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CELL WALL-DEGRADING ENZYMES AND AGGRESSIVENESS IN *STAGONOSPORA NODORUM*

ABSTRACT

Stagonospora nodorum produces cell wall degrading enzymes when grown in culture media containing cell wall components. The pathogen grew as well on minimal agar plates containing cellulose, xylan and pectin as glucose, except having sparser mycelia. Four cell wall-degrading enzymes, cellulase, xylanase, pectinase and -1,3-glucanase were coordinately induced in culture filtrates growing on xyaln and cellulose as substrates. An aggressive isolate (sn26-1) secreted more cell wall-degrading enzymes than the others. Based on isoelectric focusing profiles, six to seven xylanase isozymes were induced by cellulose and xylan. No difference was found in the high (sn26-1) and low (9074) aggressive isolates. Addition of cell wall-degrading enzyme mixtures, not high xylanase alone, to a spore suspension of a low aggressive isolate (9074) caused a limited increase in tissue necrosis. We conclude that the cell wall degrading enzymes play a role in early penetration of the host by the fungus, but they are not important elicitors for disease development.

Key words: aggressiveness, -1,3-glucanase, cellulase, isozymes, isoelectric focusing, pectinase, secretion, *Stagonospora nodorum*, xylanase

INTRODUCTION

The filamentous fungi utilize plant materials as the source of carbon and energy through the action of many cell wall-degrading enzymes. Secretion of cell wall-degrading enzymes such as xylanases (1,4- -D-xylan xylanohydrolase; EC 3.2.1.8) was detected in many plant species infected by leaf pathogens (Cooper *et al.* 1988, Waksman and Keon 1989, Mendgen *et al.* 1996, Mbwaga *et al.* 1997, Xu and Mendgen 1997, Schmidt and Wolf 1999). It was speculated that cell wall-degrading enzymes would macerate plant cell walls and facilitate fungal penetration and infection. Many fungi inciting destructive soil-borne diseases in plants also show an adaptive high level of production of these enzymes in cell wall media and infected tissues (Johansson 1988, Southerton *et al.* 1993, Dori *et al.* 1995, Bidochka *et al.* 1999, Großwindhager *et al.* 1999). In addition to enzymatic activity, fungal xylanases also act as proteinaceous elicitors of defense response reactions in dicots.

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Elicitation of ethylene biosynthesis, pathogenesis-related proteins and tissue necrosis was associated with this group of enzymes (Bailey *et al.* 1990, Lotan and Fluhr 1990, Sharon *et al.* 1993, Enkerli *et al.* 1999, Furman-Matarasso *et al.* 1999). In recent studies, there were reports that cell wall degrading enzymes were secreted by *Stagonospora nodorum*, the causing agent of glume blotch disease in wheat (Magro 1984, Lehtinen 1993, Lalaoui *et al.* 2000). Xylanase activity was highly detected in an aggressive isolate of *S. nodorum*, and was considered to play a certain role in pathogenesis (Lalaoui *et al.* 2000). In this study, we compared the xylanase and other cell wall-degrading enzyme production in some low and highly aggressive isolates of *S. nodorum* and evaluated the relation of these enzymes to fungal aggressiveness.

MATERIALS AND METHODS

Fungal isolates and growth media

Eight isolates of *S. nodorum* including four highly aggressive and the four low aggressive were studied (Table 1). Two low aggressive isolates, 9074 and 9076, were isolated from wheat and perennial grass (Krupinsky 1997a, 1997b). They caused mild symptoms on wheat and triticale (Arseniuk *et al.* 1999). Isolates 64-1 and 64-4 were progeny of a sexual cross between isolates sn26-1 and sn48-1. These two ascospore-derived cultures had low infectivity due to poor sporulation (unpublished data). Fungal cultures were maintained on V8 juice agar (18% V8 juice, 0.2% calcium carbonate and 1.5% agar) plates at 22°°C (Stevens 1974).

Table 1.

Isolates of *Stagonospora nodurum* used for cell-wall-degrading enzymes analysis.

Isolates	Original hosts	Geographic location	Aggressiveness on wheat [b]
sn26-1	Winter wheat	Rzeszów, Poland	High
sn48-1	Winter rye	Lower Silesia, Poland	High
9506	Barley	Morton, ND	High
8408	Wheat	Morton, ND	High
9074	Wheat	Gallatin, MT	Low
9076	Wheat	Richland, MT	Low
64-1[a]	-	-	Low
64-4[a]	-	-	Low

[a] Progeny of a sexual cross sn26-1xsn48-1. [b] In a 0 – 9 scale, the percentage of necrosis >4.0 is 'high' in aggressiveness. If <2.0, it is 'low' in aggressiveness (Saari and Prescott 1975).

Cell growth and enzyme assays

Fungal cultures were first transferred three times as agar blocks and maintained on yeast nitrogen base (YNB, Difco Lab, Detroit, MI) agar media containing various polysaccharides such as cellulose, xylan, and pectin as the sole carbon sources (Sigma, St.Louis, MO). The mycelial growth on glucose, cellulose, xylan and pec-

tin substrates were determined by inoculating the respective agar plates with individual agar blocks containing the mycelia, maintaining the plates at 26±2°C under 24hr fluorescent lights and measuring the colony diameter. The experiment was repeated three times, each with three plates. For enzyme assays, the agar blocks from YNB-xylan and YNB-cellulose plates were inoculated to YNB liquid media containing xylan and cellulose, respectively and grown at 26°C with constant shaking (200 rpm). The mycelial mass was harvested by centrifugation. The enzymatic activity in the supernatants was determined by measuring the amount of reducing sugars released from the substrates with glucose as the standard (Miller 1959). Cellulose (2%, W/v, pH 5.0), oat spelt xylan (2%, w/v, pH 5.0), polygalacturonic acid (0.8%, w/v, pH 4.0) and laminarin (0.5%, w/v, pH 5.0) were used as assay substrates to detect cellulase, xylanase, pectinase and -1,3-glucanase activities, respectively (Lehtinen, 1993). The protein concentration was determined with a BCA Protein Assay Kit (Pierce, Rockford, IL) using bovine serum albumin as the standard.

Isoelectric focusing (IEF)

The IEF profiles of xylanase isoenzymes from sn26-1 and 9074 grown on cellulose as substrate were compared. An acrylamide gel containing 8.25 g urea, 2ml of 30% acrylamide/1.8% bis, 0.75ml ampholine (pH3.5-10.0), 0.2ml 2%(v/v) TritonX-100, 6.0 ml water, 5 µl TEMED and 35 µl 10% ammonium persulfate was prepared. The extracellular proteins in culture filtrates were precipitated with 0-95% saturated ammonium sulfate, resuspended in 0.1M phosphate buffer (pH6.0), and desalted and concentrated by centrifugal filtration (Biomax-5 membrane with a 5,000 nominal molcular weight limit cut-off, Millipore, Bedford, MA). The partially purified protein sample with a 500 µmol/hr xylanase activity was mixed with IEF sample dye and run by electrophoresis (Hoefer SE260 Mighty Small II, Amersham Pharmacia Biotech Inc., Piscataway, NJ). A broad isoelectric point (pI) kit (pH 3.5-9.3) was used for pI determination (Amersham Pharmacia Biotech Inc.). Phosphoric acid 0.085% (v/v) and 0.02M NaOH were used as anolyte and catholyte buffers, respectively. The gel was run at 300 volts for 20 h and 1000 volts for 7 h continuously with circulated cooling water (15°C). Xylanase activity in IEF gel was detected following the method of MacKenzie and Williams (1984). The electrofocused gel was soaked in a 0.2M sodium citrate-HCl (pH 4.5) buffer at room temperature for 30 min. The gel was then placed in contact with substrate gel containing 0.2M sodium citrate-HCl (pH 4.5), 1.0% agarose and 0.75% 4-O-methyl-D-glucurono-D-xylan-remazol brilliant blue R (Sigma, St. Louis, MO) and wrapped in polyvinyl film. After incubation at 55ºC for 90 min, the gel was fixed with ethanol/sodium citrate-HCl buffer (pH 4.5) (2/1 v/v) for 48 h. The xylanase isozyme bands were displayed as clear bands in a blue background of substrate gel. For protein staining, the gel was immersed in GELCODE blue stain reagent (Pierce, Rockford, IL) for 48 h.

Inoculation tests.

To determine xylanase and cellulase activities in diseased leaves, *S. nodorum* including two highly aggressive (9506 and 8408) and two low aggressive (9074 and

9076) isolates was inoculated on wheat. The inocula were prepared by growing the cultures on V-8 juice agar at 21°C under continuous cool white fluorescent light for 16 days (Stevens 1974). Pycnidiospores (2-3x10^6/ml) were suspended in sterile deionized water with Tween20. The 10-day-old wheat seedlings of 'Fortuna' (CI 13596, susceptible) and 'Red Chief' (CI 12109, resistant) were sprayed with 3 ml of pycnidiospore suspension. The control plants were sprayed with water only. Leaves were harvested and frozen with liquid nitrogen at 0, 3, 6 and 9 days after inoculation. The leaf extraction followed the procedure described earlier (Seevers *et al.* 1971). The leaf extracts were precipitated with ammonium sulfate, desalted and concentrated by centrifugal filtration. Enzymatic activities and protein concentration in the partially purified leaf extracts were determined.

To determine the effects of cell wall-degrading enzymes on initial infection by *S. nodorum*, partially purified culture filtrates were added to spore suspensions in a detached leaf test (Baker and Smith 1978). The 5 cm-long primary leaf segments were cut from 8-day-old seedlings of wheat 'Olaf' (CI15930, moderately susceptible), and placed in a square plastic Petri plate (100 × 15mm) containing 0.015% benzimidazole and 0.5% agar. *S. nodorum* isolates 9506 and 9074 were used as inocula. A 5µl droplet of spore suspension (8,000 spores) from the low aggressive isolate 9074 containing 10 mM phosphate buffer (pH 6.0) and various enzyme concentrations was added to the center of a detached leaf. Five concentrations of high xylanase/cellulase enzyme ratio mixture (4k/15, 8k/30, 12k/45, 16k/60 and 20k/75 nmol/h) from sn26-1 grown on xylan and low xylanase/cellulase enzyme ratio mixture (4k/2.8k, 8k/5.6k, 12k/8.4k, 16k/11.2k and 20k/14k nmol/h) from sn26-1 grown on cellulose were used. The spore suspension of highly aggressive isolate 9506 was used as the control. Ten leaf segments replicates were used for each treatment. Nine days after inoculation, overall severity of infection (as percent necrosis) on the detached leaf sections was rated by direct measurements of lesion size (Shaner and Buechley 1994).

RESULTS

Cell growth and enzyme assays

All *S. nodorum* isolates except 9076 adapted to corresponding cell wall components and grew well on YNB agar media containing the cell wall components as major carbon and energy source (Fig. 1). The mycelia grew well in glucose, cellulose, and xylan, but poorly in pectin (for 8408 and 9506-1 isolates not shown). However, the mycelial growth appeared to be denser in glucose than the other three cell wall components.

It was demonstrated that cell wall-degrading enzymes, such as cellulase, xylanase, pectinase and -1,3-glucanase can be coordinately produced using either xylan or cellulose as substrates (Fig. 2). Nevertheless, xylanase activities were much higher in two highly aggressive isolates (sn26-1 and 9506) and one low aggressive isolate (64-4) grown on xylan than those on cellulose. The induction of xylanases by cellulose and xylan was probably equal in three other low aggressive isolates (9074, 9076 and 64-1) (Fig. B). When the *S. nodorum* cultures were grown on different substrates, various protein concentrations were detected in the liquid

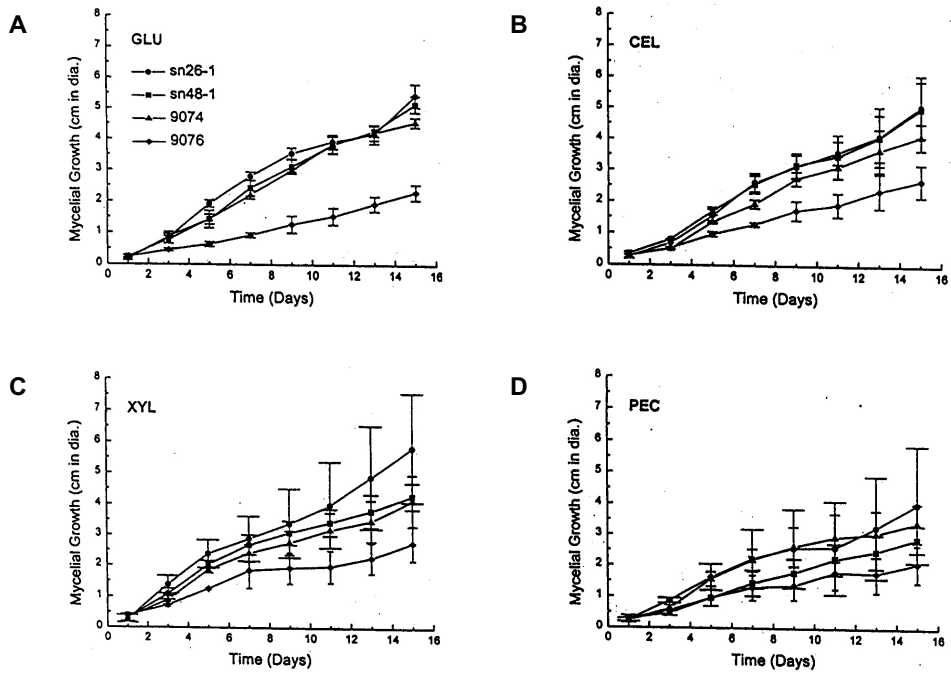

Fig. 1. Mycelial growth of *Stagonospora nodorum* isolates differing in aggressiveness on various cell wall components. Yeast nitrogen base agar plates containing 0.5% glucose (GLU) (A), cellulose (CEL) (B), xylan (XYL) (C), and pectin (PEC) (D), respectively, were used. The bars indicate the standard deviation

media. The average amount of proteins (µg/ml) secreted into culture media by six *S. nodorum* isolates was 3,059 for glucose, 467 for pectin, 432 for xylan, and 52 for cellulose. Even though the cultures grown on cellulose secreted the least amount of proteins, they induced simultaneously a reasonable amount of cell wall-degrading enzymes (Fig. 2).

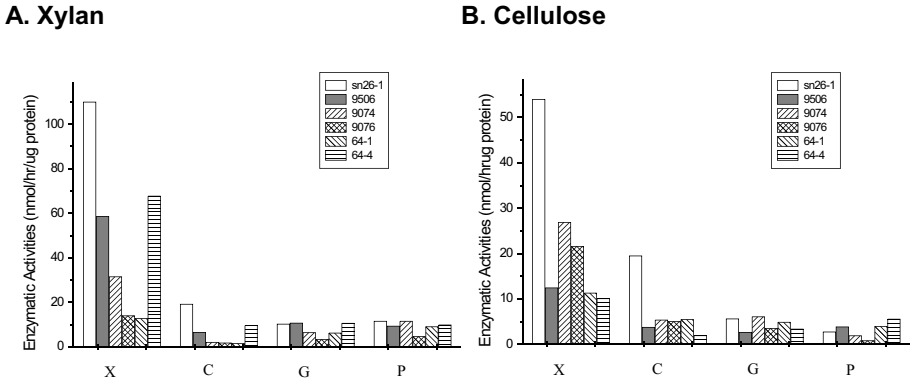

Fig 2. Secretion of cell wall-degrading enzymes by *Stagonospora nodorum* isolates differing in aggressiveness on either xylan (A) or cellulose (B). Four enzymatic activities (nmol/hr/µg protein) including xylanase (X), cellulase (C), -1,3-glucanase (G) and pectinase (P) in the liquid cultures were determined

Isoelectric focusing

Based on isoelectric focusing (IEF) profiles, the same six to seven xylanase isozymes could be detected in highly aggressive (sn26-1) and low aggressive (9074) isolates (Fig. 3). Two of the isozymes were basic pI and the others acidic.

Fig. 3. Isoelectric focusing (IEF) profiles of xylanase isozymes from two *Stagonospora nodorum* isolates differing in aggressiveness. Partially purified enzyme extracts from 9074 (1) and sn26-1 (2) were used

Table 2

Comparison of percent necrosis on the detached leaf segments infected with pycnidiospores of low aggressive *Stagonospora nodorum* isolate 9074 containing various xylanase/cellulase concentrations from the high aggressive isolate 9506.

Treatment with high (xylanase/cellulase)[a] enzyme ratio mixture	Mean difference of percent necrosis		Treatment with low (xylanase/cellulase)[a] enzyme ratio mixture	Mean difference of percent necrosis	
	9074	9506		9074	9506
9074 (-)	-	14**	9074 (-)	-	8**
9074 (4/0.015)	1	13**	9074 (4/2.8)	0	8**
9074 (8/0.030)	0	14**	9074 (8/5.6)	1	7**
9074 (12/0.045)	1	13**	9074 (12/8.4)	1	7**
9074 (16/0.060)	2	12**	9074 (16/11.2)	3	5*
9074 (20/0.075)	0	14**	9074 (20/14.0)	3	5*

[a] The unit of enzymatic activity is μmol/h.

indicates the difference between two enzyme-supplemented samples and the low aggressive isolate 9074 (control) are significant at 10% level in the t-test.

* and ** indicate the differences between highly aggressive isolate 9506 (control) and low aggressive isolate 9074 treated with various enzyme mixtures are significant at 5% and 1% levels, respectively

Inoculation tests

No significant xylanase and cellulase activity could be detected in 'Fortuna' and 'Red Chief' leaf tissue extracts infected with two highly aggressive and two low aggressive *S. nodorum* isolates (data not shown). In the detached leaf test, no significant difference was found in pycnidiospore inocula of low aggressive isolate 9074 containing high xylanase/cellulase enzyme ratio mixtures (Table 2). Nevertheless, at the 10% significance level differences were found in two high low xylanase/cellulase enzyme ratio mixtures (16/11.2 and 20/14) as compared to low

aggressive control isolate 9074. The differences between the highly aggressive control isolate 9506 and the low aggressive isolate 9074 with various enzyme ratio mixtures were significant at the 1% and 5% levels (Table 2).

DISCUSSION

Cellulases and many other cell wall-degrading enzymes, like xylanase and polygalacturonase, were coordinately induced in cultures of soil-borne fungal pathogens grown on wheat straw and other cellulose products, even though the maximal amounts of these enzymes would be produced in media containing the corresponding substrates (Lynch *et al.* 1981, Bahkali 1995). One possible induction of these cell wall-degrading enzymes in cellulose grown cultures was due to the presence of substrate contaminants in cellulose products (Lynch *et al.* 1981, Royer *et al.* 1992). The other possibility, the production of a number of cell wall-degrading enzymes is co-regulated with the presence of a single substrate, either cellulose or xylan. In some cases, the production of cell wall degrading enzymes, such as xylanase and pectinase in *Rhizoctonia solani*, mannanase in *Sclerotium* species, arabanase and laminarinase in *Rhizoctonia cerealis*, *Fusarium culmorum* and *Pseudocercosporella herpotrichoides*, and pectinases in *Sclerotinia sclerotiorum* is constitutive (Cooper *et al.* 1988, Robson *et al.* 1989, Riou *et al.* 1991, Großwindhager *et al.* 1999). In our study, none of four cell wall degrading enzymes in *S. nodorum* were detected in glucose media and were constitutively expressed. It took two days for *S. nodorum* cultures grown on YNB-glucose agar to produce xylanases for xylan hydrolysis (unpublished data). We are not sure if the pH of culture media or other environment factors would affect cell wall-degrading enzyme secretion in this fungus (Bailey *et al.* 1993).

Microbial xylanases belonging to the class of glycosyl hydrolases are assigned to two families: family 10 contains enzymes with a molecular weight over 30kD and an acidic pI, and family 11 comprises those with a molecular weight smaller than 30kD and a basic pI (Henrissat and Bairoch 1993, Biely *et al.* 1997). The number of xylanase isozymes detected in *S. nodorum* culture filtrates varied. Magro (1984) reported a single xylanase with pI 5.2 was identified in liquid cultures and diseased leaf tissues. Nevertheless, up to 13 acidic xylanase isozymes (pI range 3.6 - 6.2) were induced with oat spelt xylan and wheat cell wall substrates (Lehtinen 1993). In this study, using a broad range ampholine (pH 3.5-10.0) in IEF, 6-7 xylanase isozymes were found in *S. nodorum* culture filtrates grown in xylan and cellulose substrates. However, two low basic or near neutral pI xylanase isozymes were also found. Xylanases with high basic pI values were reported in soil-borne fungi and suggested to be important in pathogenesis (Anderson *et al.* 1993, Southerton *et al.* 1993).

In general, production of cell wall-degrading enzymes by fungal pathogens in leaf tissues is under catabolite repression. Expression of a protein kinase gene, *SNF1*, in fungal cells to release from catabolite repression is necessary for activating the cell wall-degrading enzymes, degrading and metabolizing the plant cell wall components and becoming virulent to the host plant (Tonukari *et al.* 2000). Expression of two xylanase genes was proven with cytological and molecular tech-

niques in *Claviceps purpurea*-infected rye ovarian tissues (Giesbert *et al.* 1998). In *S. nodorum,* activities of three cell wall-degrading enzymes, cellulase, pectinase and xylanase, in the diseased tissue extracts were reported (Magro 1984). However, activities of xylanase and cellulase in *S. nodorum* infected tissue extracts were not significant in our study.

Studies on the role of cell wall-degrading enzymes in plant pathogenesis are limited and controversial. Detection of these enzymes in the infected tissues suggested that secretion by fungal pathogens would facilitate hyphal penetration and increase pathogenicity (Netzr *et al.* 1979, Cooper 1989, Esquerré-Tugayé *et al.* 1989, Waksman and Keon 1989, Mendgen *et al.* 1996, Mbwaga *et al.* 1997, Xu and Mendgen 1997, Schmidt and Wolf 1999). However, in the study of expression of a single gene product, such as cellulase (Sposato *et al.* 1995), pectinases (Walton 1994) and cutinase (Kolattukuday *et al.* 1995, Oliver and Osbourn 1995), it is difficult to determine their involvement in fungal pathogenicity. The function of cell wall-degrading enzymes, particularly xylanases, in *S. nodorum* aggressiveness is not clear (Lalaoui *et al.* 2000). High xylanase producing isolate, sn26-1, did not incite severer symptoms than the other highly aggressive isolates in cereals (Arseniuk *et al.* 1999). In this study, a limited impact by cell wall-degrading enzymes on leaf necrosis indicates they may facilitate early fungal penetration but not aggressiveness. Aggressiveness in *S. nodorum* appears to be controlled by other genetic element(s).

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