from three independent experiments performed in triplicate, and analysis of variance (ANOVA) was carried out. The results are reported as the mean and standard errors (SE). *P* values <0.05 were considered significant.

Assay of endoxylanase activity

Endoxylanase catalyzes the hydrolysis of the 1,4-β-D-xylosidic linkages present in xylan, releasing β-D-xylopyranosyl oligomers and smaller molecules such as β-D-xylopyranosyl mono-, di- and tri-saccharides (Collins et al., 2005; Polizeli et al., 2005). These products can be quantified as reducing sugars using colorimetric methods. Accordingly, reaction mixtures containing 0.5 mL of 0.5% xylan (Sigma), 0.5 mL FEM and buffer A in a final volume of 1 mL were incubated at 30°C. After 10 min, the reaction was terminated by heating the samples in boiling water for 5 min and, after cooling, the amount of reducing sugars was measured using the Nelson-Somogyi method (Nelson, 1944). Activity was expressed, as µg of reducing sugars released in one min. Specific activity was referred to one milligram of protein.

Statistical analysis

Statistical analysis of data was performed using ANOVA with a GLM using a 2 x 3 factorial design (two races and three carbon sources) at each time point. The mean values of the enzyme activity of the fungus and its substrate and the amount of protein and mycelial protein were compared and grouped using the Tukey's test. All analyses were performed with STATISTICA v 10 software (Inc., 2013).

Phylogenetic analyses

Phylogenetic analyses were performed on the deduced amino acid sequence for *C. lindemuthianum* XYL1 and 11 endo-β-1,4-xylanases characterized from other ascomycetes (Table 1) and one sequence from the basidiomycete *Lentinula edodes* as an outgroup. Deduced amino acid sequences were aligned with Clustal X software (Larkin et al., 2007) using the default parameters. Before phylogenetic analyses, the signal peptide sequences, and N- and C-terminal extensions were excluded. Phylogenetic analysis was performed under Neighbor-Joining and Maximum likelihood criteria using Mega6 (Tamura et al., 2013). The JTT substitution model and gamma correction were used, 1000 bootstrap replicates were performed. The amino WAG evolution model with gamma correction was utilized for maximum likelihood analysis, and the most parsimonious trees were estimated using the heuristic search option (Nearest-Neighbor-Interchange-NNI) with random sequence addition (five random replicates). A WAG+G substitution model was used, and 1000 bootstrap replicates were performed.

RESULTS

Isolation and sequence analysis of *xyl1*

cDNA encoding an endo-β-1,4-xylanase was isolated from each race and deposited in GenBank (Accessions: KF487129, KM587707). The *C. lindemuthianum xyl1* cDNA of race 1472 has 905 bp, with a 5´ UTR of 25 bp and a 3´ UTR of 211 bp (Figure 1). The *xyl1* cDNA of race 0 has 751 bp, with a 5´ UTR of 23 bp and a 3´ UTR of 59 bp (Figure 2). At nucleotide and amino acid levels, the sequence of both races showed 100% identity. Comparison at amino acid level with corresponding sequences in GenBank showed 67, 64, 62 and 61% identity with a *xyll* of *P. tritici-repentis, xil1 of Cochliobolus carbonum, htxyl1* of *Helminthosporium turcicum*, *Xyn22 of Magnaporthe grisea,* respectively. The putative protein has an open reading frame of 222 amino acids with a signal peptide cleavage site between Ala^{19} and Ser^{20} (Figures 1 and 2), according to the SignalP 4.1 web server (Bendtsen et al., 2004), which is consistent with previously reported sequences (Apel-Birkhold and Walton, 1996; Kimura et al., 2000). The putative mature protein (residues 20 to 222) has a calculated molecular mass of 21.71 kDa and a pI of 8.94. A potential *N*-glycosylation site at Asn^{71} was found with the ExPASy Proteomics Server (Ellouze et al., 2011; Wilkins et al., 1999).

The multiple sequence alignment of the deduced amino acid sequences of *C. lindemuthianum* XYL1 with the endo-β-1,4-xylanases of other fungi revealed the conserved motif EYY where a residue corresponding to the catalytic site is found (Figure 3). In various bacteria and fungi, this motif was reported to be the key segment for enzyme catalytic activity or substrate binding (Apel et al., 1993; Degefu et al., 2001; Li and Ljungdahl, 1994). The deduced amino acid sequence of *C. lindemuthianum* XYL1 revealed two Glu residues (E^{96}, E^{187}) that are highly conserved in xylanases of family GH11 and are likely to be involved in hydrolysis of the glycosidic bond (Figures 1, 2 and 3) (Degefu et al., 2001; Kimura et al., 2000; Tanaka et al., 2005), with one acting as an acid catalyst/base and the other as a nucleophilic residue (Davies and Henrissat, 1995; Sapag et al., 2002). Additionally, XYL1 has an Asp in position 55 that may be necessary for maintaining the optimum pH of these enzymes (Ellouze et al., 2011; Lübeck et al., 1997b).

Analysis of *xyl1* expression and production of endoxylanase activity

When used as the principal carbon source, glucose sustained growth of both fungal races but the maximum growth of race 0 (501.67 mg after 10 days) was delayed by approximately two days and was 29% lower when compared with race 1472 (708 mg after 8 days) (*P*<0.0001) (Figure 4A). Extracellular endoxylanase

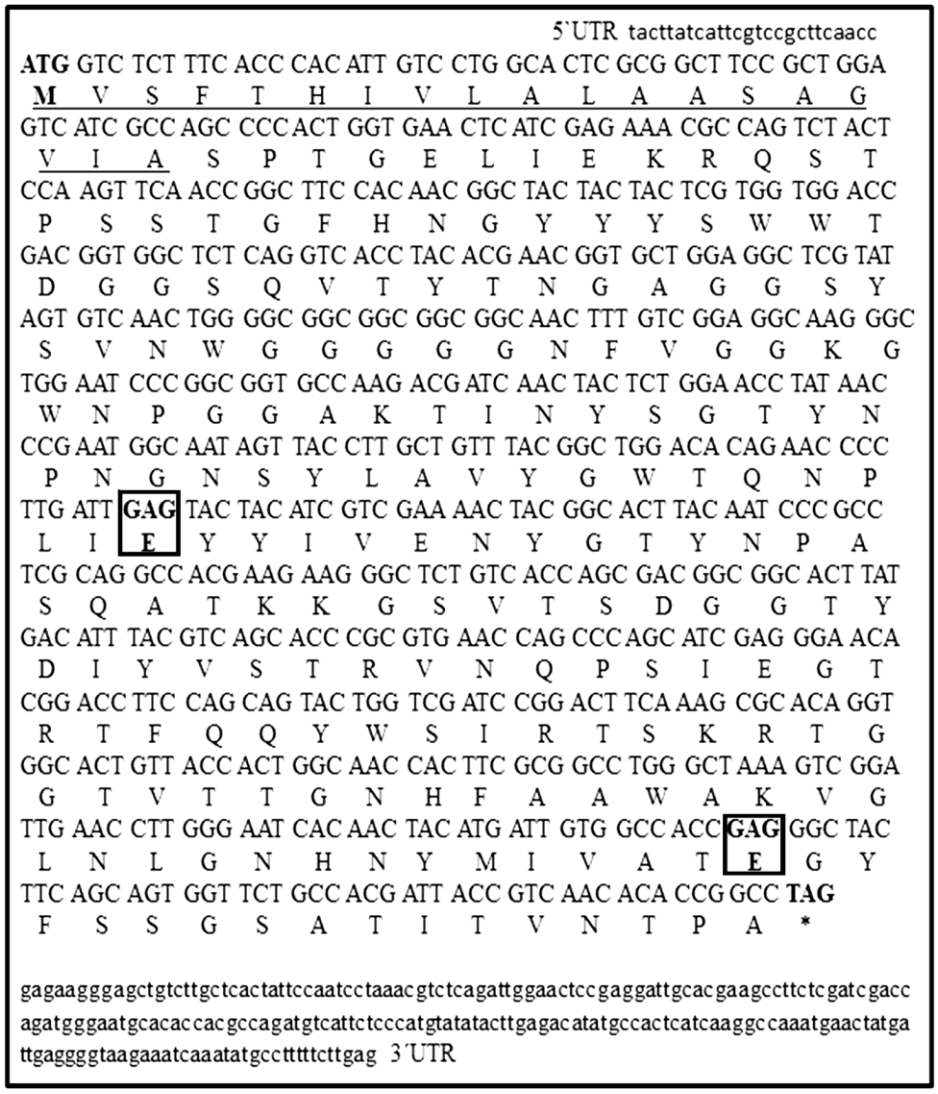

Figure 1. Nucleotide and deduced amino acid sequences of the *xyl1* gene of *C. lindemuthianum* race 1472. The signal peptide sequence is underlined, the catalytic residues (Glu-96 and 187) are boxed and an asterisk indicates the stop codon.

(XYL) production by the two races was very low, with values of specific activity in the range of 6.4 and 10.5 reducing sugars/min/mg protein after 2 and 12 days of growth, respectively, for the race 1472 (Figure 4A). Corresponding values for the race 0 were 2.5 and 11.83 (Figure 4A). However, even under these conditions, significant differences in XYL production were observed with an increase for race 1472, particularly during early days of growth. For both races, a peak was observed after seven days (P<0.0001). The *C. lindemuthianum xyl1* transcript exhibited basal levels of expression (1-fold) in both races (Figure 5A and B).

As described earlier (Hernández-Silva et al., 2007), both races utilized xylan as a carbon substrate, but growth of race 1472 (416.67 mg of mycelia after 8 days) was over 5.5-fold higher and substantially faster than that of race 0 (75 mg of mycelia after 8 days) (P<0.0001) (Figure 4B). Values of 416.67 and 75 mg represent 59 and 15% of the maxima obtained in glucose-supplemented cultures, respectively. Xylan stimulated the production of XYL activity, which was increased and peaked at 90 µg reducing sugars/min/mg protein after seven days of growth and was faster in the pathogenic race (P<0.0001) (Figure 4B). This result was by far the highest activity detected in this study and was 1.5-fold higher than that of race 0, which reached a maximum of 59.67 µg reducing sugars/min/mg protein one day later (Figure 4B). The pathogenic race strongly expressed the *xyl1* transcript after 6 to 48 h and three days (16.5-fold) and decreased over the following 4-9 days (Figure 5A). In contrast, the non-pathogenic race weakly expressed the *xyl1* transcript between 0 h and three days, increased

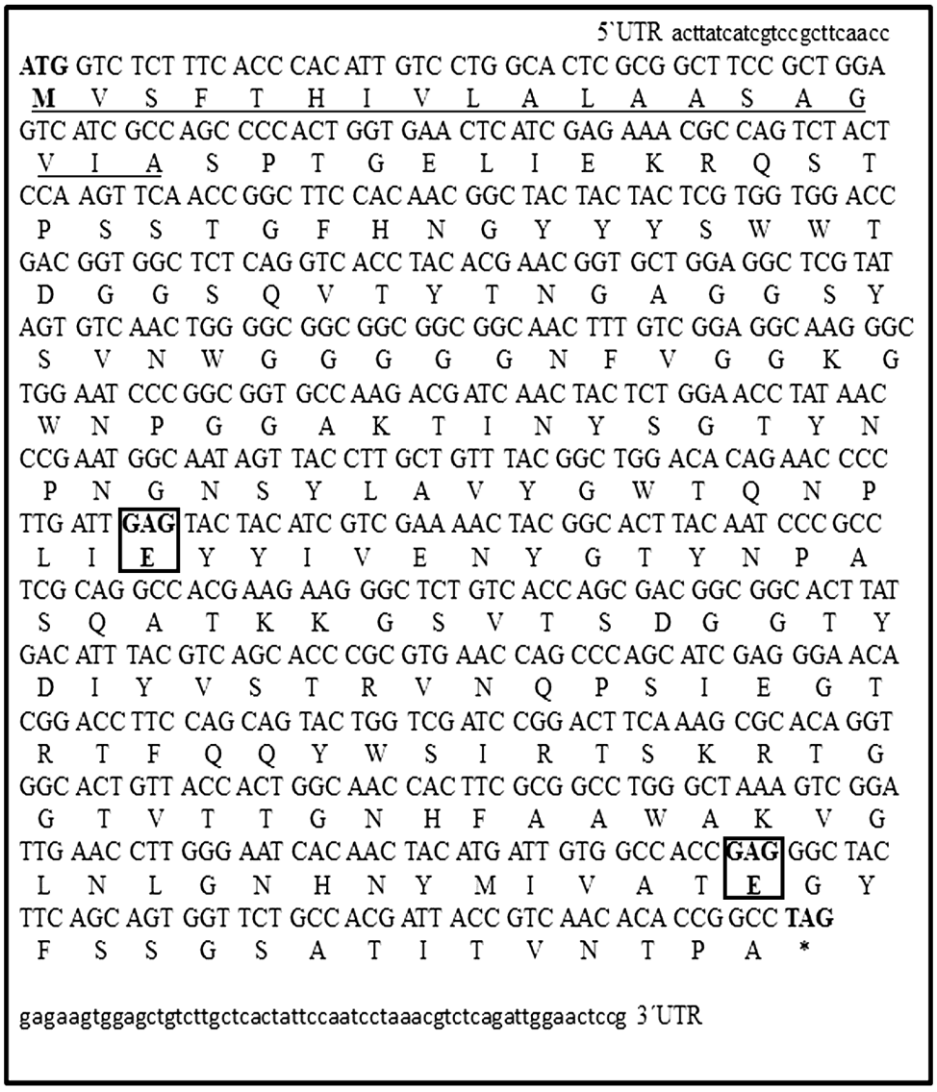

Figure 2. Nucleotide and deduced amino acid sequences of the *xyl1* gene of *C. lindemuthianum* race 0. The signal peptide sequence is underlined, and the catalytic residues (Glu-96 and 187) are boxed and an asterisk indicates the stop codon.

and peaked after four days (6.6-fold) and decreased over the following 5-9 days (Figure 5A).

The ability of cell walls fractions from *P. vulgaris* to sustain the growth of *C. lindemuthianum* and induce endoxylanase was also tested. As observed with other carbon sources, the pathogenic race grew faster and to a greater extent than the non-pathogenic race. This difference was maintained up to approximately 8-9 days of incubation ($P<0.0001$) (Figure 4C). After that, both races grew with similar rates producing comparable amounts of mycelium protein after 12 days of growth ($P>0.05$). Due to technical restrictions to measure growth on cell walls, values obtained with this substrate could not be compared with those obtained in soluble carbon substrates. The pathogenic race exhibited low expression levels of the *xyl1* transcript during early time points, then a weak peak was observed after 48 h (3.3-fold), and this increased over the following 5-9 days (9.3-fold) (Figure 5B). In contrast, the non-pathogenic race weakly expressed the *xyl1* transcript between 0-48 h, then it increased, peaked after 3 and five days (~2-fold) and finally decreased over the following 7-9 days (Figure 5B). Interestingly, after eight days of incubation, the pathogenic race produced an amount of XYL activity (80 µg reducing sugars/min/mg protein) equivalent to 89% of the maximum observed after seven days of growth on xylan (Figure 4C and B). The non-pathogenic race produced a 1.6-fold lower amount of enzymatic activity(50 µg reducing sugars/min/mg protein) that corresponded to 67% of the maximum induced by xylan (Figure 4C and B). It should be noted that in this case, the maximum XYL activity was produced by the

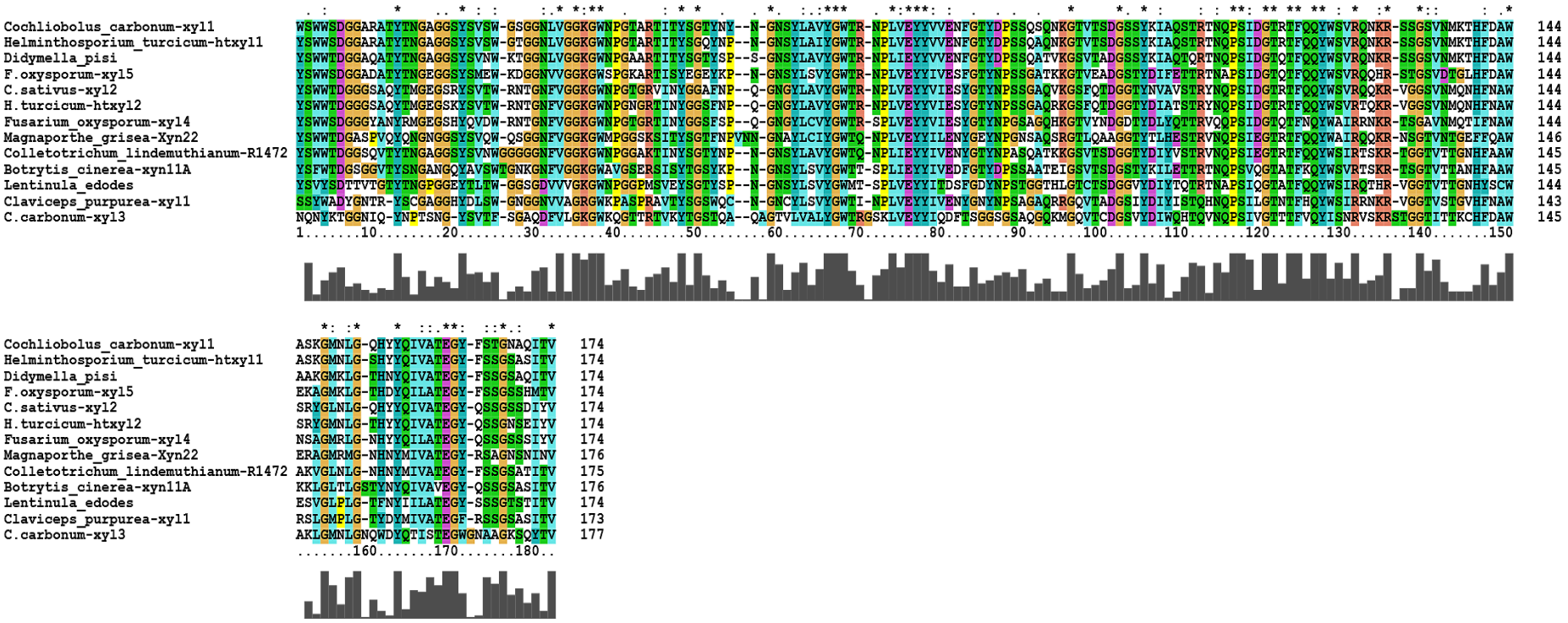

Figure 3. Clustal alignment of endoxylanases. Identical residues and conserved catalytic residues are marked with an asterisk (*). Dots indicate the change of one amino acid for another of the same group.

non-pathogenic race two days after the maximum produced by the pathogenic race.

The regulation of lytic enzymes in some fungi (St. Leger et al., 1988) and gene expression in several species of *Colletotrichum* can be modulated by ambient pH (Prusky et al., 2001) and this mechanism of regulation operates in a vast diversity of organisms (Denison, 2000). Therefore, we considered it necessary to determine whether the pH of the extracellular medium varied during the period of incubation under the different culture conditions. Throughout this study, the initial pH of all culture media was adjusted to 5.5. The growth of the pathogenic race in the presence of glucose, xylan or cell walls resulted in a rather irregular profile of pH variation. After 12 days, the pH reached values of 6.0, 6.5 and 7.2, respectively (Table 2). On cell walls, an abrupt acidification of the medium was observed after two days and then it became increasingly alkaline. The growth of the non-pathogenic race on glucose or xylan resulted in a steadier pattern of pH alkalinization to final corresponding values of 6.2 and 6.5. Alkalinization of the medium to pH 7.0 by race 0 grown on plant cell walls followed a more irregular profile.

Phylogenetic analyses

Comparison of amino acid sequences of endoxylanases showed 40 to 67% identity suggesting a diversification process that gave rise to proteins that shared identity mainly in sequence and the structure of the catalytic site. Clustal alignment identified the location of amino acids

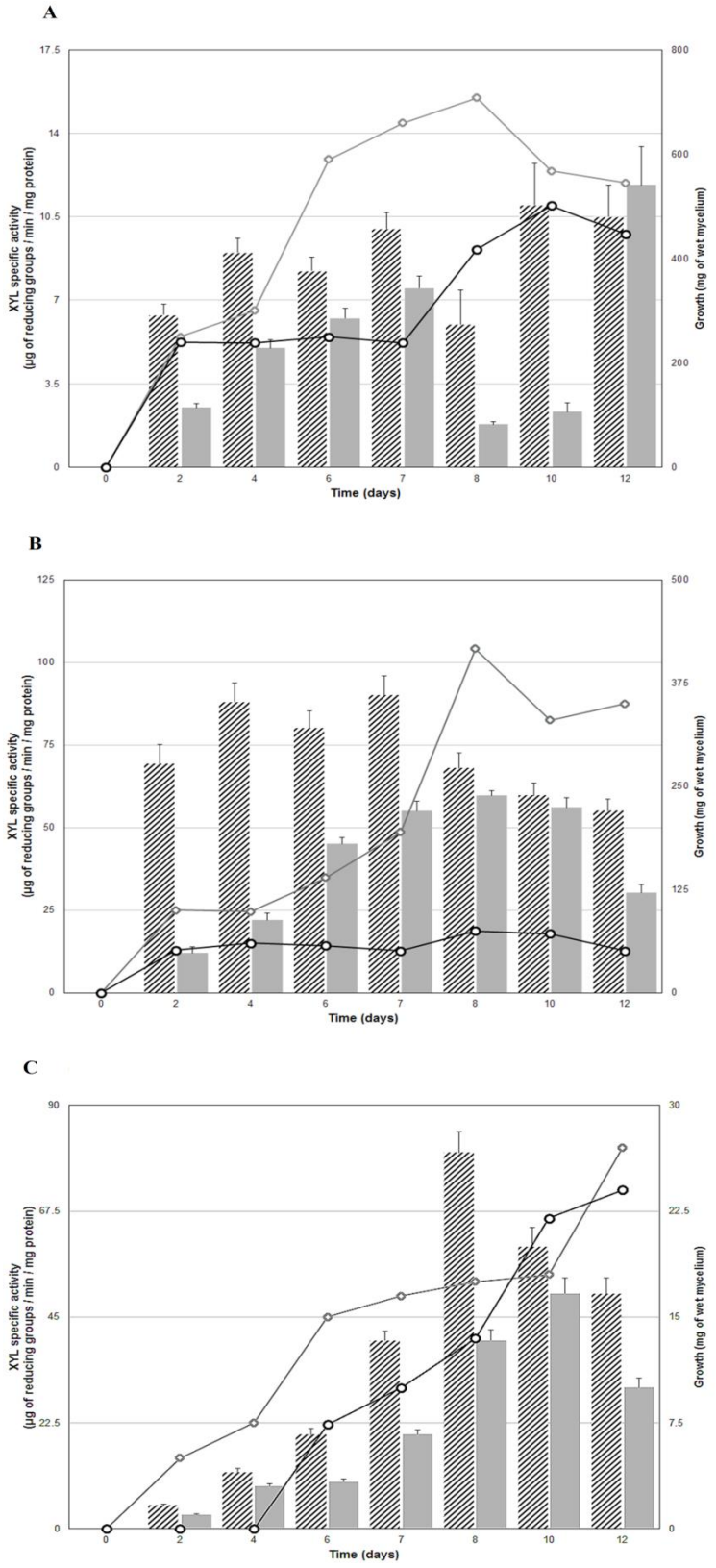

Figure 4. Growth and production of extracellular endoxylanase activity (XYL) by race 1472 and race 0 cultivated in the presence of glucose (A), xylan (B), or plant cell walls (C) as the sole carbon sources. Diamonds, the growth of race 1472; circles, the growth of race 0; stripped bars, XYL activity of race 1472; gray bars, XYL activity of race 0.

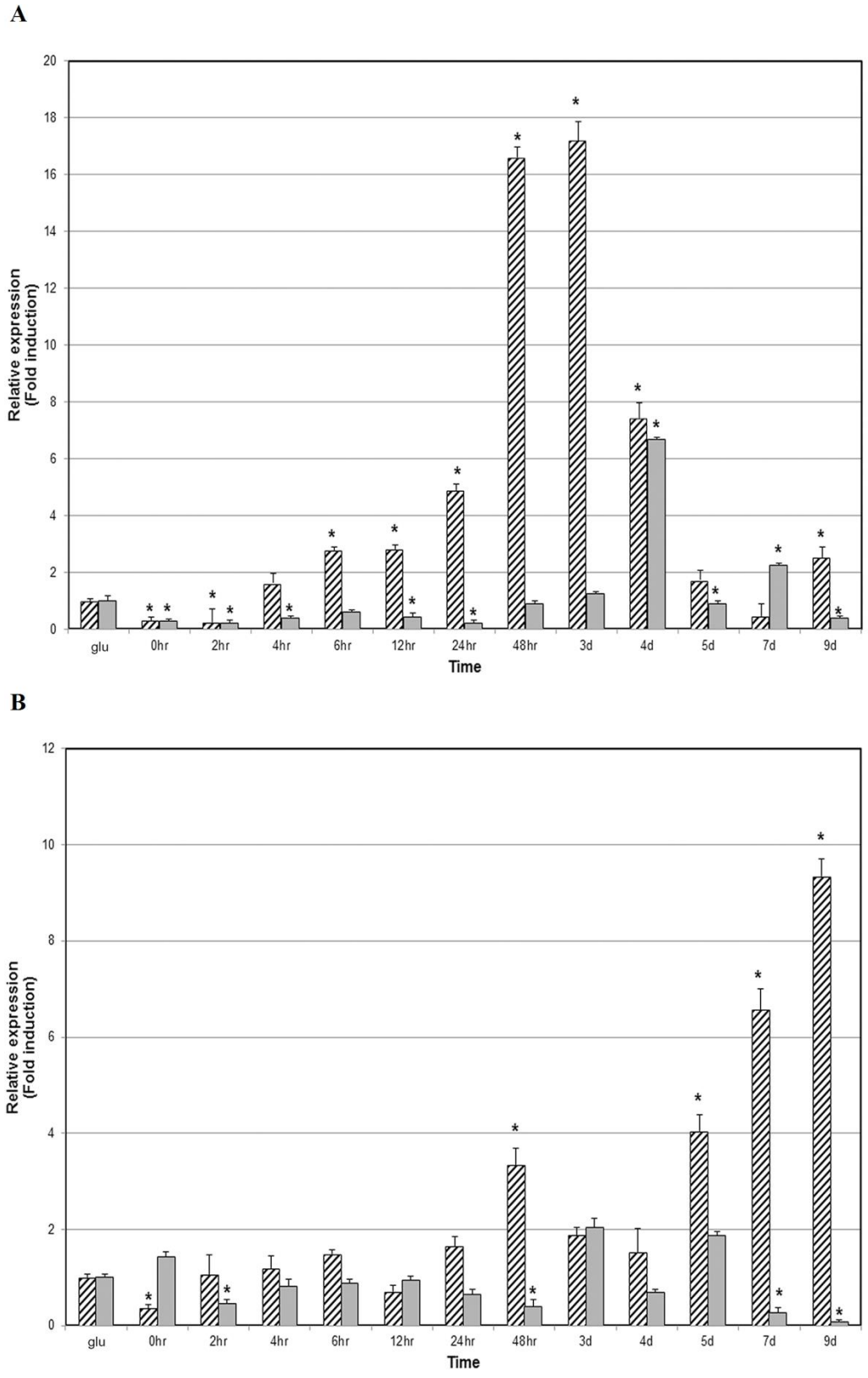

Figure 5. Expression analysis of *xyl1* by RT-qPCR induced with xylan (A) or bean cell walls (B). Stripped bars, show *xyl1* expression in race 1472; gray bars, show *xyl1* expression in race 0. Each bar indicates the mean of triplicates ± SE of three independent experiments. The symbol "*" indicates significant changes (P<0.05) about the control (glu, glucose).

Table 2. Kinetic data of pH analysis in races 1472 and 0 of *C. lindemuthianum,* grown with 2.5% of glucose, xylan or *P. vulgaris* cell walls.

Time*	Race 1472			Race 0		
	Glucose	Xylan	Cell walls	Glucose	Xylan	Cell walls
0	5.5	5.5	5.5	5.5	5.5	5.5
2	5.66	5.585	4.43	5.52	5.605	5.5
4	6.79	6.04	6.5	5.63	6.015	6.08
6	5.6	6.88	6.93	5.81	6.09	7.06
7	5.92	6.485	6.91	5.93	6.295	6.36
8	4.83	7.215	7.99	6.13	6.655	6.66
10	4.95	6.705	8.05	6.39	6.79	7.21
12	6.03	6.66	7.41	6.18	6.725	7.15

*Time expressed in days.

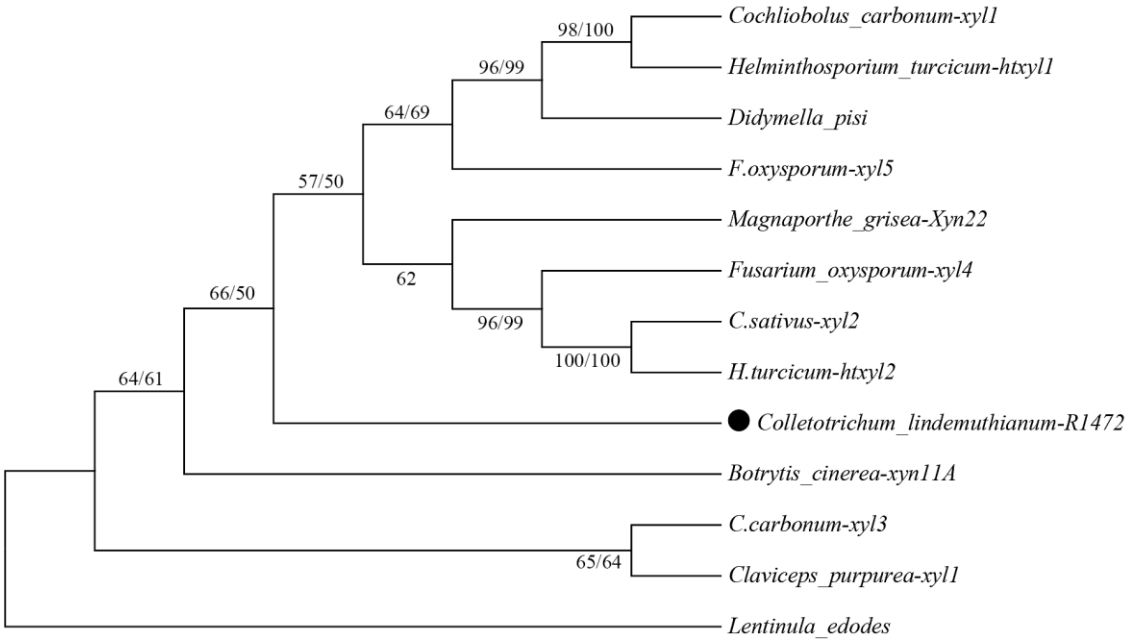

Figure 6. Phylogenetic analyses of endoxylanases from *C. lindemuthianum* and other fungal species. The tree was constructed using the Neighbor-Joining (NJ) and *Maximum* likelihood methods and includes only the conserved region of the catalytic domain (190 aa) of the proteins used in the analysis. General topology obtained is represented by the 50% of majority rule consensus tree, in which the NJ posterior probabilities and ML bootstrap support are indicated on the branches. The numbers at the nodes indicate bootstrap values based on 1,000 bootstrap replications.

in endoxylanases expected to have a catalytic role (Figure 3) (Ellouze et al., 2011; Sapag et al., 2002). The phylogenetic analyses revealed xyl3 of *Cochliobolus carbonum* and xyl1 of *Claviceps purpurea* in a basal clade (Figure 6). Next, xyn11A of *Botrytis cinerea* was separated as the next version of these enzymes. Later, in an evolutionary progression, the rest of enzymes were grouped into a widely diversified clade with XYL1 of *C. lindemuthianum* as basal of two sub-clades or lineages. In one of these sub-clades, other protein (xyl1) of *C. carbonum* grouped with htxyl1 of *H. turcicum*, the enzyme of *Didymella pisi* and xyl5 of *Fusarium oxysporum,* was found. In the other sub-clade, htxyl2 of *Helminthosporium turcicum* grouped with xyl2 of *Cochliobolus sativus*, xyl4 of *Fusarium oxysporum* and xyn22 of *Magnaporthe grisea* were found (Figure 6).

DISCUSSION

No differences were found in the coding region of the endo-β-1,4-xylanase of the non-pathogen and pathogen

races of *C. lindemuthianum.* The *xyl1* sequence analysis and Clustal alignment with xylanases reported for other fungi revealed the characteristic elements of genes coding for endo-β-1,4-xylanases of family GH11.

Induction of cell wall degrading enzymes by different carbon substrates has been studied in some phytopathogenic fungi such as *Sclerotinia sclerotiorum* (Riou et al., 1991), *Sclerotium rolsfii* (Sachslehner et al., 1998) and *Penicillium* sp. (Rahman et al., 2003), among others. The involvement of some of these enzymes in the development of bean anthracnose by *C. lindemuthianum* race ɣ was first described by Wijesundera et al. (1989). Results presented here show a clear difference between the non-pathogenic (0) and pathogenic (1472) races of *C. lindemuthianum* regarding growth, induction of *xyl1* transcript expression and production of extracellular endoxylanase activity when they are challenged with different carbon substrates. Accordingly, though maximum growth was obtained on glucose, a readily metabolizable nutrient, basal expression of *xyl1* and only trace amounts of XYL were produced by both races; however, although low, a higher XYL production was observed for race 1472, particularly early during incubation. A similar basal production of xylanase and endoglucanase (Sachslehner et al., 1998; Tuncer et al., 2004), pectinases (Hernández-Silva et al., 2007; Oyeleke et al., 2012; Riou et al., 1991) and cellulases (Acosta-Rodriguez et al., 2005; Carle-Urioste et al., 1997; Sharada et al., 2013) has been described in the fungi *S. rolsfii*, *S. sclerotiorum* and *C. lindemuthianum*, respectively.

In fungi, the expression of extracellular hydrolytic enzymes is coordinately regulated by transcriptional activators and repressors (Aro et al., 2005; Tani et al., 2014). The expression of genes encoding xylanolytic enzymes is subject to catabolic repression through the action of CreA under a preferred carbon source and the activation through the action of XlnR under carbon limitation (Cho and Choi, 1999; de Vries and Visser, 2001; Tani et al., 2014).

In this study, significant levels of the *xyl1* expression and endoxylanase were produced in pathogenic race 1472 only when the enzyme substrate was available in a medium lacking other nutrients of easier assimilation, such as glucose, indicating that expression of *xyl1* can be regulated by the carbon source. It has been proposed that basal levels of endoxylanase commence degradation of xylan generating products that induce further enzymatic activity. Xylose, as the final product of xylan degradation, functions as a regulator of the expression of xylanases, acting as an inducer at low concentrations and as a repressor at high levels (de Vries et al., 1999; de Vries, 2003; Kulkarni et al., 1999; Mach-Aigner et al., 2010, 2012; Stulke and Hillen, 2000). These results support this idea as degradation of xylan occurred when basal levels of endoxylanase activity produced low levels of xylose, which then induced expression of higher levels of expression of *xyl1* and enzyme production in the pathogenic race 1472. At later time points, a reduction of activity was observed most likely due to repression by the accumulation of xylose.

The non-pathogenic race of *C. lindemuthianum* used in this work is unable to infect *P. vulgaris*, and thus its lifestyle is closer to that of a saprophytic fungus. Therefore, it is possible that the differences found between the non-pathogenic and pathogenic races of *C. lindemuthianum* are related to the speed of activation of the lytic enzyme genes during the interaction with the host. Additionally, the pathogenic race degrades xylan faster and grows better than the non-pathogenic race, suggesting a different ability in the degradation of this polysaccharide and the use of oligo- or monomeric sugars. As previously described (Hernández-Silva et al., 2007; Lara-Marquez et al., 2011), the expression of *Clpnl2* gene and activity of pectin lyase between the two races were similar to that observed in this study when 92% esterified pectin was utilized as the sole carbon source. In other words, the pathogen requires a rapid and higher level expression of endoxylanase activity and other related lyticases for successful interaction with the live plant tissue, which implies an energy cost and the non-pathogen does cannot invest because it feeds on dead plant tissue.

The response of the fungus to cell walls from *P. vulgaris* is interesting if it is considered that only a certain proportion of the provided substrate corresponds to xylan. A typical primary cell wall contains 9-25% cellulose, 25-50% hemicellulose (whose main structural polymer is xylan), 10-35% pectin and 10% proteins (Cosgrove, 1997). The authors previously demonstrated that *P. vulgaris* cell walls also induced pectin lyase (PNL) activity in the pathogenic race to levels that represent up to 46% of the maximum obtained with 92% esterified pectin, the best PNL inducer. This value was approximately 2.8-fold higher than that produced by race 0 (Hernández-Silva et al., 2007). This difference is close to that observed for endoxylanase activity in this study. These data indicate that polysaccharides present in the cell wall structure cooperate efficiently to induce a range of polysaccharidases specific for the types of glycosidic linkages present in the cell wall components, thus granting the fungus the ability to degrade the wall barrier efficiently.

A number of evidences indicate a role of ambient pH in the regulation of production of pectinolytic enzymes in fungi such as *Penicillium paxilli* (Szajer and Czajer, 1985), *P. itallicum* (Alaña et al., 1989), the avocado-pathogen *C. gloeosporioides* (Drori et al., 2003; Kramer-Haimovich et al., 2006; Yakoby et al., 2000), *Trametes trogii* (Levin and Forchiassin, 1998) and *Aspergillus oryzae* (Fontana and Silveira, 2012). In the latter, alkalinization during fruit infection is necessary for the conversion of the biotrophic stage into the necrotrophic stage (Kramer-Haimovich et al., 2006). Also, ambient pH

has been described as a regulatory factor related to the pathogenesis of *S. sclerotiorum* (Rollins and Dickman, 2001), *C. gloesporoides* (Alkan et al., 2013) and *C. acutatum* (You et al., 2007). Contrary to these findings, an effect of the pH on growth, expression of *xyl1* gene and production of XYL activity by *C. lindemuthianum* was not observed, which is consistent with previous results on PNL activity (Hernández-Silva et al., 2007).

The results, allow us to hypothesize that the regulation of enzyme expression and nature of the assimilatory carbon substrates processed by these organisms play a determinant role in their lifestyle. The differences in growth, *xyl1* expression and production of enzymatic activity between the two races of *C. lindemuthianum* suggest an adaptation of race 1472 that results in a rapid degradation of xylan, induction of increased activity and utilization of depolymerization products as carbon nutrients. Race 0 does not seem to prefer xylan as a carbon source but instead grows better with bean cell walls, suggesting that differences exist in the utilization of mono- or oligosaccharides on race 1472. The authors observed a similar behavior of other enzymes of the complex involved in the degradation of the cell wall suggesting that it may be a general phenomenon (Acosta-Rodriguez et al., 2005; Hernández-Silva et al., 2007; Lara-Marquez et al., 2011). The differences at this level can be part of the general response of fungi to host components. However, future studies comparing the enzymatic complexes of degradation of more fungal species with different lifestyles will be required to confirm this hypothesis.

Finally, phylogenetic analyses showed a diversification of endo-β-1,4-xylanases and separation of proteins from the same fungal species into various groups or lineages. Similar results were described after a phylogenetic analysis of the nucleotide sequences of the *htxyl1* and *htxl2* xylanase genes from the corn pathogen, *H. turcicum.* These genes showed differential expression related to the substrate type (xylan and/or xylose) or stages of infection of maize, suggesting a role in saprophytic or pathogenic phases (Degefu et al., 2004; Ellouze et al., 2011). Here, a phylogenetic separation of other xylanases with differential expression was found; *xyl1* and *xyl3* in *C. carbonum* (Apel-Birkhold and Walton, 1996) and *xyl4* and *xyl5* in *F. oxysporum* (Gomez-Gomez et al., 2001, 2002), suggesting diversifying selection (Brunner et al., 2013). In this context, the differential expression of cutinases, cellulases, hemicellulases and pectinases related to different stages of the life cycle, namely, biotrophic, necrotrophic and saprophytic, has been reported in the hemibiotrophic pathogen *Zymoseptoria tritici* (Brunner et al., 2013). Also, purified selection has been detected in many genes, which can be related to the optimization of enzymatic activity. In some of these genes, diversifying selection has also been detected, which is possibly related to the adaptation to the host and/or the life cycle of the fungus (Brunner et al., 2013).

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors thank the financial support provided by SEP-CONACyT (Project 2012-01, code 182755 to MGZP), CONACyT for scholarships granted to UCS and MGVR and UNAM for a postdoctoral fellowship program 2012-14 to ALM.

REFERENCES

Acosta-Rodriguez I, Pinon-Escobedo C, Zavala-Paramo MG, Lopez-Romero E, Cano-Camacho H (2005). Degradation of cellulose by the bean-pathogenic fungus *Colletotrichum lindemuthianum*. Production of extracellular cellulolytic enzymes by cellulose induction. Antonie Van Leeuwenhoek 87:301-310.

Ahmed S, Riaz S, Jamil A (2009). Molecular cloning of fungal xylanases: an overview. Appl. Microbiol. Biotechnol. 84:19-35.

Alaña A, Gabilondo A, Hernando F, Moragues MD, Dominguez JB, Llama MJ, Serra JL (1989). Pectin Lyase Production by a *Penicillium italicum* Strain. Appl. Environ. Microbiol. 55:1612-1616.

Alkan N, Meng X, Friedlander G, Reuveni E, Sukno S, Sherman A, Thon M, Fluhr R, Prusky D (2013). Global aspects of pacC regulation of pathogenicity genes in *Colletotrichum gloeosporioides* as revealed by transcriptome analysis. Mol. Plant Microbe Interact. 26:1345-1358.

Apel PC, Panaccione DG, Holden FR, Walton JD (1993). Cloning and targeted gene disruption of XYL1, a beta 1,4-xylanase gene from the maize pathogen *Cochliobolus carbonum*. Mol. Plant Microbe Interact. 6:467-473.

Apel-Birkhold PC, Walton JD (1996). Cloning, disruption, and expression of two endo-beta 1,4-xylanase genes, XYL2 and XYL3, from *Cochliobolus carbonum*. Appl. Environ. Microbiol. 62:4129-4135.

Aro N, Pakula T, Penttilä M (2005). Transcriptional regulation of plant cell wall degradation by filamentous fungi. FEMS Microbiol. Rev. 29:719-739.

Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004). Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340:783-795.

Biely P (1985). Microbial xylanolytic systems. Trends Biotechnol. 3:286-290.

Biely P, Vrsanska M, Tenkanen M, Kluepfel D (1997). Endo-beta-1,4-xylanase families: differences in catalytic properties. J. Biotechnol. 57:151-166.

Brito N, Espino JJ, Gonzalez C (2006). The endo-beta-1,4-xylanase xyn11A is required for virulence in *Botrytis cinerea*. Mol. Plant Microbe Interact. 19:25-32.

Brunner PC, Torriani SF, Croll D, Stukenbrock EH, McDonald BA (2013). Coevolution and life cycle specialization of plant cell wall degrading enzymes in a hemibiotrophic pathogen. Mol. Biol. Evol. 30:1337-1347.

Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B (2009). The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. Nucleic Acids Res. 37:D233-238.

Carle-Urioste JC, Escobar-Vera J, El-Gogary S, Henrique-Silva F, Torigoi E, Crivellaro O, Herrera-Estrella A, El-Dorry H (1997). Cellulase induction in *Trichoderma reesei* by cellulose requires its own basal expression. J. Biol. Chem. 272:10169-10174.

Cho SG, Choi YJ (1999). Catabolite repression of the xylanase gene (xynA) expression in *Bacillus stearothermophilus* no. 236 and B.

subtilis. Biosci. Biotechnol. Biochem. 63:2053-2058.
Collins T, Gerday C, Feller G (2005). Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiol. Rev. 29:3-23.
Cosgrove DJ (1997). Assembly and enlargement of the primary cell wall in plants. Annu. Rev. Cell Dev. Biol. 13:171-201.
Davies G, Henrissat B (1995). Structures and mechanisms of glycosyl hydrolases. Structure 3:853-859.
de Vries RP (2003). Regulation of *Aspergillus* genes encoding plant cell wall polysaccharide-degrading enzymes; relevance for industrial production. Appl. Microbiol. Biotechnol. 61:10-20.
de Vries RP, Visser J (2001). *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. Microbiol. Mol. Biol. Rev. 65:497-522.
de Vries RP, Visser J, de Graaff LH (1999). CreA modulates the XlnR-induced expression on xylose of *Aspergillus niger* genes involved in xylan degradation. Res. Microbiol. 150:281-285.
Dean R, Van Kan JA, Pretorius ZA, Hammond-Kosack KE, Di Pietro A, Spanu PD, Rudd JJ, Dickman M, Kahmann R, Ellis J, Foster GD (2012). The Top 10 fungal pathogens in molecular plant pathology. Mol. Plant Pathol. 13:414-430.
Degefu Y, Lohtander K, Paulin L (2004). Expression patterns and phylogenetic analysis of two xylanase genes (htxyl1 and htxyl2) from *Helminthosporium turcicum*, the cause of northern leaf blight of maize. Biochimie 86:83-90.
Degefu Y, Paulin L, Stephensen Lübeck P (2001). Cloning, Sequencing and Expression of a Xylanase Gene from the Maize Pathogen *Helminthosporium Turcicum*. Eur. J. Plant Pathol. 107:457-465.
Denison SH (2000). pH regulation of gene expression in fungi. Fungal Genet. Biol. 29:61-71.
Dodds PN, Rafiqi M, Gan PH, Hardham AR, Jones DA, Ellis JG (2009). Effectors of biotrophic fungi and oomycetes: pathogenicity factors and triggers of host resistance. New Phytol. 183:993-1000.
Drori N, Kramer-Haimovich H, Rollins J, Dinoor A, Okon Y, Pines O, Prusky D (2003). External pH and nitrogen source affect secretion of pectate lyase by *Colletotrichum gloeosporioides*. Appl. Environ. Microbiol. 69:3258-3262.
Ellouze OE, Loukil S, Marzouki MN (2011). Cloning and molecular characterization of a new fungal xylanase gene from *Sclerotinia sclerotiorum S2*. BMB Rep. 44:653-658.
Emami K, Hack E (2001). Characterisation of a xylanase gene from *Cochliobolus sativus* and its expression. Mycol. Res. 105:352-359.
Flor HH (1971). Current status of gene for gene concept. Annu. Rev. Phytopathol. 9:275.
Fontana RC, Silveira MM (2012). Influence of pectin, glucose, and pH on the production of endo- and exo-polygalacturonase by *Aspergillus oryzae* in liquid medium. Braz. J. Chem. Eng. 29:683-690.
Fry SC (2006). The growing plant cell wall chemical and metabolic analysis. Blackburn Pr., Caldwell, NJ.
Giesbert S, Lepping HB, Tenberge KB, Tudzynski P (1998). The Xylanolytic System of Claviceps purpurea: Cytological Evidence for Secretion of Xylanases in Infected Rye Tissue and Molecular Characterization of Two Xylanase Genes. Phytopathology 88:1020-1030.
Gilkes NR, Henrissat B, Kilburn DG, Miller RC, Jr., Warren RA (1991). Domains in microbial beta-1,4-glycanases: sequence conservation, function, and enzyme families. Microbiol. Rev. 55:303-315.
Gomez-Gomez E, Isabel M, Roncero G, Di Pietro A, Hera C (2001). Molecular characterization of a novel endo-beta-1,4-xylanase gene from the vascular wilt fungus *Fusarium oxysporum*. Curr. Genet. 40:268-275.
Gomez-Gomez E, Ruiz-Roldan MC, Di Pietro A, Roncero MI, Hera C (2002). Role in pathogenesis of two endo-beta-1,4-xylanase genes from the vascular wilt fungus *Fusarium oxysporum*. Fungal Genet. Biol. 35:213-222.
Gonzalez M, Rodriguez R, Zavala ME, Jacobo JL, Hernandez F, Acosta J, Martinez O, Simpson J (1998). Characterization of Mexican Isolates of *Colletotrichum lindemuthianum* by Using Differential Cultivars and Molecular Markers. Phytopathology 88:292-299.
Griekspoor AGT (2012). 4Peaks. p. Computer program distributed by the authors.
Hernández-Silva L, Piñón-Escobedo C, Cano-Camacho H, Zavala-Páramo MG, Acosta-Rodríguez I, López-Romero E (2007). Comparison of fungal growth and production of extracellular pectin lyase activity by pathogenic and non-pathogenic races of *Colletotrichum lindemuthianum* cultivated under different conditions. Physiol. Mol. Plant Pathol. 70:88-95.
Inc. (2013). STATISTICA v 10 software, Statistical Software.
Kimura T, Suzuki H, Furuhashi H, Aburatani T, Morimoto K, Karita S, Sakka K, Ohmiya K (2000). Molecular cloning, overexpression, and purification of a major xylanase from *Aspergillus oryzae*. Biosci. Biotechnol. Biochem. 64:2734-2738.
King B, Waxman K, Nenni N, Walker L, Bergstrom G, Gibson D (2011). Arsenal of plant cell wall degrading enzymes reflects host preference among plant pathogenic fungi. Biotechnol. Biofuels 4:4.
Kramer-Haimovich H, Servi E, Katan T, Rollins J, Okon Y, Prusky D (2006). Effect of ammonia production by *Colletotrichum gloeosporioides* on pelB activation, pectate lyase secretion, and fruit pathogenicity. Appl. Environ. Microbiol. 72:1034-1039.
Kulkarni N, Shendye A, Rao M (1999). Molecular and biotechnological aspects of xylanases. FEMS Microbiol. Rev. 23:411-456.
Lara-Marquez A, Zavala-Paramo M, Lopez-Romero E, Calderon-Cortes N, Lopez-Gomez R, Conejo-Saucedo U, Cano-Camacho H (2011). Cloning and characterization of a pectin lyase gene from *Colletotrichum lindemuthianum* and comparative phylogenetic/structural analyses with genes from phytopathogenic and saprophytic/opportunistic microorganisms. BMC Microbiol. 11:260.
Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007). Clustal W and Clustal X version 2.0. Bioinformatics 23:2947-2948.
Lee CC, Wong DW, Robertson GH (2005). Cloning and characterization of the xyn11A gene from *Lentinula edodes.* Protein J. 24:21-26.
Levin L, Forchiassin F (1998). Culture conditions for the production of pectinolytic enzymes by the white-rot fungus *Trametes trogii* on a laboratory scale. Acta Biotechnol. 18:157-166.
Li XL, Ljungdahl LG (1994). Cloning, sequencing, and regulation of a xylanase gene from the fungus *Aureobasidium pullulans* Y-2311-1. Appl. Environ. Microbiol. 60:4647.
Lombard V, Golaconda Ramulu H, Drula E, Coutinho PM, Henrissat B (2014). The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res. 42:D490-495.
Lübeck PS, Paulin L, Degefu Y, Lübeck M, Alekhina I, Bulat SA, Collinge DB (1997a). PCR cloning, DNA sequencing and phylogenetic analysis of a xylanase gene from the phytopathogenic fungus *Ascochyta pisi.* Lib. Physiol. Mol. Plant Pathol. 51:377-389.
Lübeck PS, Paulin L, Degefu Y, Lübeck M, Alekhina IA, Bulat SA, Collinge DB (1997b). PCR cloning, DNA sequencing and phylogenetic analysis of a xylanase gene from the phytopathogenic fungus *Ascochyta pisi* Lib. Physiol. Mol. Plant Pathol. 51:377-389.
Mach-Aigner AR, Omony J, Jovanovic B, van Boxtel AJB, de Graaff LH (2012). d-Xylose Concentration-Dependent Hydrolase Expression Profiles and the Function of CreA and XlnR in *Aspergillus niger*. Appl. Environ. Microbiol. 78:3145-3155.
Mach-Aigner AR, Pucher ME, Mach RL (2010). d-Xylose as a Repressor or Inducer of Xylanase Expression in *Hypocrea jecorina* (*Trichoderma reesei*). Appl. Environ. Microbiol. 76:1770-1776.
Münch S, Lingner U, Floss DS, Ludwig N, Sauer N, Deising HB (2008). The hemibiotrophic lifestyle of *Colletotrichum* species. J. Plant Physiol. 165:41-51.
Nelson N (1944). A Photometric adaptation of the somogyi method fro the determination of glucose. J. Biol. Chem. 153:375-380.
Oblessuc PR, Borges A, Chowdhury B, Caldas DGG, Tsai SM, Camargo LEA, Melotto M (2012). Dissecting *Phaseolus vulgaris* Innate Immune System against *Colletotrichum lindemuthianum* Infection. PLoS ONE 7:e43161.
O'Connell RJ, Bailey JA (1988). Differences in the extent of fungal development, host cell necrosis and symptom expression during race-cultivar interactions between *Phaseolus vulgaris* and *Colletotrichum lindemuthianum*. Plant Pathol. 37:351-362.
Oyeleke SB, Oyewole OA, Egwim EC, Dauda BEN, Ibeh EN (2012). Cellulase and Pectinase Production Potentials of *Aspergillus Niger* Isolated from Corn Cob. Bayero J. Pure Appl. Sci. 5:78-83.
Paës G, Berrin JG, Beaugrand J (2012). GH11 xylanases:

Structure/function/properties relationships and applications. Biotechnol. Adv. 30:564-592.
Perfect SE, Hughes HB, O'Connell RJ, Green JR (1999). *Colletotrichum*: A model genus for studies on pathology and fungal-plant interactions. Fungal Genet. Biol. 27:186-198.
Polizeli ML, Rizzatti AC, Monti R, Terenzi HF, Jorge JA, Amorim DS (2005). Xylanases from fungi: properties and industrial applications. Appl. Microbiol. Biotechnol. 67:577-591.
Pollet A, Delcour JA, Courtin CM (2010). Structural determinants of the substrate specificities of xylanases from different glycoside hydrolase families. Crit. Rev. Biotechnol. 30:176-191.
Prusky D, McEvoy JL, Leverentz B, Conway WS (2001). Local modulation of host pH by *Colletotrichum* species as a mechanism to increase virulence. Mol. Plant Microbe Interact. 14:1105-1113.
Rahman AK, Sugitani N, Hatsu M, Takamizawa K (2003). A role of xylanase, alpha-L-arabinofuranosidase, and xylosidase in xylan degradation. Can. J. Microbiol. 49:58-64.
Riou C, Freyssinet G, Fevre M (1991). Production of Cell Wall-Degrading Enzymes by the Phytopathogenic Fungus *Sclerotinia sclerotiorum*. Appl. Environ. Microbiol. 57:1478-1484.
Rodríguez-Guerra R, Acosta-Gallegos JA, González-Chavira MM, Simpson J (2006). Pathotypes of *Colletotrichum lindemuthianum* and their implications for the development of resistant bean cultivars. Agric. Téc. Méx. 32:101-114.
Rollins JA, Dickman MB (2001). pH signaling in *Sclerotinia sclerotiorum*: identification of a pacC/RIM1 homolog. Appl. Environ. Microbiol. 67:75-81.
Sachslehner A, Nidetzky B, Kulbe KD, Haltrich D (1998). Induction of Mannanase, Xylanase, and Endoglucanase Activities in *Sclerotium rolfsii*. Appl. Environ. Microbiol. 64:594-600.
Sánchez-García BM, Flores-Olivas A, Sánchez-Arizpe A, Pineda-Rodríguez S, López-Jiménez G, Fraire-Velásquez S, Garrido-Ramírez ER, Acosta-Gallegos JA, Simpson Williamson J, Rodríguez-Guerra R (2009). *Colletotrichum lindemuthianum* pathotypes in Oaxaca and San Luis Potosi, Mexico, and resistance in common bean. Agric. Téc. Méx. 35:49-60.
Sapag A, Wouters J, Lambert C, de Ioannes P, Eyzaguirre J, Depiereux E (2002). The endoxylanases from family 11: computer analysis of protein sequences reveals important structural and phylogenetic relationships. J. Biotechnol. 95:109-131.
Sharada R, Venkateswarlu G, Venkateshwar S, Rao MA (2013). Production of cellulase-a review. Int. J. Pharm. Chem. Biol. Sci. 3:1070-1090.
Sokolovsky V, Kaldenhoff R, Ricci M, Russo VEA (1990). Fast and reliable mini-prep RNA extraction from *Neurospora crassa*. Fungal Genet. Newsl. 37:41-43.
St. Leger RJ, Durrands PK, Cooper RM, Charnley AK (1988). Regulation of production of proteolytic enzymes by the entomopathogenic fungus *Metarhizium anisopliae*. Arch. Microbiol. 150:413-416.
Stulke J, Hillen W (2000). Regulation of carbon catabolism in Bacillus species. Annu. Rev. Microbiol. 54:849-880.
Sunna A, Antranikian G (1997). Xylanolytic enzymes from fungi and bacteria. Crit. Rev. Biotechnol. 17:39-67.
Szajer I, Czajer C (1985). Formation and release of pectin lyase during growth of Penicillium paxilli. Biotechnol. Lett. 7:105-108.
Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol. Biol. Evol. 30:2725-2729.
Tanaka H, Nakamura T, Hayashi S, Ohta K (2005). Purification and properties of an extracellular endo-1,4-beta-xylanase from *Penicillium citrinum* and characterization of the encoding gene. J. Biosci. Bioeng. 100:623-630.
Tani S, Kawaguchi T, Kobayashi T (2014). Complex regulation of hydrolytic enzyme genes for cellulosic biomass degradation in filamentous fungi. Appl. Microbiol. Biotechnol. 98:4829-4837.
Thon MR, Royse DJ (1999). Evidence for two independent lineages of shiitake of the americas (*Lentinula boryana*) based on rDNA and beta-tubulin gene sequences. Mol. Phylogenet. Evol. 13:520-524.
Tuncer M, Kuru A, Isikli M, Sahin N, Çelenk FG (2004). Optimization of extracellular endoxylanase, endoglucanase and peroxidase production by *Streptomyces sp*. F2621 isolated in Turkey. J. Appl. Microbiol. 97:783-791.
van den Brink J, de Vries RP (2011). Fungal enzyme sets for plant polysaccharide degradation. Appl. Microbiol. Biotechnol. 91:1477-1492.
Wijesundera RLC, Bailey JA, Byrde RJW, Fielding AH (1989). Cell wall degrading enzymes of *Colletotrichum lindemuthianum*: their role in the development of bean anthracnose. Physiol. Mol. Plant Pathol. 34:403-413.
Wilkins MR, Gasteiger E, Bairoch A, Sanchez JC, Williams KL, Appel RD, Hochstrasser DF (1999). Protein identification and analysis tools in the ExPASy server. Methods Mol. Biol. 112:531-552.
Wu SC, Kauffmann S, Darvill AG, Albersheim P (1995). Purification, cloning and characterization of two xylanases from *Magnaporthe grisea*, the rice blast fungus. Mol. Plant Microbe Interact. 8:506-514.
Yakoby N, Kobiler I, Dinoor A, Prusky D (2000). pH regulation of pectate lyase secretion modulates the attack of *Colletotrichum gloeosporioides* on avocado fruits. Appl. Environ. Microbiol. 66:1026-1030.
You BJ, Choquer M, Chung KR (2007). The *Colletotrichum acutatum* gene encoding a putative pH-responsive transcription regulator is a key virulence determinant during fungal pathogenesis on citrus. Mol. Plant Microbe Interact. 20:1149-1160.
Zhao Z, Liu H, Wang C, Xu JR (2013). Comparative analysis of fungal genomes reveals different plant cell wall degrading capacity in fungi. BMC Genomics 14:274.