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Plant Cell Wall Degradation with a Powerful *Fusarium graminearum* Enzymatic Arsenal

Phalip, Vincent[1]*, Florence Goubet[2†], Raphaël Carapito[1], and Jean-Marc Jeltsch[1]

[1]*UMR 7175, ESBS, Université Louis Pasteur, F-67413 Ilkirch, Strasbourg, France*
[2]*Department of Biochemistry, University of Cambridge, CB2 1GA Cambridge, U.K.*

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The complex enzyme pool secreted by the phytopathogenic fungus *Fusarium graminearum* in response to glucose or hop cell wall material as sole carbon sources was analyzed. The biochemical characterization of the enzymes present in the supernatant of fungal cultures in the glucose medium revealed only 5 different glycosyl hydrolase activities; by contrast, when analyzing cultures in the cell wall medium, 17 different activities were detected. This dramatic increase reflects the adaptation of the fungus by the synthesis of enzymes targeting all layers of the cell wall. When the enzymes secreted in the presence of plant cell wall were used to hydrolyze pretreated crude plant material, high levels of monosaccharides were measured with yields approaching 50% of total sugars released by an acid hydrolysis process. This report is the first biochemical characterization of numerous cellulases, hemicellulases, and pectinases secreted by *F. graminearum* and demonstrates the usefulness of the described protein cocktail for efficient enzymatic degradation of plant cell wall.

Keywords: Cell wall degrading enzyme, xylanase, cellulase, fungus, polysaccharide, bioethanol

When *Fusarium graminearum*, a very destructive plant pathogen, is grown on plant cell wall, half of the 84 known proteins of its exoproteome are most likely implicated in cell wall polysaccharide degradation [24]. Furthermore, of the 32 genes encoding putative hemicellulases, 30 are transcribed at variable levels in these conditions [13]. Although genomic, transcriptomic, and proteomic studies are convergent concerning the diversity of cell wall degrading enzymes CWDEs secreted by *F. graminearum* in response to plant material; the studies' conclusions about their activities remain putative. *In silico* prediction cannot ascertain enzyme activities, nor describe specificities and other properties.

Although it is well-known that fungi produce many CWDEs it is erroneous to assume that the information available on glycosyl hydrolases is exhaustive [23]. To date, in *Fusarium*, only a limited number of glycosyl hydrolases have been characterized for their enzymatic behaviour. Roncero *et al.* [26] have purified and measured the activities of an endopolygalacturonase (endo-PG), an endoxylanase, and a pectate lyase from *F. oxysporum*. In *F. graminearum*, a total of 5 xylanases have been characterized [3, 4]. Finally, only the 3D structures of an endoglucanase from *F. oxysporum* [29] and an endo-PG from *F. moniliforme* [7] are known. Apart from the wheat pathogen *Mycosphaerella graminicola*, in which 6 major cell wall hydrolyzing activities (xylanase, β-1,3-glucanase, polygalacturonase, cellulase, β-glucosidase, and β-galactosidase) were detected [5], to date no other organism has been investigated with respect to all of its CWDE activities.

Climbing crude oil prices exceeding USD 140 per barrel in 2008, as well as growing awareness of the deleterious impact of petrol consumption on the environment, have led to a renewed interest in CWDEs. Hence, as reviewed recently [23], glycosyl hydrolases are considered as an attractive option for the conversion of plant biomass to fermentable sugars. The latter could be used by yeasts to produce ethanol intended to partially replace fossil fuels. Furthermore, as recently stated by Lynd *et al.* [17], R&D-driven improvements of biomass conversion are crucial for the emergence and the development of the biofuels industry. The preferred organism for bioethanol production is *Trichoderma reesei* since it produces large amounts of cellulases [6]. Unfortunately, this fungus tends to have weak hemicellulase [8] and facilitating activities that are necessary for the access of endo- and exohydrolases to polysaccharides. Consequently, *T. reesei* does not seem to be the panacea for complete conversion

**Corresponding author*
Phone: +33 (3) 90 24 47 94; Fax: +33 (3) 90 24 46 83;
E-mail: phalip@esbs.u-strasbg.fr

*Present address: Université de Strasbourg, Institut Gilbert Laustriat CNRS - UMR 7175, ESBS, Bld Sébastien Brant, BP 10413, 67412 Illkirch Cedex, France
†Present address: Bayer BioScience N.V., Technologie Park 38, B-9052 Zwijnaarde, Belgium

of biomass to monomeric sugars. To improve the efficiency of the process, complementation of *Trichoderma* hydrolases with recombinant enzymes arising from other fungi were performed [30]. An alternative strategy could be the use of a fungus that has been shown to be rich in enzymes able to efficiently degrade every layer of the cell wall. The results of previous studies on *F. graminearum* [4, 13, 24] indicate that this phytopathogenic fungus could be a good candidate for monosaccharides production from plant biomass.

The goal of the present study was to identify the enzymatic potentialities of the CWDE cocktails produced by *F. graminearum* and to assess its efficiency for biomass hydrolysis. In this report, we show that *Fusarium* secreted 17 different CWDEs acting on many oligo- and polysaccharides when grown on plant cell wall. Conversely, in the presence of glucose alone, the enzyme mix was very limited in diversity (5) and quantity. Moreover, the capacity of the enzyme mix produced on plant cell wall to convert plant biomass into a variety of fermentable sugars was demonstrated. Therefore, for the first time, *F. graminearum* is shown to be a promising supplier of CWDE usable for biomass bioconversion.

Materials and Methods

Strain and Growth Conditions

Fusarium graminearum (*Gibberella zeae*) strain F9 was isolated in our laboratory from diseased hop and identified by CABI Bioscience (United Kingdom). Hop cell wall preparation was performed as described

Table 1. Detection of CWDE activities in supernatant of *Fusarium graminearum* grown in the presence of glucose or hop cell wall and summary of results obtained.

Substrate used	Analysis by	Activities detected in glucose medium supernatant	Activities detected in cell wall medium supernatant
PGA (citrus)[a]	Reducing sugars / PACE, lyase[c]	3.2.1.15	3.1.1.11, 3.2.1.15, 3.2.1.67
Pectin with 63–66% DM (citrus)[a]	Reducing sugars	3.2.1.15	3.1.1.11, 3.2.1.15
Pectin with 70–76% DM (apple)[a]	Reducing sugars	3.2.1.15	3.1.1.11, 3.2.1.15
Pectin with 89% DM[a]	PACE	nd	3.1.1.11, 3.2.1.15
Rhamnogalacturonan-I (potato)[b]	PACE	nd	Hydrolase or lyase[d]
α-1,5-Arabinan (sugar beet)[b]	PACE	nd	3.2.1.55
β-1,4-Galactan (lupin)[b]	PACE	nd	3.2.1.23, 3.2.1.89
*p*NP-α-L-Ara*f*[a]	*p*NP assay	nd	3.2.1.55
Arabinoxylan (wheat)[b]	PACE	nd	3.2.1.55
Xylan (birchwood)[a]	Reducing sugars / PACE	3.2.1.8	3.2.1.8
AZCL-arabinoxylan (wheat)[b]	AZCL assay	3.2.1.8	3.2.1.8
AZCL-xylan (oat)[b]	AZCL assay	3.2.1.8	3.2.1.8
*o*NP-β-D-Xyl*p*[a]	*o*NP assay	nd	3.2.1.37
Mannnan (ivory nut)[b]	PACE	nd	3.2.1.78
Glucomannan (konjac)[b]	PACE	nd	3.2.1.78
Galactomannan (mung bean)[a]	PACE	nd	3.2.1.78
*p*NP-β-D-man*p*[a]	*p*NP assay	nd	3.2.1.25
Gum arabic[a]	PACE	nd	nd
Xyloglucan (tamarind)[b]	PACE	nd	3.2.1.21
Lichenan (Iceland moss)[a]	Reducing sugars	nd	3.2.1.6, 3.2.1.39, 3.2.1.73 ?
Laminarin[a]	PACE	nd	3.2.1.21
β-Glucan (barley)[b]	Reducing sugars / PACE	nd	3.2.1.6, 3.2.1.21, 3.2.1.39, 3.2.1.73
Crystalline cellulose[a]	Glucose	3.2.1.4	3.2.1.4
Avicel[a]	Glucose	nd	3.2.1.4
Carboxymethyl cellulose[a]	Glucose	3.2.1.4	3.2.1.4
Cellobiose[a]	Glucose	3.2.1.21	3.2.1.21
*p*NP-β-D-cellobioside[a]	*p*NP assay	3.2.1.91	3.2.1.91
*p*NP-β-D-Glc*p*[a]	*p*NP assay	nd	3.2.1.21
*p*NP-α-D-Glc*p*[a]	*p*NP assay	nd	nd

Techniques are described in Material and Methods. The substrate concentration was 1% for the assay in the presence of AZCL-substrate (OD 585 nm) and for the determination of glucose and reducing sugars. The concentration of *p*NP or *o*NP-derived substrates was 0.1% (OD 420 nm). In PACE, 50 µg was used. The activities are specified according to their E.C. (Enzyme Classification) numbers.

[a]Products from Sigma (France); [b]products from Megazyme (Ireland); [c]pectate lyase was measured also according to Guo *et al.* [11]; [d]the analysis performed cannot distinguish between those two activities (see Discussion).

DM, degree of methylation; nd, not detected.

earlier [28]. Cultivations were conducted at 25°C on M3 medium [20] supplemented with either glucose (M3-glucose) or hop cell wall (M3-cell wall), at 10 g/l [24]. A culture flask (Falcon 175 cm^2) was filled with 125 ml of medium and inoculated with 1,250±50 macroconidia. Cultivations were performed for 6 and 9 days for M3-glucose and M3-cell wall, respectively. This experiment was repeated three times.

Enzyme Cocktail Preparation

Supernatants from cultures of 125 ml were separated from the fungus by centrifugation and filtrated through a 0.2-µm polyethersulfone membrane (Millipore, France). Proteins from culture supernatants were precipitated with 95% ammonium sulfate (Sigma, U.S.A.) at 4°C and resuspended in 1 ml of 100 mM sodium phosphate buffer, pH 7. These solutions were diluted with 15 ml of 100 mM sodium phosphate buffer, pH 7, and concentrated to 1 ml with a concentration unit (Vivaspin 20, MW cutoff 10 kDa; Vivascience, Germany). After dilution with 7 ml of fresh buffer, the solution was finally concentrated as described above to a final volume of 0.6 ml. The resulting concentration factor of all these steps was about 200. For pretreated biomass hydrolysis, the fungal cultures and the sample preparations were performed in the same conditions except that the supernatants were only concentrated up to 10 ml (concentration factor: 12.5). Each enzyme preparation was supplemented with an antiproteases cocktail (Complete EDTA-free; Roche, Germany).

Enzyme Assays

The substrates were prepared in 100 mM sodium phosphate buffer, pH 7 (previous unpublished observations indicate that pH 7 is an acceptable compromise for an "average CWDE action"), at concentrations indicated in Table 1. Then, 15 µl of concentrated supernatant, prepared as described above, was added to 300 µl of substrate/buffer solution. Immediately after mixing, 100 µl was collected and frozen in liquid nitrogen to obtain the t_0 of the reaction. To determine the enzymatic activities, aliquots were recovered after 3 and 18 h of incubation at 37°C. This allows accurate enzymatic activities determination for both fast and/or abundant enzymes (3 h) and slow and/or less abundant ones (18 h).

Glucose concentration was estimated using the chromogen 2,2'-azino-bis(3-ethyl benzthiazoline-6-sulfonate) [30]. Reducing sugars were measured by the dinitrosalicylic acid method using xylose or galacturonic acid (GalU) as standards [18]. Enzyme activities measured with chromogenic substrate were calculated using the molar extinction coefficient of ε=13,250/M.cm at 420 nm for *p*-nitrophenol (*p*NP) and *o*-nitrophenol (*o*NP). Reactions were rapidly stopped by adding one volume of 2% sodium carbonate before freezing.

All spectrophotometric measurements were performed using an Uvikon 922 spectrophotometer (Kontron Instrument, Italy). The activities were expressed in nmol of product formed per hour and per milliliter of supernatant. The molar extinction coefficient of AZCL (azurine cross-linked) being unknown, the enzyme activities were

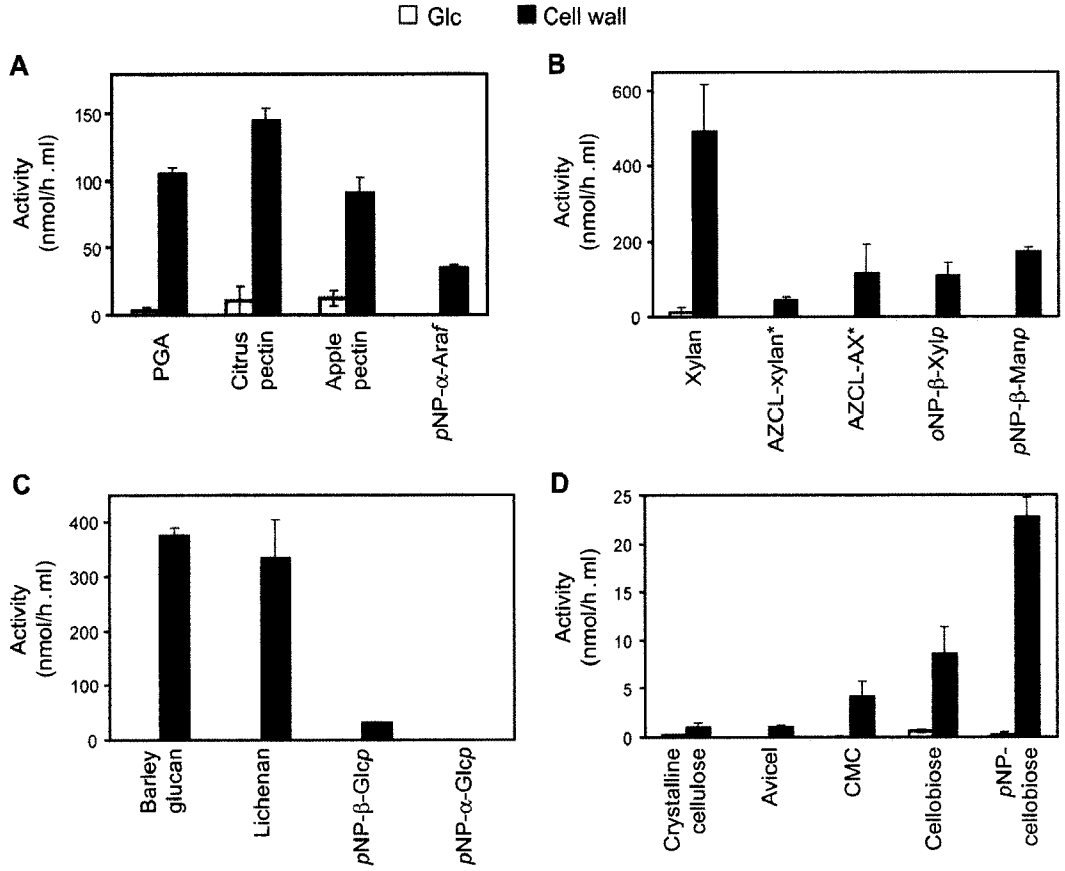

Fig. 1. CWDE activities detected in the supernatants of *F. graminearum* grown in glucose or cell wall medium.

Supernatants from cultures on glucose and plant cell wall were incubated at 37°C with the substrates prepared in 0.1 M sodium phosphate buffer, pH7. Aliquots were recovered after 0, 3, and 18 h, products were assayed, and the activities were calculated at each of these time points. The experiment from fungal growth to enzymatic products quantification was repeated three times independently. The indicated values correspond to the means of these repetitions. AX, arabinoxylan. *, arbitrary units. **A**. Pectin substrates; **B**. Xylans; **C**. Non-cellulosic glucans; **D**. Celluloses.

expressed in arbitrary units corresponding to optical density variation per hour and per milliliter of supernatant. Unlike considering the protein concentrations, reporting the activities to a supernatant volume allows direct comparison of the enzymes secreted in each culture condition and highlights the metabolic response of the fungus to its environment. The pectate lyase activity was assayed according to Guo *et al.* [12].

Polysaccharide Analysis by Carbohydrate Gel Electrophoresis PACE

To complement the activities analysis, reaction products were identified by PACE [9]. Degradation was performed using 50 μg of highly purified substrate polysaccharides (Table 1) in either 50 mM ammonium acetate, pH 5, or 50 mM Tris-HCl, pH 8, and 0.2 mM $CaCl_2$ in the presence of either 2 or 40 μl of supernatant containing enzymes for 2 h and one night, respectively. The reactions were boiled for 30 min and the samples were dried before derivatization. The derivatization, the electrophoresis, and the analysis were performed as previously described [10, 15, 22].

Pretreatment, Biomass Hydrolysis, and Sugar Assays

Stems, leaves, and cones (female flower) from dried hop were crushed with a Retsch SK100 apparatus (grid 2.0 mm). To facilitate enzymatic hydrolysis, the plant material was pretreated with ammonia according to Kim *et al.* [16]. Briefly, 10 g was soaked in 80 ml of 20% aqueous ammonia, heated to 100°C in a pressure-cooker for 1 h, and dried at 80°C overnight to remove residual ammonia. Pretreated biomass 0.5 g was incubated with 5 ml of concentrated supernatants resulting from cultures of *F. graminearum* on M3-glucose and M3-cell wall (prepared as described above). Blank reactions were performed by adding 5 ml of 100 mM sodium phosphate buffer, pH 7, to the biomass. After adding streptomycin at 50 μg/ml, the mixture was incubated at 37°C under agitation (80 rpm). Aliquots were recovered after 0, 20, and 48 h for sugar analysis. To determine total sugars for yield calculations, 0.5 g of pretreated biomass was subjected to acid hydrolysis by incubation in 5 ml of 0.4 M HCl at 100°C for 2 h [27]. Reducing sugars and glucose were assayed as described above [18, 30]. The xylose concentration was measured with the D-Xylose assay kit (Megazyme, U.K.). The level of galactose and arabinose was determined by the Lactose/D-Galactose kit (R-biopharm, Germany), which could not discriminate arabinose from galactose. Sugar quantities obtained with blanks at 0, 20, and 48 h were subtracted from the sample values. The whole experiment was performed in duplicate.

Results

CWDE in Culture Supernatant of *F. graminearum* Grown in Glucose Medium

In supernatant produced from glucose growth, very few enzymatic activities were detected. Reducing sugars analysis revealed a polygalacturonan (PGA) degrading activity (Fig. 1A). As indicated by the presence of oligogalacturonans (OGA) of degree of polymerisation (DP) 2-5, this activity corresponds to endo-PG (Fig. 2). The presence of endo-β-1,4-xylanases is shown by a low level of xylan hydrolysis (Fig. 1B) and is confirmed by weak PACE bands corresponding to oligoxylans of DP 3-6 (Fig. 3). Eventually, a slight degradation of cellulose and cellobiose was detected with 0.2 and 0.6 units, respectively (Fig. 1D). This indicates that small quantities of β-1,4-glucanases, cellobiohydrolases, and/or β-glucosidases were present in the supernatant. Table 1 summarizes all the detected activities described above.

CWDE in Culture Supernatant of *F. graminearum* Grown in Cell Wall Medium

The plant cell wall is composed of pectins, hemicelluloses, and cellulose. In supernatant of cell wall-based cultures, enzymes targeting each of these polysaccharides were present.

Pectin is the outermost layer of the cell wall and is mainly constituted by PGA and rhamnogalacturonan I (RGI). PGA is predominantly composed of galacturonic acid. This

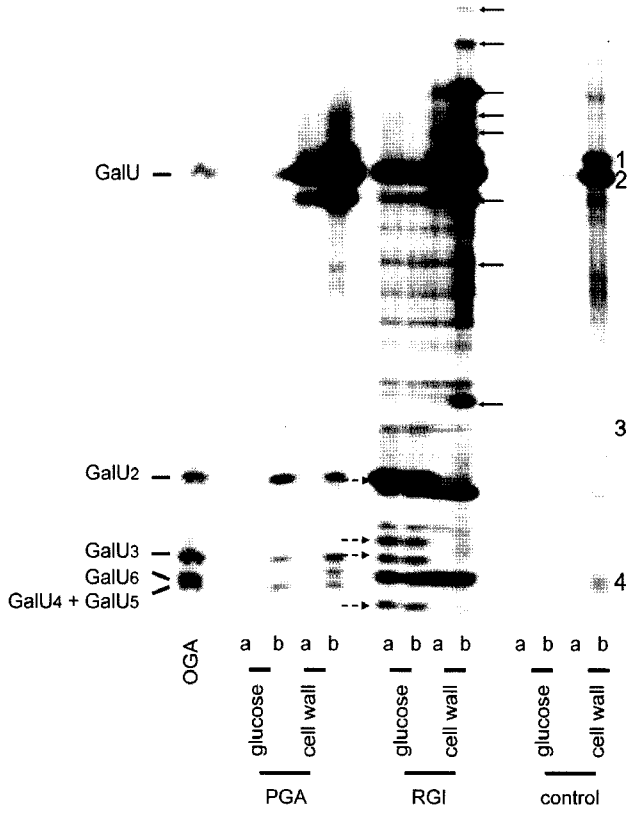

Fig. 2. PACE analysis of PGA and RGI degradation by enzymes secreted by *F. graminearum* grown in glucose or cell wall medium.

Oligogalacturonic acids (OGA) were loaded as standards. The control was performed without polysaccharides to detect any unspecific signal present in the supernatant (bands 1-4). High amounts (40 μl) of supernatant from cell wall-based cultures showed a high background, indicating that some OGA were present in the supernatant (particularly bands 1 and 4). Therefore, the pectins present in the hop cell wall were not completely degraded during fungal growth. Dotted arrows correspond to oligosaccharides present in the RGI batch that can be degraded by RG lyases or hydrolases of the fungal supernatant. Plain arrows refer to the specific oligosaccharides produced by RG lyase or hydrolase activity. PGA and RGI (50 μg) were incubated with 2 μl (a) or 40 μl (b) of fungal supernatant for 2 h or one night, respectively.

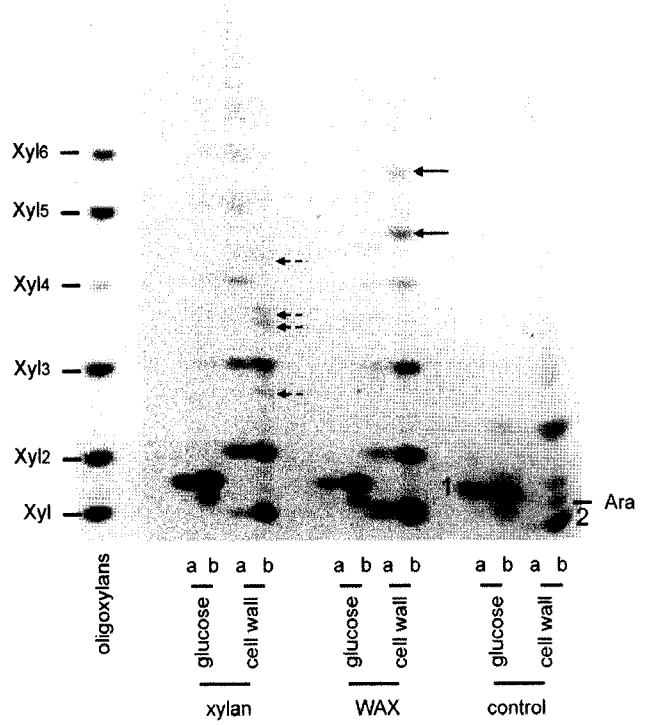

Fig. 3. PACE analysis of xylan degradation by enzymes secreted by *F. graminearum* grown in glucose or cell wall medium.
Oligoxylans were used as standards. The control was performed without polysaccharides to detect any unspecific signal present in the supernatant. The differences between these control fingerprints compared with those of Fig. 2 are due to another derivatization methodology. Unspecific bands were detected in supernatants of fungus grown in the presence of glucose (band1) or cell wall (band 2). Band 1 comigrated with glucose, showing that not all the glucose contained in the medium had been used by the fungus. Dotted arrows correspond to oligoglucuronoxylans. Plain arrows correspond to oligoarabinoxylans. β-1,4-Xylan and wheat arabinoxylan (WAX, 50 µg) was incubated with 2 µl (a) or 40 µl (b) of fungal supernatant for 2 h or one night, respectively.

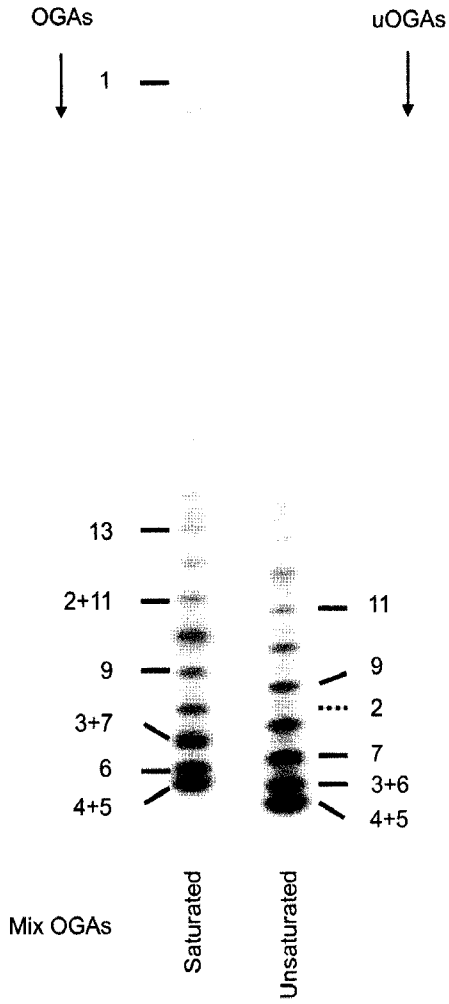

Fig. S1. PACE analysis of the separation of saturated OGA and unsaturated OGA.
The samples were prepared by cleavage of PGA with either 50 mU endo-PG or 0.2 mg of pectate lyase from *B. subtilis* [generously provided by K. Johansen (Novozymes, Denmark)] at pH 8 without $CaCl_2$ for 10 min. The endo-PG produced saturated OGA whereas the pectate lyase produced unsaturated OGA. The equivalent of 1 mg of PGA was loaded onto a gel as described in the paper. Sizes (DP) of OGA were determined using either standards or samples extracted from a band and analyzed by MS (Goubet *et al.* 2006). The unsaturated OGA of DP 2 was not present in the oligosaccharide mix, but its migration position is shown by a dotted line.

polysaccharide can be cleaved by exo-PG, endo-PG, and pectin/pectate lyases or de-esterified by pectin methylesterase (PME). The PG and pectin/pectate lyase activities can be distinguished by using two specific reaction conditions; namely, pH 5 and pH 8 in the presence of $CaCl_2$, respectively. With a subsequent PACE analysis, it is possible to differentiate saturated and unsaturated OGA produced by hydrolases and lyases, respectively (Fig. S1). In both reaction conditions, only one type of fingerprint corresponding to saturated OGA was obtained (data not shown). Moreover, no unsaturated OGAs were detected by absorbance measurements at 235 nm. Thus, only PGs were secreted by the fungus. As indicated by the high amounts of released GalU and the presence of OGA (Fig. 2), these PGs were exo-PG (E.C. 3.2.1.67) and endo-PG (E.C. 3.2.1.15).

RGI is a pectin with a complex structure displaying many side chains of arabinan, arabinogalactan, and galactan. To study the presence of enzymes able to degrade its backbone, we used a commercial debranched, de-esterified, and de-acetylated RGI (Table 1). The fingerprint observed on the PACE gel reveals that RGI backbone cleaving enzymes were present in the secreted mix (Fig. 2). These products could be the result of the action of RG hydrolases, lyases, or both. Concerning the activities on the side chains of RGI, three different enzyme types were identified. Endo-β-1,4-galactanases (E.C. 3.1.1.89) and β-galactosidases (E.C. 3.2.1.23) were identified by PACE (Fig. 4) and *p*NP-gal degradation (data not shown), respectively. Analysis of wheat arabinoxylan (WAX, Fig. 3) and *p*NP-arabinofuranosidase (Fig. 1A) degradation products revealed arabinosidase activity (E.C. 3.2.1.55). No endo-α-1,5-arabinases were detected in the supernatant.

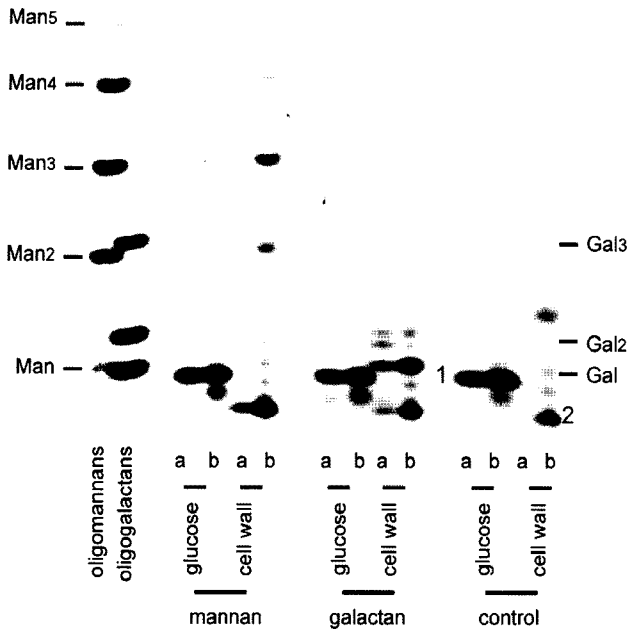

Fig. 4. PACE analysis of mannan and galactan degradation by enzymes secreted by *F. gramimearum* grown in glucose or cell wall medium.

Oligomannan and oligogalactan were used as standards. The control was performed without polysaccharides to detect any unspecific signal present in the supernatant. Unspecific bands were detected in supernatants of fungus grown in the presence of glucose (band1) or cell wall (band 2). Band 1 co-migrated with glucose showing that not all the glucose contained in the medium had been used by the fungus. β-1,4-mannan and β-1,4-galactan (50 μg) was incubated with 2 μl (a) or 40 μl (b) of fungal supernatant for 2 h or one night, respectively.

Hemicellulose is the second main polysaccharide of the cell wall and is composed of β-1,4-xyloglucan, β-1,4-arabinoxylan, and β-1,4-mannan [24] that can be substituted by different side groups such as L-arabinose, D-mannose, D-galactose, or glucuronic acid. Xylanase activities were identified by the degradation of xylan and arabinoxylan (Fig. 3). The release of oligoxylan (DP 1-4) (Fig. 3) and the hydrolysis of *o*NP-xylopyranoside (Fig. 1B) demonstrate the presence of endo-1,4-β-xylanase (E.C. 3.2.1.8) and β-xylosidase (E.C. 3.2.1.37), respectively. As no oligoglucuronoxylans were detected after degradation of xylan, the enzyme mix contained no glucuronidase acting on this specific substrate (see dotted arrows in Fig. 3). This absence could be explained by the high specificity of this type of enzyme with respect to the positions of the glucuronosyl substituants on the oligoxylans [21]. The substituted positions of birchwood xylan used for the enzymatic tests may be different from those of the cell wall material in the growth medium. Ivory nut mannan was predominantly degraded to oligomannans of DP 3-5, but also to larger oligosaccharides (Fig. 4), indicating endomannanase activity (E.C. 3.2.1.78) in the supernatant. As *p*NP-Man was hydrolyzed (Fig. 2B), β-mannosidases (E.C. 3.2.1.25) were also present. The fungus did not secrete any enzymes able to cleave the galactosyl residue of galactomannan (data not shown). No other activity than cleavage of arabinose by arabinosidases was observed when the enzymes were incubated with arabinogalactan (data not shown).

Cellulose is the third layer of the plant cell wall and is composed of β-1,4-glucans. All cellulose compounds tested [crystalline cellulose, avicel, and carboxymethylcellulose (CMC)] were hydrolyzed to glucose by the supernatant (Fig. 1D), suggesting that endocellulases (E.C. 3.2.1.4) were produced by the fungus. The activity on *p*NP-cellobioside shows the presence of cellobiohydrolases (E.C. 3.2.1.91), and the one on cellobiose attests that β-glucosidases (E.C. 3.2.1.21) were secreted by *F. graminearum* to fully degrade cellulose to glucose.

Some glucans other than cellulose contain β-1,3 and β-1,4 linkages. Barley glucan and lichenan, both β-1,4, β-1,3 glucans, were hydrolyzed by the CWDE of the supernatant. This substrate degradation can be attributed to enzymes acting on both β-1,3 and β-1,4 linkages (endoglucanases, E.C. 3.2.1.6 and/or lichenase, E.C. 3.2.1.73) and/or enzymes acting only on β-1,3 linkage (E.C. 3.2.1.39). In the presence of glucans, laminarin, cellulose, barley glucan, and xyloglucan (all containing β-glucan backbone with different linkages or substitutions), the supernatant displayed a high β-glucosidase activity masking all other enzyme activities when using PACE analysis (data not shown). With *p*NP-α-D-glucopyranoside as a substrate, no α-glucosidases were detected (Fig. 1C).

Biomass Hydrolysis

Pretreated hops were used as a substrate to study the biomass hydrolysis by enzyme pools produced by *F. graminearum* grown on glucose or on cell wall medium. To evaluate the efficiency of biomass breakdown, the enzyme cocktails were incubated with crude pretreated plant material and sugar measurements were performed after 0, 20, and 48 h at 37°C (Fig. 5). To express the sugar formed during 20 and 48 h of enzymatic treatment, the t_0 values were subtracted. The ratios of values obtained by enzymatic degradations to those generated by acid hydrolysis correspond to the reaction yields.

Enzymes secreted on glucose inefficiently produced monosaccharides from the biomass. The only sugar detected was glucose with a yield of 3.5±1.6% after 48 h. This result is consistent with the low levels of enzymatic activities described above.

By contrast, with the enzyme cocktail produced on the cell wall medium, high reducing sugars and monosaccharide yields were observed. Thirty-eight±3% of reducing sugars,

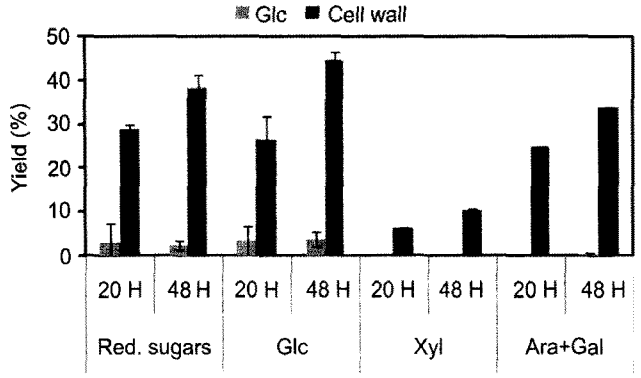

Fig. 5. Sugar yields obtained by digestion of pretreated biomass using CWDE of *F. graminearum* grown in glucose or cell wall medium.

Five ml of supernatant from cultures on glucose or plant cell wall was incubated at 37°C in the presence of 0.5 g of pretreated hop biomass. Aliquots were analyzed after 0, 20, and 48 h of incubation. The sugars present in the biomass were quantified by acid hydrolysis. Different assays were performed to determine reducing sugar (Red. sugars), glucose (Glc), xylose (Xyl), and arabinose+galactose (Ara+Gal). The yields correspond to the ratios of the sugar level generated by enzymatic digestion (*i.e.*, t_0 values subtracted) to the level obtained by acid hydrolysis. The experiment from fungal growth to sugar assays was repeated twice. The indicated values correspond to the means±standard deviation of these repetitions. The acid hydrolysis of pretreated hops generated 123 mM of reducing sugars, 76 mM of glucose, 21 mM of xylose, and 17 mM of Ara+Gal.

44±1.7% of glucose, 10.5±0.3% of xylose, and 34±0.07% of arabinose and galactose were released after 48 h of enzymatic treatment. Yields were better after 48 h than after 20 h, indicating that the hydrolysis was still in progress and that the enzyme cocktail was stable for at least 48 h.

DISCUSSION

Fusarium graminearum is a pathogen of many plants [1, 31]. Consequently, it likely possesses a very active system to attack the plant cell wall. The degradation of the cell wall allows the fungus to access the plant cell as well as to obtain nutrients. CWDE diversity can be deduced from *in silico*-based experiments like genome, transcriptome [13], or proteome [24] analyses. However, homology-based studies only provide predictions that should be validated by enzymatic identifications and are not sufficient to fully characterize CWDE. In this paper, numerous enzymatic activities of *F. graminearum* grown on glucose or on cell wall were identified and studied.

The presence of plant cell wall induced the production of many highly active CWDEs (Table 1). The probable presence of enzymes acting on pectins such as endo-PG, exo-PG, RG hydrolases, or RG lyases in the supernatant was deduced from activity measurements. As endo-PGs generally do not cut highly esterified pectins [9], the fact that both PGA (Fig. 1) and highly esterified pectin (Table 1) were accessible to the enzyme suggests that PME (E.C. 3.1.1.11) may also be present in the enzyme mix. Contrary to natural RGI that can be acetylated [25], the polysaccharide used in this study harbored no acetyl groups. However, the presence of RG lyases or RG hydrolases, the activities of which are hindered by acetylations, indicate that the enzyme cocktail probably also contained RG acetylases. Other enzymes presumably secreted by *F. graminearum* and targeting pectins are β-1,4-galactanases and arabinosidases that were shown to digest arabinogalactans, arabinoxylans, and galactans.

High mannanases, endo-xylanases and β-xylosidases activities were measured in this work. Previously, Hogg *et al.* [15] compared two different families of mannanases: GH5 and GH26. Both classes of enzymes were able to cleave mannan, but with GH26 enzymes being inhibited by glucosyl or galactosyl residues, only mannanases of family GH5 were able to hydrolyze glucomannan and galactomannan. The nature of the products obtained in our experiments suggests the presence of mannanases of family GH5. This hypothesis is in accordance with the two putative GH5 mannanases found in the exoproteome of *F. graminearum* grown on plant cell wall [24]. Moreover, the detected mannosidase activity completes the mannan digestion potentiality of the enzyme cocktail.

Cellulose is known to be very difficult to hydrolyze [32]. Many enzymes are required to achieve its complete degradation. Using enzymes from cell wall supernatant, insoluble crystalline cellulose and avicel were hydrolyzed in addition to soluble substrates, indicating the ability of the mix to completely breakdown cellulose. Interestingly, β-1,4, β-1,3 glucanase activity was evidenced using dicotyledon plant material that does not contain β-1,4, β-1,3 glucan. This could be an explanation for the wide host spectrum of *F. graminearum* and suggests that CWDE production is not only plant specific.

Aside from the absence of pectate lyase activity, the results of this study are in accordance with the findings of the exoproteome of *F. graminearum* grown on plant cell wall [24]. It is possible that the assay conditions were not optimal for the detection of pectate lyases. Other very active enzymes degrading the same substrate (*i.e.*, exo-PG) may also have masked their action. Note that a few activities like mannosidases or β-galactosidases were identified in this paper, whereas no protein with one of these putative functions was observed in the exoproteome.

When *F. graminearum* was grown in the presence of glucose, only limited CWDE activities (xylanases, glucanases, and endo- and exo-PG) were observed (Table 1). This is in agreement with the findings indicating that most fungal CWDEs are subjected to catabolic repression [2]. As described for *Sclerotinia sclerotiorum* [14], the few enzymes secreted on glucose may be involved in a detection mechanism of the host plant. In *S. sclerotiorum*, the activities of some CWDEs generate signalling molecules, which in turn induce the secretion of a second large wave of CWDEs able to

digest all plant tissue. Even though the hypothesis of such behavior in *F. graminearum* needs further investigation, this could explain why few hydrolase activities were measured with the glucose medium.

The enzymatic identifications reported here confirm unequivocally that when *F. graminearum* grows on plant material, it secretes a powerful arsenal of enzymes potentially active on each polysaccharide layer of the plant cell wall. This profusion of enzymes opens new ways to study the implication of fungal CWDE in plant cell wall digestion and plant disease. Some of the enzymes evidenced in this paper could be investigated to determine their 3D structure and their sensitivity to polysaccharide-inhibiting proteins secreted by plants [19]. Such studies could lead to the rational design of specific inhibitory molecules useful for disease treatments.

Enzyme cocktails produced by the fungus on glucose and cell wall medium were also tested for their ability to degrade pretreated hop biomass. As expected, the fungal enzymes produced on hop cell wall were very efficient to release monosaccharides. The yields obtained for glucose (~45%), reducing sugars (~40%), and arabinose+galactose (~35%) demonstrated the high efficiency of the system. Although the monosaccharide recovery was encouraging, the yields could be augmented by changing the reaction conditions (*i.e.*, incubation time, temperature, enzyme loading, pH). Furthermore, pretreatment conditions could be optimized by testing different ammonia concentrations and temperatures, for instance [16], and other protocols and/or biomasses may be used to recover more monosaccharides. Unfortunately, the amount of xylose released was relatively low (~10%). In a process intended to be transposed to industry using yeast strains able to convert hexoses and pentoses into ethanol, the xylose yield should be significantly increased. A promising way to achieve this would be to complement the concentrated supernatant by selected enzymes – currently being studied in the laboratory – acting on xylan. Further ongoing experiments aim at the valorization of these findings in an industrial process for bioethanol production in satisfactory economic conditions.

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