FULL LENGTH RESEARCH ARTICLE

EFFECTS OF CULTURE AGES ON THE PRODUCTION AND ACTIVITIES OF POLYGALACTURONASE AND CELLULASE (CX) ENZYMES PRODUCED BY *Pythium aphanidermatum* (EDSON FITZPAT.) ISOLATED FROM SOFT STEM ROT DISEASE OF COWPEA.

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ABSTRACT

Effect of culture ages was investigated on the production and activities of polygalacturonase (PG) and Cx cellulase enzymes. *Pythium aphanidermatum* was isolated from cowpea seedlings infected by soft stem rot disease and prepared into pure culture through successive culturing. Two discs (2 mm each) of two-day-old culture of *P. aphanidermatum* were put into 21 Erlenmeyer flasks containing 50 ml of Reese and Levinson's solution, consisting of Sodium Polypectate (NaPP) as carbon source, and incubated for 7 days at 38ºC. At one-day interval, content of 3 flasks was independently centrifuged at 10,000g for 30 min. The PG enzyme activity was determined viscometrically and expressed as the Relative Viscometric Unit (RVU). Data collected were analysed using analysis of variance (Anova) and the means separated by the Duncan Multiple Range Test. Activity of Cx cellulase enzyme was also evaluated by the same method used for PG enzyme but using, carboxylmethyl cellulose (CMC) as the carbon source. The results showed that there was significant difference in the production and activities of PG and Cx enzymes in the various days of the incubation. The PG and Cx enzymes were produced progressively from the 1st day of incubation and reached their peak on the 5th day for PG (RVU 105) and 4th day for Cx (RVU 49), after which, production and activities of the enzymes started declining. Results suggested that soft stem rot disease in cowpea is a monocyclic disease, whereby any attacked plant that is able to withstand the disease is capable of surviving after the peak of the infection.

Key words: *Pythium aphanidermatum,* polygalacturonase, Cx cellulose,enzymes, viscometric, rot, disease.

INTRODUCTION

Polygalacturonase (PG) and cellulase are pectin and cellulase modification enzymes, respectively, and the first cell-wall degrading enzymes synthesized by phytopathogenic fungi (Copper & Wood, 1975; deVries & Visser 2001) as well as secreted by bacteria and nematode (Mahalingam *et al.* 1999).

Bateman & Basham (1976) stated that polygalacturonases that breakdown polygalacturonate chain in plants in a random manner into small chains of oligogalacturonate are the endo-polygalacturonases, whereas the exo-polygalacturonases act on the polygalacturonate chain in terminal manner and produce monomeric products such as galacturonic acids. There are strong correlative evidences supporting the involvement of endo-polygalacturonase in causing symptoms in diseases characterized by soft-rotting or tissue maceration (Bateman & Basham 1976; Huang & Allen 2001; Scott *et al.* 2005).

When a pathogen confronts a plant cell wall, it faces a complex barrier composed of polymers with different chemical linkages that require specific enzymes for their degradation. The degradation of each of these components of the cell wall is brought about by the action of one or more sets of enzymes secreted by the pathogen, principally cellulose and pectin enzymes. Micro-organisms capable of utilizing native cellulase have been termed cellulolytic and are believed to produce a cellulase enzyme designated C1 (Mendels & Reese 1957) which destroys the crystalline structure of cellulose, thus exposing the glucan chain to β-1-4 glacanase enzyme known as Cx.

P. aphanidermatum (Edson Fitzpat.) is a pathogenic fungus that causes soft stem rot disease in some plants, including cowpea (Onuorah 1973; Moorman 2002; Emechebe & Florini 1997). Production of pectolytic and cellulolytic enzymes, the major agents of pathogenicity of any organism, have been identified in several *Pythium* species, including *P. aphanidermatum* (Janardhanan & Husain 1974; Weinstead & Mccombs, 1961; Sutton *et al*. 2006) and *P. debaryanum* (Wood & Gupta 1958). Secretion of polygalacturonase appears to be one of the key requirements for establishing infection in plants (Clausen & Green 1996; Ohazurike, 1996) and differences in polygalacturonase levels and mycelial growth rates of isolates of a pathogen are highly correlated with differences in virulence (Wei-Chen *et al*. 1998; Ohazurike & Arinze 1999; Owen-Going *et al*. 2004).

Enzyme production in culture is usually affected or modified by the prevailing cultural conditions or factors. Ohazurike (1996) observed that the types of pectic and cellulase enymzes present in culture filtrates of *Fusarium solani* differ with the age of the culture and pH. The same author reported that young cultures of *F. solani* with an acidic pH contain primarily polygalacturonase while older cultures of this organism with alkaline pH contain only polygalacturonase lyase. These underline the necessity to test for the enzymes at various culture ages to determine enzyme activities so as to avoid or minimize misleading conceptions concerning pathogenicity of organisms.

This paper investigated the effects of culture ages on the production and activities of polygalacturonase and cellulase enzymes produced by *P. aphanidermatum* isolated from infected soft stem rot cowpea seedlings.

MATERIALS AND METHODS

Diseased cowpea seedlings planted in the Teaching and Research Farm of the Faculty of Agriculture and Veterinary Medicine, Imo State University, Owerri Nigeria were collected and the affected stem cut off, washed under running water and surface-sterilized by blotting the lesion surface with cotton wool soaked in 90 % alcohol. Small pieces of the rotted area of the stem were cut with a sterilized scissors, and the cut pieces immersed in 1 % Sodium hypochlorite solution for 5 min and after rinsed with distilled water and plated on Potato Dextrose Agar (PDA) mixed with 5 ml of Streptomycin (bacteriocide) and 5 ml of Benomyl (fungicide) solutions. The plate was incubated for 5 days at 38oC. The organism growing in the culture was identified as P. aphanidermatum by the techniques of Koleosho *et al.* (1987) and pure isolates of the pathogen obtained by repeated sub-culturing.

Assay of Polygalacturonase enzyme production and activities: Two discs (2 mm each) of two-day-old culture of *P. aphanidermatum* growing on the PDA were added into 21 Erlenmeyer flasks (250 ml capacity each), each containing 50 ml of Reese and Levinson's liquid medium (consisting of Sodium Polypectate (NaPP) as Carbon source). The flasks were incubated for seven days at 38oC within which, at one-day interval, contents of three flasks were centrifuged at 10,000g for 30 min. The polygalacturonase enzyme activity of the filtrate obtained at each day interval was determined viscometrically and presented in Relative Viscometric Unit (RVU), using the formula 1000/t, where; t = time for 50% loss in viscosity of the reaction mixture. The reaction mixture consisted of 4 ml of 1 percent NaPP solution in 0.1M Citrate buffer pH 5.0, 1 ml of distilled water, and 2 ml of the culture filtrate of PG enzyme obtained at each day interval. Data collected on the RVU values of the 3 flasks for the various days were analysed by the analysis of variance method, using the completely randomized design procedure, while the mean RVU values were separated by the Duncan Multiple Range Test (Onuh & Igwemma 1998).

Assay of Cx enzyme production and activities: Two discs (2 mm each) of two-day-old culture of *P. aphanidermatum* growing on PDA were added into 21 Erlenmeyer flasks (250 ml capacity each), each containing 50ml of Reese and Levinson's medium with Carboxylmethyl Cellulose (CMC) as the Carbon source. The flasks were incubated for seven days at 38oC. At one-day-interval, contents of three flasks were filtered over several layers of Muslin cloth and the filtrates were centrifuged at 10,000g for 30min. The Cx cellulase activity of the filtrate obtained at each day interval was determined viscometrically and expressed as RVU, using the formula, 1000/t, where; t = time for 50% loss of viscosity of the reaction mixture. Data collected on the RVU values of the 3 flasks for the various days were analysed by the analysis of variance method, using the completely randomized design procedure, while the mean RVU values were separated by the Duncan Multiple Range Test (Onuh & Igwemma 1998).

RESULTS

Table 1 showed maximum polygalacturonase enzyme production and activity recorded on the 5th day of incubation with RVU of 105. This value was significantly different from the RVU recorded in the 1st day of incubation. The RVU values increased progressively from the 1st day of incubation until the 5th day, which was the maximum. The RVU value started dropping from the 6th day with RVU value of 89, while the 7th day recorded 70 RVU value (Fig.1), though these values were not significantly different from the 5th day's value.

Results on the production and activity of Cx cellulase enzyme indicated that production and activity the enzyme was highest on the 4th day of incubation (Table1). The RVU value for this day was significantly different from the RVU values recorded for the other incubation days. Similar to the observation in the ages of culture on the production and activity of PG enzyme, Cx cellulase production and activity rose progressively from the 1st day of incubation and reached its peak at the 4th day. After the 4th day, Cx cellulase activity started to decline with RVU values of 40, 34 and 28 for the 5th, 6th and 7th day, respectively (Fig.2).

TABLE 1. MEAN RELATIVE ENZYME ACTIVITY (RVU) OF POLYGALACTURONASE (PG) AND CX CELLULASE ENZYMES PRODUCED BY *P. aphanidermatum* INCUBATED FOR 7 DAYS AT 38oC

	Mean* relative PG activity (RVU)	Mean* relative Cx enzyme activity (RVU)
1	52^{b}	15^{c}
2	67^{ab}	26^{bc}
3	88^{a}	37^{ab}
4	97^{a}	49^{a}
5	105^{a}	42^{a}
6	89^{a}	34^{b}
7	70^{a}	28^{bc}

*Means with the same letter(s) are not different at P = 0.05, according to Duncan Multiple Range Test.

FIG. 1. EFFECT OF AGE OF CULTURE OF *P.aphanidermatum* ON PG ENZYME PRODUCTION AND ACTIVITY.

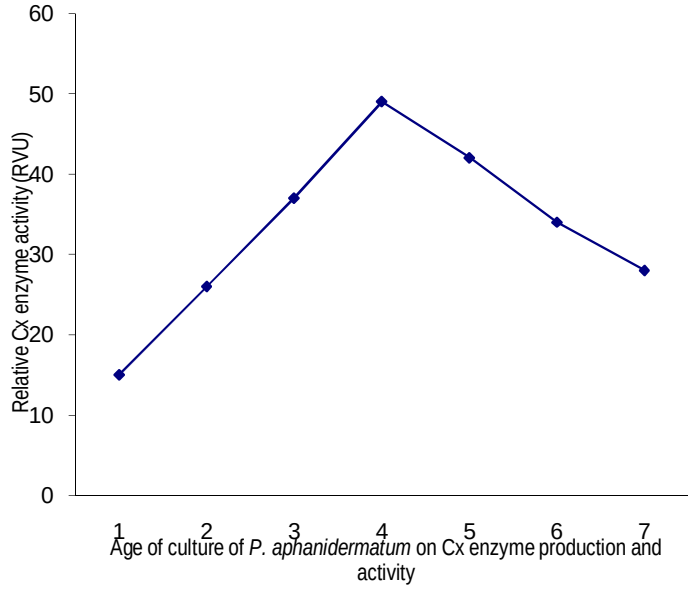

FIG. 2. EFFECT OF AGE OF CULTURE OF *P. aphanidermatum* ON CX CELLULOSE ENZYME PRODUCTION AND ACTIVITY

DISCUSSION

Results observed in this experiment indicated that *P. aphanidermatum* as a pathogen of soft stem rot disease, released Cx (cellulase) and polygalacturonase (pectinase) enzyme which, enable the pathogen entry into the host tissues. This observation was in consonance with (Janardhanan & Husain 1974, Weinstead & Mccombs, 1961 & Sutton, *et al.* 2006), who reported the ability of *P. aphanidermatum* to produce cellulolytic and pectolytic enzymes. Production and activity of polygalacturonase and Cx cellulase enzymes were assayed and it was observed that ages of the culture had influence in the quantity and activity of either the pectinase and cellulase enzymes produced by *P. aphanidermatum.* There was significant difference in the quantity of cellulase and pectolytic enzymes produced by *P. aphanidermatum* in the different days of the culture. Polygalacturonase enzyme was progressively produced in increased level until after the 5th day of culturing, when the production level started declining. Similarly, the Cx cellulase enzyme was produced in an increasing trend from the first day, until the 5th day of incubation when the level started to decrease. These observations conform to the report of Allen *et al.* (2004) who found out that conidium production in *Drechslera avenacea* increased with culture age. Similarly, Ohazurike (1996) reported that the types of the pectic and cellulose enzymes present in culture filtrates of *Fusarium solani* differed with the age of the culture. According to Onuh & Ohazurike (2007 in press), some cowpea seedlings infected by *P. aphanidermatum* and expressed symptoms of soft stem rot disease were able to recover after 2 wks of infection. The observations in this study gave credence to the fact that soft stem rot disease caused by *P. aphanidermatum* is a dynamic monocyclic disease, which starts and progressively reach the peak in its attack and then drops its pathogenicity (Onuh & Ohazurike 2007 in the press). Results from the production and activity curves of polygalacturonase and Cx cellulase enzymes suggests that any cowpea plant infected by *P. aphanidermatum* that is able to tolerate the disease within 14 days of infection, stands the chance of surviving the attack. This, however, will depend on the level of disease inhibitory substances that the plant is able to produce.

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