Correlative analysis of *Fusarium* species pathogenicity and \textit{in vitro} xylanase activity

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**ABSTRACT**

Fusarium head blight (FHB) of wheat is one of the most destructive diseases caused by a number of *Fusarium* species worldwide. In this study, the correlation between aggressiveness and \textit{in vitro} xylanase production of 20 *Fusarium* isolates (6 *Fusarium culmorum*, 6 *F. solani* and 8 *F. verticillioides*) was investigated. Isolate aggressiveness was evaluated as a percentage of each head which is diseased and calculate the average severities for all the heads in wheat plants. Additionally, the isolates were compared for their ability to produce \textit{in vitro} significant levels of xylanase activities when grown in a solid medium. Aggressive isolates released more xylanase of weakly aggressive isolates. Correlation tests analysis revealed a significant relationship ($r = 0.72$, $r = 0.92$, $r = 0.78$; $P < 0.01$; and $r = 0.60$, $r = 0.51$, $r = 0.70$; $P < 0.01$) between the xylanase produced by *F. verticillioides*, *F. culmorum* and *F. solani* isolates and aggressiveness on the two wheat cultivars Cham4 and Hourani, respectively. Correlation between the production of this enzyme and the origin of the isolates was not found. The results indicate that the production of xylanase influences the aggressiveness of the isolates of *Fusarium* spp. towards wheat heads.

**Keywords**: *Fusarium* spp, aggressiveness, xylanase, \textit{in vitro}.

**INTRODUCTION**

*Fusarium* is a diverse genus consisting of an array of species responsible for damping-off, root rot, and vascular wilt in a multitude of economically important plant species (Summerell et al., 2001). The importance of *Fusarium* species in the current context is that infection may sometimes occur in developing seeds, especially in cereals (Clear et al., 1996; Fakhfakh et al., 2011). Because of this unusually wide range of symptoms, cell wall degrading enzymes (CWDEs) could have a diversity of important functions for the fungus in penetration, in heads, and in saprotrophic growth in dead tissue. Thus, *Fusarium* species has the potential to provide an instructive system for analysing the contribution of CWDEs to pathogenesis.

Fusarium head blight (FHB) or scab of wheat (*Triticum aestivum*) is one of the most destructive diseases in humid and semi-humid areas. The disease is caused by a number of *Fusarium* species, except *F. acuminatum* and *F. equiseti* (Stack and McMullen, 1985; Salas et al., 1999). The mechanism and pathogenicity of symptom induction by this fungus are poorly understood despite ultrastructural, biochemical and genetic studies (Kang and Buchenauer, 2000a; Arabi and Jawhar, 2010).

Xylanases are thought to be particularly important CWDEs in interactions between pathogens of gramineous and their hosts (Jaroszuk et al., 2011). Several recent studies have provided new genetic evidence for the importance of CWDEs in the host–
pathogen interaction outcome (Vorwerk et al., 2004; Douaiher et al., 2007). Because of their expected importance, we are examining the role of xylanases as aggressiveness factors in some *Fusarium* spp., which has been reported to produce several different CWDE activities, including xylanase (Alconada and Martínez, 1994; Jenczmionka and Schafer, 2005). Some of these CWDEs were shown to be correlated with the aggressiveness of *Phaeosphaeria nodorum* (Lalaoui et al., 2000), *Cochliobolus sativus* (Bakri et al., 2009) and *Mycosphaerella graminicola* isolates (Douaiher et al., 2007).

The aim of this study was to investigate, under controlled conditions, whether or not a relationship between xylanase production *in vitro* and FHB severity exists in twenty isolates of three *Fusarium* spp. (*Fusarium culmorum*, *F. solani* and *F. verticillioides*) differing in their aggressiveness.

**MATERIALS AND METHODS**

**Fungal growth conditions**

Over several years, more than 105 isolates of *Fusarium* spp. were obtained from seeds of wheat showing FHB symptoms. Seeds were sterilized in 5% sodium hypochlorite (NaOCl) for 5 min. After three washings with sterile distilled water, the seeds were transferred onto Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI, USA) amended with 13 mg/l kanamycin sulphate added after autoclaving, and incubated for 10 days, at 23 ±1ºC in the dark to allow mycelial growth and sporulation. All isolates were identified morphologically according to Nelson et al. (1983). In previous studies, different wheat genotypes had been inoculated with 105 fungal isolates, evaluating host-pathogen reactions. Emphasis was placed on selecting isolates that induced differential reactions on specific genotypes, leading to the selection of the 20 monosporic isolates (six belonging to *F. culmorum*, six to *F. solani* and eight to *F. verticillioides*) used in this study. The *Fusarium* isolates, their host plants, and geographic origin are listed in Table 1. The cultures were maintained on silica gel at 4 ºC until needed.

**Analysis of xylanase activity**

Enzyme production by the selected isolates was carried out in 250 ml Erlenmeyer flask containing 5 g of solid substrate and nutrients (based on 100 ml of liquid medium) plus distilled water to adjust the moisture content to 80%. The fermentation medium consisted of: (g/l) Na₂HPO₄ × 2H₂O 10; KCl 0.5; MgSO₄ × 7H₂O 0.15 and yeast extract 5, as a nitrogen source. Fresh fungal spores have been used as inoculums and 1-ml spore suspension (10⁶ spores/ml) was added to sterilized medium and incubated at 30ºC.

Flasks were removed after cultivation and the enzyme was extracted by adding distilled water containing 0.1% Triton x 100 to make the volume in flask equivalent to 100 ml. The flasks contents were stirred for 1.5 hours on a magnetic stirrer. The clear supernatant was obtained by centrifugation (5000 × g for 15 min) followed by filtration (Whatman no. 1. paper).

Xylanase was assayed by the optimized method described by Bailey et al. (1992), using 1% birchwood xylan as the substrate. The xylan solution and the enzyme at appropriate dilution were incubated at 55°C for 5 min and the reducing sugars were determined by the dinitrosalicylic acid procedure (Miller, 1959), with xylose as the standard. The released xylose was measured spectrophotometrically at 540 nm. One unit (U) of enzyme activity is defined as the amount of enzyme releasing 1 µmol xylose/ml per minute under the described assay conditions. All the experiments were repeated twice.
Table 1. Xylanase production by 20 isolates of *Fusarium* species in solid culture

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Host</th>
<th>Location</th>
<th>Year of collection</th>
<th>Xylanase (U/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F.SY1</td>
<td>wheat seeds</td>
<td>north</td>
<td>2005</td>
<td>20.3</td>
</tr>
<tr>
<td>2</td>
<td>wheat seeds</td>
<td>north</td>
<td>2005</td>
<td>96.36</td>
</tr>
<tr>
<td>3</td>
<td>wheat seeds</td>
<td>north</td>
<td>2005</td>
<td>163.69</td>
</tr>
<tr>
<td>12</td>
<td>wheat seeds</td>
<td>north</td>
<td>2005</td>
<td>115.9</td>
</tr>
<tr>
<td>13</td>
<td>wheat seeds</td>
<td>north</td>
<td>2004</td>
<td>90.64</td>
</tr>
<tr>
<td>14</td>
<td>wheat seeds</td>
<td>middle region</td>
<td>3003</td>
<td>19.52</td>
</tr>
<tr>
<td>F.SY9</td>
<td>wheat seeds</td>
<td>ICARDA</td>
<td>2005</td>
<td>138.72</td>
</tr>
<tr>
<td>10</td>
<td>wheat seeds</td>
<td>ICARDA</td>
<td>2003</td>
<td>19.52</td>
</tr>
<tr>
<td>15</td>
<td>wheat seeds</td>
<td>ICARDA</td>
<td>2003</td>
<td>61.92</td>
</tr>
<tr>
<td>17</td>
<td>wheat seeds</td>
<td>ICARDA</td>
<td>2003</td>
<td>16.56</td>
</tr>
<tr>
<td>18</td>
<td>wheat root</td>
<td>middle region</td>
<td>2005</td>
<td>129.92</td>
</tr>
<tr>
<td>19</td>
<td>wheat seeds</td>
<td>middle region</td>
<td>3004</td>
<td>108.56</td>
</tr>
<tr>
<td>21</td>
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<td>middle region</td>
<td>3003</td>
<td>102.3</td>
</tr>
<tr>
<td>25</td>
<td>wheat root</td>
<td>middle region</td>
<td>2003</td>
<td>140.3</td>
</tr>
<tr>
<td>F.SY7</td>
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<td>middle region</td>
<td>2003</td>
<td>908.2</td>
</tr>
<tr>
<td>26</td>
<td>wheat root</td>
<td>north</td>
<td>2005</td>
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</tr>
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<tr>
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<td>181.2</td>
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<td>north</td>
<td>2004</td>
<td>234.96</td>
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<td>24</td>
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<td>north</td>
<td>2004</td>
<td>40.1</td>
</tr>
</tbody>
</table>

LSD (5%)

**Aggressiveness tests**

The aggressiveness of the 20 isolates was conducted under growth room conditions using two different cultivars (Cham4 and Hourani) of wheat, were chosen for their different reaction. Cham4 is a local cultivar selected as susceptible to FHB, whereas Hourani is a differential resistant genotype as described by Alazem (2007). Seeds were surface-sterilized with 5% sodium hypochlorite solution for 5 min and then washed three times in sterile distilled water. They were sown into plastic pots (15-cm) filled with sterilized peatmoss, and arranged in a randomized complete block design with three replicates. Each experimental unit consisted of five pots of 20 seedlings per genotype. A full replicate consisted of the plots of two cultivars; this full test was repeated three times. Pots were placed in a growth chamber at temperatures at 23-25°C during the day and 18-20°C at night. Supplemental light was provided by 300-W metal halide lamps to ensure a 16 h photoperiod and a minimum intensity of 350 µmol m⁻² s⁻¹. Following emergence, plants were thinned to three per pot and fertilized with a 1% solution of 20-20-20 (N-P-K) once a week starting 5 weeks after planting.

**Inoculation procedure**

Inoculum was prepared as described by Xue et al. (2004): 0.5 ml of a concentrated conidial suspension from the single spore culture was spread over the surface of PDA in 9-cm Petri dishes and incubated as above for 48 h. Each
dish then received 10 ml of sterile distilled water containing 0.01% Tween 20 (polyoxyethylene sorbitan monolaurate) and was scraped gently with a sterile microscope slide to dislodge spores. The resulting spore suspension was filtered through two layers of cheesecloth and adjusted to $5 \times 10^4$ spores/ml for inoculation.

The two wheat cultivars were inoculated with each of the 20 isolates 10-14 d after heading. Prior to inoculation, a maximum of 12 spikes per pot were randomly selected, while the remainder and those from lateral tillers were removed. Plants were sprayed with the spore suspension at 0.2 ml per spike using a hand-held spray bottle. After the inoculum dried for 30 min, plants were transferred to a polyethylene humidity chamber in a growth chamber for 48 h. The growth chamber was operated at 25°C with a 12-h photoperiod. The humidity chamber was maintained at or near 100% RH by the continuous operation of two ultrasonic humidifiers. After incubation, plants were returned to the greenhouse bench. For each isolate and genotype combination, three replicate pots per genotype were used.

Symptoms of FHB were rated percentage of infected spikelets after 21 d, when plants were at the soft dough stage. Disease severity was estimated visually for each inoculated spike on a 0 (no visible FHB symptoms) to 9 (severely diseased, spike dead) scale described by Xue et al. (2004). Each head was assessed separately in all experiments. The experiment was repeated twice.

**Statistical analysis**

The percentages of infected spikelets from all plants in each pot were averaged and the means per pot of percent infected spikelets were used in the analysis of variance (Anonymous, 1996) using the super ANOVA computer package to determine whether there was a significant test × genotype interaction.

Correlations between data for *in vitro* enzyme production and aggressiveness were calculated with the SAS General Linear Models Procedure (SAS Institute; Cary, NC, USA).

**RESULTS AND DISCUSSION**

All the 20 isolates of *Fusarium* species caused disease symptoms on the wheat cultivars, but there was a large variation in aggressiveness. Moreover, the results demonstrated that neither of the two tested cultivars was immune from disease. However, the disease symptoms induced by the 20 isolates of *Fusarium* species were clear and easy to score. The disease symptoms were always more severe in the susceptible cultivar Cham4. The mean FHB severity for isolates is shown in Fig. 1. The analysis of variance of the combined results of wheat tests demonstrated a significant ($P < 0.05$) test x genotype interaction for each isolate.

On the other hand, significant differences ($P < 0.05$) in the mean xylanase yield values were detected among isolates, with high values being consistently higher in the isolates *F. solani* F.SY7 and F.SY41 with mean value 908.2 and 234.96 U/g, respectively. Whereas, low enzyme of 12.16 and 16.56 U/g was detected for *F. solani* F.SY26 and *F. verticillioides* F.SY17, respectively (Table 1). Correlation between the production of this enzyme and the origin of the isolates was not found ($r = -0.42$; $P < 0.01$). A significant correlation ($r = 0.72$, $r = 0.92$, $r = 0.78$; $P < 0.01$; for cv. Cham 4 and $r = 0.60$, $r = 0.51$, $r = 0.70$ $P < 0.01$ for cv. Hourani) was found between xylanase production of *F. verticillioides*, *F. culmorum* and *F. solani* isolates and FHB severity, respectively (Fig.1). This gives us reason to believe that the ability of *Fusarium* spp. to produce xylanase may be of importance in the aggressiveness of this fungus towards wheat heads. However, the relative differences
between the correlation values observed for the two cultivars is mainly because in the susceptible cultivar Cham 4 all the isolates producing high xylanase activity are relatively highly pathogenic on this cultivar. Gebruers et al. (2001) reported that susceptible wheat cultivar does not have endoxylanase inhibitor proteins which may be involved in plant defense mechanisms. Moreover, enzyme- and immunogold-labelling investigations confirmed involvement of extracellular enzymes that is cellulases, xylanases and pectinases, in degradation of cell wall components in F. culmorum-infected wheat spikes (Kang and Buchenauer, 2002).

The degradation of host cell walls by pathogenic fungi is based on the coordinated excretion of a number of enzymes that depend on the parasitic features of the pathogens (Xue et al., 2004). The Fusarium spp. isolates

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**Fig. 1.** Correlative analysis between disease severity of 20 *Fusarium* isolates (a: *F. verticillioides*; b: *F. culmorum* and c: *F. solani*) on two wheat cultivars, Cham4 (1) and Horani (2), and their xylanase activities *in vitro*. Disease severity was assessed as a percentage of each head which is diseased and calculate the average severities for all the heads (include the zero ratings as well) according to Xue et al. (2004).
studied here produced significant levels of xylanase, activity in vitro. The results indicate that the production of xylanase influences the aggressiveness of the isolates of *Fusarium* spp. towards wheat heads. Kang and Buchenauer (2000b) showed that *Fusarium culmorum* infects the wheat ovary usually through the junctions between the epidermal cell walls. These junctions may be a more preferable site for entry of the pathogen, allowing a quicker establishment of infection. The same phenomenon was described by Clay et al. (1997) for *Cochliobolus sativus*, which enters barley leaf tissue through the pectin-rich junction between the epidermal cell wall. In *Phaeosphaeria nodorum*, xylanase was the most active polymer-degrading enzyme produced in vitro, suggesting a key role during pathogenesis (Lalaoui et al., 2000). However, in the graminaceous plants, cell walls contain up to 40% of β-1, 4-xylans found in dicotyledonous cell walls (Buckeridge et al., 2004). The fungal pathogens of graminaceous hosts tend to secrete enzymes that degrade arabinoxylans and glucans rather than pectic polymers, whereas pectic enzymes predominate for fungal pathogens of dicotyledons (Lehtinen, 1993).

In this work, the results suggest that xylanase is likely to be key determinants of aggressiveness in *Fusarium* spp. The results also confirm the variability of xylanase activities produced in vitro among *Fusarium* species. Efforts to increase plant host resistance might be aided by new information on aggressiveness determinants. In the future, attempts to elucidate the role of xylanases in the aggressiveness of *Fusarium*, and an increased focus on the chemotype of the isolates used will undoubtedly be of value.

**ACKNOWLEDGEMENTS**

The authors thank the Director General of AECS and the Head of the Molecular Biology and Biotechnology Department for their continuous support throughout this work.

**REFERENCES**


