EXTRACELLULAR ENZYME ACTIVITIES POTENTIALLY INVOLVED IN *FUSARIUM CULMORUM* AGGRESSIVENESS

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ABSTRACT

Fusarium culmorum (*Fc*) is the causal agent of Fusarium head blight (FHB) of wheat worldwide. It has an ability to produce a range of extracellular hydrolytic enzymes capable of degrading plant cell wall components which are important for analyzing their contribution to pathogenesis. In this work, two *F. culmorum* strains *Fc1* and *Fc6* widely deferred in aggressiveness were compared for their production of hydrolytic enzymes undersolid state fermentation. Both isolates produced xylanase, lipase, amylase, polygalacturonase, filterpase and carboxy-methyl cellulase. The aggressive strain *Fc6* released more enzymes than the weak one, *Fc1*. Correlation analysis revealed a significant relationship (r =0.62, $P<$ 0.01) between the production of hydrolytic enzymes andthe strains aggressiveness on susceptible wheat plants. Moreover, the aggressive strain *Fc6*has a significantly better ability to produce protein than the weak one, *Fc1*. Results indicate that the production of hydrolytic enzymes may contribute to *Fc1* and *Fc6*aggressiveness on wheat spikelets.

Key words: *Fusarium culmorum*; aggressiveness; wheat; hydrolytic enzymes; *in vitro*

No: of Tables: 1 **No: of Figures: 3** **No: of References: 26**

INTRODUCTION

Fusarium culmorum is one of the most globally important pathogen on cereals that causes serious diseases, such as crown rot, ear rot, foot rot, and head blight (Scherm et al. 2013). Several putative pathogenicity factors of this fungus have been described, including secreted hydrolytic enzymes (Rucka et al. 1998; Jaroszuk-Scisel et al. 2011). Therefore, hydrolytic enzymes excreted extracellularlyby this fungus can play an important role in the colonization of host plant tissues.

Extracellular enzymes are thought to be particularly important in interactions between *Fusarium* spp. and their hosts (Bakri et al. 2014; Sidaoui et al. 2018), however, the role of these enzymes in the infection process is poorly understood. Studies conducted on enzymes production by phytopathogenic fungi are complicated due to the presence of plant enzymes and microbial enzyme inhibitors (Schaller et al. 2005). Therefore, the most practical way to study enzymes production by a fungus is to study them on artificial growth media that contain no plant or enzyme inhibitors.

Solid-state fermentation (SSF) is considered an attractive alternative method for the production of industrially demanded enzymes that employ microorganisms. It has generated great interest in recent years because it can be used for a variety of purposes, supported by some authors who have even indicated numerous advantages over the submerged fermentation (Lizardi-Jiménez and Hernández-Martínez 2017).The importance of SSF in enzyme production is due to the agro-industrial residues that are generally used for this purpose. However, among microorganisms that are capable of growing on solid substrates, filamentous fungi are the most distinguished producers of enzymes involved in the degradation of lignocellulosic material (Kumar and Kanwar 2011).

Despite the economic importance of *F. culmorum* incited disease, knowledge on its pathogenicity factors is still limited. In the present investigation, hydrolase enzymes including xylanase, lipase, amylase, polygalacturonase, filterpase, and carboxy-methyl cellulase by a weak aggressive strain *Fc1*and a high-aggressive one *Fc6* was monitored under SSF culture. Hydrolases from the two strains were compared for differences in activity to determine if a linkage exists between production of various hydrolases by *F. culmorum* and its degree of invasiveness in wheat.

Materials and Methods

Fungal strains

The two major strains of *F. culmorum* in Syria, *Fc1* and *Fc6* were used in this study (Table 1). They differed widely in DNA patterns and aggressiveness (Alazem 2007; Arabi and Jawhar 2010). Each strain was grown separately in 9 cm Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI. USA) and incubated for

10 days, at 23 ±1°C in the dark to allow mycelial growth and sporulation.

Extraction of enzymes from solid-state cultures

Enzyme production by the strains was carried out in 250 ml Erlenmeyer flasks containing 5 g of solid substrate and nutrients (based on 100 ml of liquid medium) plus distilled water to adjust the moisture content to 75%. Fresh fungal spores were used as inoculums and1 mL spore suspension (containing around 10^6 spores/mL) was added to a sterilized medium and incubated at 30°C. The enzyme was extracted by adding distilled water containing 0.1% Tritonx100 to make the in-flask volume equivalent to 100 mL. Flask contents were stirred for 1.5 h on a magnetic stirrer. The clear supernatant was obtained by centrifugation(5000 x g for 15 min) followed by filtration (Whatman no. 1. Paper).

Amylolytic activity

Amylase activity was measured according to the method described by Okolo et al. (1995). The reaction mixture consisted of1.25 ml of % soluble starch, 0.25 of 0.1 M acetate buffer (pH5.0), 0.25 ml distilled water and 25 ml of crude enzyme extract. After 10 min of incubation at 50°C, the liberated reducing sugars (glucose equivalent) were estimated by the dinitrosalicylic acid method of Miller (1959).One unit (IU) of a-amylase is defined as the amount of enzyme that releases 1 µmol of glucose equivalent per min under the assay conditions.

Carboxy-methyl cellulase (CMCase) and filter paperase (Fpase) activity

The CMCase and Fpase activity were measured using the methods described by Refaz et al. (2013). One unit of enzyme activity (IU) was defined as the amount of enzyme that released 1µmol of glucose per ml per minute.

Pectinolytic activity

Polygalacturonase (PGase) activity was determined according to Marcia et al. (1999) by measuring the release of reducing groups using the dinitrosalicylic acid reagent (DNS) assay (Miller 1959). The reaction mixture containing 0.8 ml of 1% citric pectin with 67% of metoxilation in 0.2M citratephosphate, pH 6.0 buffer and 0.2 ml of culture supernatant was incubated at 40 °C for 10 min. One unit of enzymaticactivity (U) was defined as 1 µmol of galacturonicacid release per minute.

Xylanolytic activity

Xylanase activity was measured based on themethod described by Bailey et al. (1992), using 1% birchwood xylan as substrate. The solution of xylan and the enzyme at appropriate dilution were incubated at 55°C for 5min and the reducing sugars were determined by the dinitrosalicylic acid method described by Miller (1959), with xylose as standard. The released xylose was measured spectrophotometrically at 540 nm. One unit of xylanase is defined as the amount of enzyme required to release 1µmol of reducing sugar as xylose equivalent per min under the above assay conditions.

Lipolytic activity

Lipase activity was determined using 1 ml sunflower oil, 5 ml of 50 mM phosphate buffer pH 7.0 and 1 ml enzyme solution. The assay was carried out according to the method of Park et al. (1988). One unit of lipase activity was defined as the amount of enzyme liberating 1µ mol of fatty acid per min under the experimental conditions.

Protein determination

Total protein was determined according to the method of Lowry et al. (1951). Soluble protein was measured from the standard curve as mg of protein per ml oftest samples.

Aggressiveness tests

The aggressiveness of the two strains *Fc1* and *Fc6* was conducted under growth room conditions using the Syrian susceptible cultivar'Cham4 of wheat (Alazem 2007). Seeds 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. Pots were placed in a growth chamber at temperatures 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 $\mu molm^{-2}ms^{-1}$. 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 was performed at 10-14 d after heading as described by Bekele (1987) using a little piece of cotton soaked with Fusarium suspension was placed between the glumes of a spikelet in contact with the anthers and the stigma of a floret. The size of the cotton was about one fifth of a glume and the amount of the soaked inoculums about 16 µl. The growth chamber was operated at 25°C with a 12-h photoperiod and 80-90% RH. Disease severity was estimated after 21 d, when plants were at the soft dough stage as described by Xue et al. (2004).

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.

Results and Discussion

F. culmorum Fc1 and *Fc6* strains caused FHB symptoms on wheat plants, but there was a large variation in aggressiveness (data not shown). The aggressive strain *Foc6* caused severe symptoms on the susceptible wheat plants as compared with the weak one *Fc1*. The mean severity for both strains is shown in Fig. 1. Analysis of variance of the combined results of wheat tests demonstrated a significant ($P< 0.01$) test × genotype interaction for each strain.

On the other hand, significant differences ($P< 0.01$) were found in the

main hydrolytic enzymes such as xylanase, lipase, amylase and polygalacturonase between the two strains, with high values being consistently higher in the high aggressive strain*Foc6*, whereas, both isolates produced CMCase and Fpase in low levels(Fig. 2).Correlation tests analysis revealed a significant relationship (r = 0.62, P< 0.01) between the production of hydrolytic enzymes and the aggressivenesson the susceptible wheat plants. This result can be supported by the finding of Kang and Buchenauer (2002) who reported that cellulases, xylanases and pectinases had active roles in the degradation of cell wall components in *F. culmorum*-infected wheat spikes. In addition, increased amylase and protease activity, as seen in the high aggressive strain *Fc6*, should enhance fungal ability to degrade wheat cell membranes, use starch as a growth substrate, and evenproduce atoxins (Woloshuk et al. 1997; Jaroszuk-Scisel et al. 2011).

On the other hand, protein contents also showed significant (P<0.05) differences between *Fc1* and *Fc6* strains (Fig. 3) which was higher in the aggressive strain *Fc6*. These proteins are important since they could be considered active at the exact moment of fermentation and responsible for the enzymatic activities detected in this work (Horta et al. 2018). However, the high enzymatic activity released by the aggressive strain *Fc6*was related to proteins secreted into the mediumby the cells through different protein export or lysis mechanisms,

Data showed that strain *Fc6* was more aggressive than*Fc1*on the susceptible wheat cultivar Cham 4 which was accompanied with differences in their hydrolytic enzymes produced under SSF. Gebruers et al. (2001) reported that susceptible wheat cultivar does not have endoxylanase inhibitor proteins which may be involved in plant defense mechanisms. However, 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. The two strains of *F. culmorum* studied here produced significant levels of hydrolytic activity *invitro*. The results indicate that the production of these enzymes mightinfluence the aggressiveness of the strains towards wheat heads. Kang and Buchenauer (2000) showed that *F. culmorum* infects the wheat ovary usually through thejunctions between the epidermal cell walls. These junctionsmay be 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 epidermalcell wall. In addition, 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; Sidaoui et al. 2018).

Results of this study confirm the variability of hydrolytic activities produced *in vitro* between the highly aggressive and weakly aggressive *F. Culmorum* strains, and the production of these enzymes correlated with the aggressiveness towards wheat spikelets. However, further investigation is needed to determine if these differences can be expressed in vivo.

Table 1. Source and pathogenicity rating of the two strains *Fc1* and *Fc6* of *F. culmorum* used in the study

Isolate	Host	Location	Year of collection	Colony colour
Foc1	wheat seeds	Aleppo (north)	2005	yellow mycelia -brown base
Foc6	wheat seeds	*ICARDA	2005	yellow mycelia -red base

*ICARDA, International Center for Agricultural Research in Dry Areas, Syria.

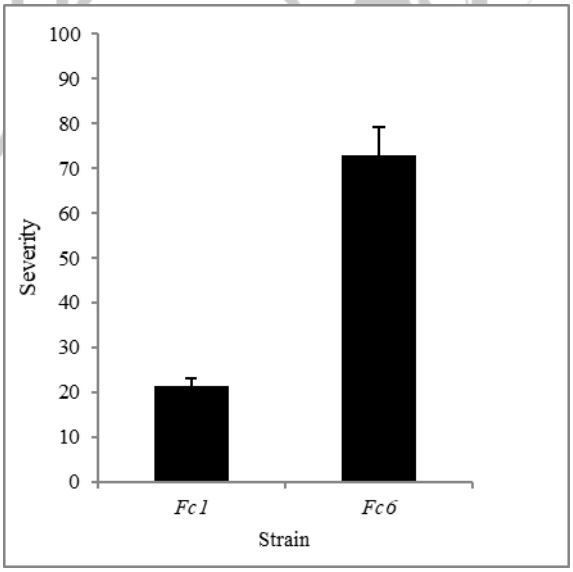

Fig. 1. Frequency of disease reactions incited on the wheat cv. Cham4 by the *Fc1* and *Fc6* strains. Significance at $P < 0.001$ between the two strains.

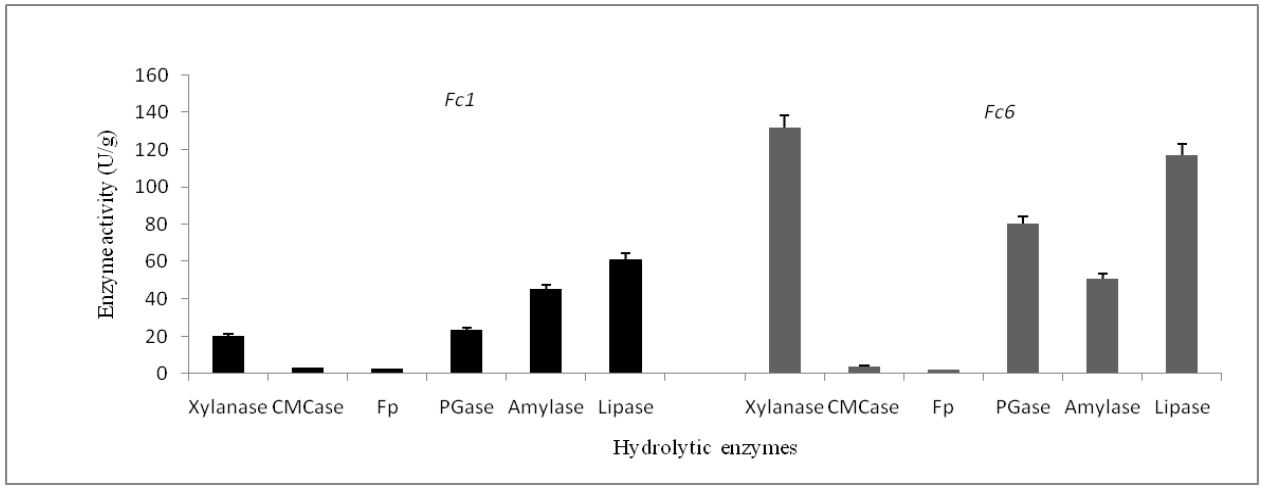

Fig. 2. Enzyme activity of the *Fc1* and *Fc6* strains of *F. culmorum* used in this study

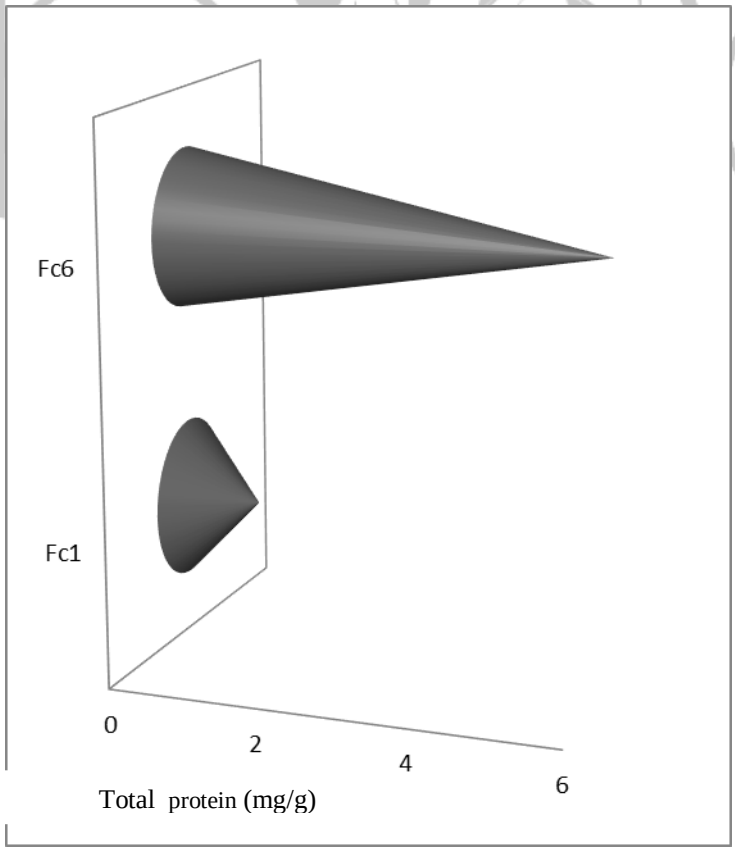

Fig. 3. Total protein of *F. Culmorum* strains *Foc1* and *Foc6* under SSF.Significance at $P< 0.05$ between the two strains.

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