

In Vitro Evaluation of Extracellular Hydrolytic Cell Wall Degrading Enzymes of *Fusarium oxysporum* f. sp. *elaeidis*, Pathogen of Oil Palm Vascular Wilt

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Abstract— *Fusarium oxysporum* f. sp. *elaeidis* (Foe), the causal agent of vascular wilt disease of oil palm, is known to secrete a range of extracellular cell wall-degrading enzymes during infection, yet the enzyme profiles of geographically distinct isolates have received little comparative attention. This study examined the production of endoglucanase, exoglucanase, β -glucosidase and pectinase by four pathogenic strains of Foe originating from Nigeria (isolate N), Ghana (isolate G) and Cameroon (isolates CA and C1), grown in submerged culture under a range of physico-chemical conditions. Enzyme activity was assayed colorimetrically using the dinitrosalicylic acid and O-toluidine methods, and total extracellular protein was determined by the Lowry method. All four isolates produced measurable activity of all four enzymes on both carboxymethylcellulose and pectin as sole carbon sources, although the two substrates did not differ significantly in the overall enzyme yield they supported. Pectin favored slightly higher endoglucanase and exoglucanase output, whereas carboxymethylcellulose favoured β -glucosidase and pectinase. Enzyme production peaked on the fourth day of incubation, at pH 5.5 (except β -glucosidase, which peaked at pH 7.5), and at 30°C, with activity declining markedly outside these optimal ranges. These results confirmed that Foe possesses the enzymatic machinery required to breach the oil palm cell wall, and show that carbon source, incubation time, pH and temperature all shape how much of that machinery the fungus expresses. This information should assist future work on isolate characterization and on host-pathogen interaction in vascular wilt disease.

Keywords— *Fusarium oxysporum* f. sp. *elaeidis*; oil palm; vascular wilt; cell wall-degrading enzymes; cellulase; pectinase.

I. INTRODUCTION

The oil palm, *Elaeis guineensis* Jacq, remains the world's most productive source of vegetable oil, yielding far more oil per hectare than any other oil crop (Hartley, 1988; Oil World, 2008). Production is nonetheless constrained by several diseases, of which vascular wilt caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *elaeidis* (Foe) is the most economically damaging in West and Central Africa, where losses of 25–50% of a plantation are not unusual (Renard and De Franqueville, 1989; Flood, 2006). Foe colonizes the root and vascular system, and disease severity is closely tied to the balance between the pathogen's invasive capacity and the host's ability to wall off infected xylem tissue (Corley and Tinker, 2003; Cooper, 2011). Selection of tolerant planting material remains the most effective control measure currently available (Renard and De Franqueville, 1989), which makes a clearer understanding of how the pathogen actually breaches host tissue directly relevant to breeding and screening programs.

Central to that invasive capacity is the fungus's ability to secrete extracellular cell wall-degrading enzymes (CWDEs). The plant cell wall, built mainly from cellulose, hemicellulose, pectin and lignin, is the first physical barrier a pathogen must overcome, and necrotrophic and hemibiotrophic fungi typically breach it by secreting a battery of hydrolytic enzymes that

loosen and macerate the wall (Bateman and Millar, 1966; Carpita and Gibeau, 1993; Annis and Goodwin, 1997). Cellulose is broken down synergistically by endoglucanases, exoglucanases (cellobiohydrolases) and β -glucosidases (Henrissat, 1994; Dashtban et al., 2009; Maki et al., 2009), while pectin is degraded by pectinases, which are usually produced earliest and in the largest amount because they alone are capable of macerating living plant tissue (Carpita and Gibeau, 1993; Annis and Goodwin, 1997). The amount and mix of enzymes a given isolate secretes are not fixed: they respond to the available carbon source, incubation time, pH and temperature, and this physiological plasticity is thought to contribute to differences in aggressiveness between isolates (Naessens and Vandamme, 2003).

Foe has previously been shown to produce pectin esterase, polygalacturonase and Cx-cellulase (Osagie and Obuekwe, 1991), but comparative data on the four principal hydrolases examined here—endoglucanase, exoglucanase, β -glucosidase and pectinase—across geographically distinct isolates, and on how their production responds to culture conditions, remain limited. Reducing sugar assays such as the dinitrosalicylic acid (DNS) method (Miller, 1959) and the O-toluidine method for glucose remain the most widely used tools for quantifying this activity (Alexander et al., 2011), and were adopted here for consistency with earlier work on Foe enzymology (Osagie and Obuekwe, 1991).

This study therefore set out to (i) determine the extracellular hydrolytic enzymes endoglucanase, exoglucanase, β -glucosidase and pectinase (endo-polymethylgalacturonase) produced by four pathogenic strains of Foe from Nigeria, Ghana and Cameroon; (ii) establish the culture conditions (carbon source, incubation period, pH and temperature) that optimize production of each enzyme; and (iii) quantify the amount of each enzyme produced under these conditions.

II. MATERIALS AND METHODS

2.1 Fungal isolates

Four pathogenic strains of *F. oxysporum* f. sp. *elaedis*, accession numbers Abak 508.1746 (Nigeria), OPC 2 (Ghana), and Ndian CI0 and Ndian 3AR6 (Cameroon), hereafter referred to as isolates N, G, CA and C1 respectively, were obtained from the Plant Pathology Division, Malaysian Institute for Oil Palm Research (MIFOR), and maintained on potato dextrose agar slants.

2.2 Culture medium

Enzyme production medium was a modified Czapek Dox broth (sodium nitrate 2 g/L, potassium nitrate 1 g/L, potassium chloride 0.5 g/L, magnesium sulphate 0.5 g/L, ferrous sulphate 0.01 g/L) in which the standard sucrose component was replaced with either carboxymethylcellulose (CMC) or citrus pectin at 30 g/L (Okunowo et al., 2010).

2.3 Enzyme production and optimisation

Enzymes were produced by submerged fermentation: spore inocula were added at 1% (v/v) to the production medium, which was autoclaved at 121°C for 15 minutes prior to inoculation, and all cultures were run in duplicate (Singh and Hayashi, 1995). The effect of carbon source was assessed by growing each isolate on CMC- or pectin-based medium for 7 days at 30°C on an orbital shaker at 150 rpm (Hussain et al., 2012). Using pectin broth as the base medium, the effect of incubation period was then assessed over 8 days, with activity measured every 48 hours; the effect of pH was assessed across the range 4.5–7.5 (adjusted with 1–5 M NaOH or 1 N HCl) at 30°C for 4 days; and the effect of temperature was assessed across 25–40°C, at 5°C intervals, for 4 days (Hussain et al., 2012).

2.4 Enzyme extraction

Culture filtrates were passed through nylon cloth and the filtrate centrifuged at 12,000 rpm for 10 minutes to remove mycelial fragments and substrate debris (Shamala and Sreekantiah, 1986). The resulting supernatant served as the crude enzyme extract for all subsequent assays.

2.5 Enzyme assays

Endoglucanase (EC 3.2.1.4) activity was determined using carboxymethylcellulose as substrate, following Zaldivar et al. (2001): 1 mL of crude enzyme extract was incubated with 10 mg CMC in 1 mL of 0.05 M sodium acetate buffer (pH 5.0) at 50°C for 30 minutes, and the reducing sugar released was quantified by the DNS method, with absorbance read at 540 nm against a glucose standard curve. Exoglucanase (EC 3.2.1.91) activity was assayed identically, substituting microcrystalline cellulose (Avicel) for CMC (Okunowo et al., 2010). β -Glucosidase (EC 3.2.1.21) activity was measured using cellobiose as substrate and the O-toluidine method (Zaldivar et al., 2001): the glucose released was reacted with trichloroacetic acid and O-

toluidine and absorbance read at 620 nm. Pectinase activity was assayed by incubating enzyme extract with 1% pectin in 0.1 M citrate buffer (pH 5.8) at 50°C for 30 minutes, terminating the reaction with DNS reagent and reading absorbance at 540 nm (Mandels, 1985; Minjares-Carranco et al., 1997). In each case, one unit of activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar (or glucose, or galacturonic acid, as appropriate) per minute under the assay conditions. Total extracellular protein was determined by the Lowry method using bovine serum albumin as standard, with absorbance read at 750 nm (Lowry et al., 1951).

2.6 Statistical analysis

Data were analyzed by analysis of variance (ANOVA), Duncan's multiple range comparison test and least significant difference, using GenStat version 8.1.0.

III. RESULTS

3.1 Effect of carbon source

All four isolates produced endoglucanase, exoglucanase, β -glucosidase and pectinase on both carbon sources (Table 1), although Duncan's test showed no statistically significant difference in overall enzyme yield between the two substrates. Pectin medium generally supported higher endoglucanase and exoglucanase activity, while carboxymethylcellulose favoured β -glucosidase and pectinase. Isolate CA produced the highest endoglucanase and exoglucanase activity on both substrates (4.92 and 6.42 $\mu\text{g/mL}$ on CMC; 5.72 and 7.45 $\mu\text{g/mL}$ on pectin). β -glucosidase activity was highest for isolate G on CMC (0.24 $\mu\text{g/mL}$) and isolate N on pectin (0.19 $\mu\text{g/mL}$), while pectinase activity was highest for isolate N on CMC (3.80 $\mu\text{g/mL}$) and isolate G on pectin (2.80 $\mu\text{g/mL}$). Total extracellular protein was consistently higher in pectin-grown cultures than in CMC-grown cultures across all four isolates.

TABLE 1

EFFECT OF CARBON SOURCE ON TOTAL EXTRACELLULAR PROTEIN AND ENZYME ACTIVITY OF FOUR *FUSARIUM OXYSPORUM* F. SP. *ELAEIDIS* ISOLATES IN SUBMERGED CULTURE

Isolate	Carbon source	Total protein ($\mu\text{g/mL}$)	Endoglucanase ($\mu\text{g/mL}$)	Exoglucanase ($\mu\text{g/mL}$)	β -glucosidase ($\mu\text{g/mL}$)	Pectinase ($\mu\text{g/mL}$)
<i>F. oxysporum</i> (N)	CMC	323.08	3.63	6.27	0.225	3.8
<i>F. oxysporum</i> (N)	Pectin	480.77	4.27	7.06	0.194	1.98
<i>F. oxysporum</i> (G)	CMC	401.92	2.91	2.18	0.241	2.81
<i>F. oxysporum</i> (G)	Pectin	409.62	3.67	3.11	0.19	2.8
<i>F. oxysporum</i> (CA)	CMC	286.54	4.92	6.42	0.233	2.57
<i>F. oxysporum</i> (CA)	Pectin	476.92	5.72	7.45	0.191	2.46
<i>F. oxysporum</i> (C1)	CMC	332.69	2.82	5.95	0.235	3.74
<i>F. oxysporum</i> (C1)	Pectin	648.08	4.95	6.36	0.172	1.19

3.2 Effect of incubation period

Enzyme production changed markedly over the 8-day incubation period, with the clearest overall peak on day 4: endoglucanase reached its highest recorded value in isolate C1 (10.32 $\mu\text{g/mL}$), exoglucanase and pectinase peaked together in isolate CA (9.89 and 10.69 $\mu\text{g/mL}$), and β -glucosidase was highest in isolate N (0.25 $\mu\text{g/mL}$). Activity on day 2 was generally lower, fell further by day 6, and rose slightly again by day 8, although Duncan's test confirmed that only day-4 values differed significantly from the other three time points, which did not differ significantly from one another.

3.3 Effect of pH

Enzyme production was highest at pH 5.5 for endoglucanase, exoglucanase and pectinase, with isolate C1 recording the greatest endoglucanase and pectinase activity (10.10 and 11.80 $\mu\text{g/mL}$) and isolate G the greatest exoglucanase activity (11.75 $\mu\text{g/mL}$) at this pH. β -Glucosidase behaved differently, reaching its highest activity at pH 7.5 rather than pH 5.5. Activity at the extremes of the range tested (pH 4.5 and 7.5) was generally lower than at pH 5.5–6.5, and pH 5.5 differed significantly from all other pH levels tested.

3.4 Effect of temperature

Enzyme activity peaked at 30°C for all four enzymes, with isolate C1 recording the highest endoglucanase and pectinase activity (8.59 and 9.29 µg/mL) and isolate N the highest exoglucanase activity (9.24 µg/mL) at this temperature. Activity fell progressively as incubation temperature rose above 30°C, with the lowest values recorded at 40°C across all four isolates, indicating that temperature exerts a direct and fairly consistent influence on enzyme output in this pathogen.

IV. DISCUSSION

The four isolates examined here, despite originating from three different countries, behaved consistently: each grew on both carbon sources and secreted measurable endoglucanase, exoglucanase, β-glucosidase and pectinase activity, confirming that cellulolytic and pectinolytic capacity is a general, rather than isolate-specific, feature of *F. oxysporum* f. sp. *elaeidis* (Bateman and Basham, 1976). This is broadly consistent with the CWDE profile reported for the same forma specialis by Osagie and Obuekwe (1991), and supports the wider observation that necrotrophic pathogens typically secrete pectinases earliest and in the largest amount, since these enzymes alone can macerate living plant tissue (Carpita and Gibeau, 1993; Annis and Goodwin, 1997).

The absence of a statistically significant difference between CMC and pectin as carbon sources, despite the modest differences in which enzyme each substrate favored, likely reflects differences in substrate heterogeneity and in each isolate's relative efficiency at degrading the two polymers (Bateman and Basham, 1976). The higher total protein recorded in pectin-grown cultures than in CMC-grown cultures, even where pectinase activity itself was not correspondingly higher, suggests that other extracellular proteins—potentially additional cell wall-degrading enzymes not assayed here—were also being secreted (Chinedu et al., 2011).

Enzyme production peaking on day 4 of incubation is consistent with earlier work on cellulolytic fungi (Aurangzeb et al., 1997) and is generally attributed to fungal cultures reaching a stationary or late-exponential growth phase around this point (Hussain et al., 2012). The comparatively high pectinase activity recorded as early as day 2 fits the broader pattern in which pectinases are produced first during infection, weakening host cells and opening the way for further colonization (Carpita and Gibeau, 1993; Annis and Goodwin, 1997).

The optimum pH of 5.5 recorded here for endoglucanase, exoglucanase and pectinase sits close to the pH 3.0–5.0 range reported for maximal cellulase production by Juhasz et al. (2004), although the shift of β-glucosidase's optimum to pH 7.5 diverges from the pH 5.5 optimum reported for the same enzyme by Maria et al. (2014)—a discrepancy that may simply reflect differences between isolates or assay conditions rather than a general rule.

The 30°C optimum for enzyme production also agrees with reports for other cellulolytic fungi, including *Trichoderma reesei* (28–30°C; Ahmed et al., 2005) and *T. harzianum* Rut C-8230 (28°C; Kocher et al., 2008), and is consistent with Hussain et al. (2012). The steady decline in activity above 30°C across all four isolates confirms that temperature is a direct and fairly universal determinant of CWDE output in this pathogen, and together with the pH and incubation-time optima identified here provides a practical reference point for standardising conditions in future comparisons of CWDE production across *Foe* isolates.

V. CONCLUSION

This study demonstrates that four pathogenic strains of *F. oxysporum* f. sp. *elaeidis*, drawn from Nigeria, Ghana and Cameroon, all produce endoglucanase, exoglucanase, β-glucosidase and pectinase in submerged culture, and that the amount of each enzyme produced is shaped by carbon source, incubation period, pH and temperature, with production generally maximal around day 4, pH 5.5 (pH 7.5 for β-glucosidase), and 30°C. These optimized conditions offer a practical reference point for further work characterising CWDE production across *Foe* isolates and for exploring how these enzymes contribute to the host-pathogen interaction underlying oil palm vascular wilt disease.

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All authors contributed equally to the study. Deekermue Paye conceptualized the study idea. Numei Wamah made the data collection instrument. Oluwatosin Oladeji did the data analysis. All the authors did the manuscript drafting and approval.

AUTHOR CONTRIBUTIONS

Deekermue Paye: Conceptualization, Data collection, Formal analysis, Methodology, Writing original draft, Writing review and editing; Numei Wamah: Conceptualization, Methodology, Supervision, Writing original draft, Writing review and editing; Oluwatosin Oladeji: Conceptualization, Data collection, Formal analysis, Funding acquisition, Resources, Writing original draft, Writing review and editing.

DISCLOSURE STATEMENT

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CONSENT TO PUBLISH DECLARATION

Not applicable.

CONSENT TO PARTICIPATE

All participants agreed to participate in the study after an informed consent was granted.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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