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EFFECT OF pH ON ENZYME PRODUCTIVITY BY THREE PATHOTYPES OF *CHOANEPHORA CUCURBITARUM*

E. J. UMANA and TUNDE IKOTUN

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ABSTRACT

Three pathotypes of *Choanephora cucurbitarum* isolated from *Abelmoschus esculentus, Amaranthus hybridus* and *Vigna unguiculata* were investigated for their *in vitro* ability to produce pectolytic enzymes at different pH levels. Results obtained show that all the three pathotypes did not vary in their ability to produce enzyme *in vitro*. The enzymes produced were exo-polygalacturonase, endo-polygalacturonase and endo-peptate lysate. The endo-polygalacturonase was the predominant enzyme at a lower pH of 4.0 - 6.0, with the highest activity at pH 5.0. The relative activities of 2RVu/ml, 11 RVu/ml and 6RVu/ml were obtained from the pathogens of *Amaranthus, Vigna* and *Abelmoschus* respectively occurring at pH of 4.0 in three day-old cultures. The relative activities of 14RVu/ml (*Amaranthus*), 22 RVu/ml (*Vigna*) and 10 RVu/ml (*Abelmoschus*) were obtained at pH of 6.0 in three day-old cultures. In six day-old cultures at pH, the following relative activities of 75 RVu/ml (*Amaranthus*), 3.0 RVu/ml (*Vigna*) and 25RVu/ml (*Abelmoschus*) were obtained and 72 RVu/ml (*Amaranthus*), 37 RVu/ml (*Vigna*) and 5 RVu/ml (*Abelmoschus*) at pH of 5.0. These values were reduced to 60 RVu/ml (*Amaranthus*), 25 RVu/ml (*Vigna*) and 12 RVu/ml (*Abelmoschus*) at pH 6.0. The above patterns were also observed in nine, twelve, fifteen and eighteen day-old cultures. The endo-pectate lysate which has optimal pH of 8.0 occurred less often. The exo-polygalacturonase had a lower pH of between 3.0 and 5.0. The amount of enzymes produced varied with the age of culture.

KEY WORDS: *Choanephora cucurbitarum, in vitro*, pathotypes, exo-polygalacturonase, endo-polygalacturonase, endo-pectate lyase.

INTRODUCTION

Choanephora cucurbitarum is a facultative saprobe that belongs to the subdivision Zygomycotina, order Mucorales and family Choanephoraceae (Alexopoulous and Mins, 1979). The fungus is more successful under humid conditions (Oladiran, 1988), thrives best at temperature of 25°C and relative humidity of about 100%. A temperature of about 31% stimulates the production of large sporangia, but unfavourable for conidial formation (Barnet and Lilly, 1955).

C. cucurbitarum is a causal agent of rot disease of most plants especially vegetables such as *Amaranthus hybridus, Vigna unguiculata, Abelmoschus esculentus* and other cucurbits (Odebunmi-Osikanlu, 1977; Rao and Thirupthaiah, 1978, Ikediugwu, 1981). It is known to attack several other crops which include cereals such as millet, rice and sorghum. The fungus is involved in the storage rot of cowpea while in transit. It has been observed to cause wet rot disease of castor plant (*Ricinus communis*).

Many fungi are known to produce enzymes which are implicated in the degradation of plant cell wall polysaccharides and cytoplasmic materials (Guillen *et al.*, 1987).

Although, some work have been carried out on the enzymic activity of some fungi, the effect of pH on the enzyme productivity by pathotypes of *C. cucurbitarum* has not been reported. This paper reports on the effect pH on the enzyme productivity by the three pathotypes of *C. cucurbitarum*.

MATERIALS AND METHODS

C. cucurbitarum used in this study was isolated from *A. hybridus, A. esculentus* and *V. unguiculata* plants isolated from the students project farm of Faculty of Agriculture and Forestry of University of Ibadan, Nigeria.

The isolates were inoculated on potato dextrose agar (PDA) and allowed to grow at room temperature of 28°C ± 2°C. Subculturing was carried out until axenic cultures were obtained.

E. J. UMANA, Department of Biological Sciences, University of Calabar, Calabar, Nigeria.
TUNDE IKOTUN, Department of Agricultural Biology, University of Ibadan, Ibadan, Nigeria.

The standard basal salt medium, with pectin as the carbon source, was prepared using the method described by Punja and Jenkins (1984). Thirty millilitres of the medium were dispensed into 100 ml conical flasks, the flasks were plugged with non-absorbent cotton wool and covered with aluminium foil and then autoclaved at 121°C (1.05 kg/cm^3) for 20 min and allowed to Jenkins (1984). Thirty millilitres of the medium were dispensed into 100 ml conical flasks, the flasks were plugged with non-absorbent cotton wool and covered with aluminium foil and then autoclaved at 121°C (1.05 kg/cm^3) for 20 min and allowed to cool.

The pure culture isolates were inoculated into the basal medium with two discs (4 mm in diameter) of mycelia taken from the margin of seven day old culture. The inoculated flasks were incubated at room temperature(28°C ± 2°C) as still culture and harvested every three days for 18 days.

Isolates from each of the crop were treated separately. The cultures were filtered through eight-layer cheese cloth and the filtrate used for the bioassay.

The enzyme precipitation was carried out according to the method described by Ikotun and Balogun (1987). The filtrates obtained were centrifuged differently at 5000 x g for 20 min to remove fungal debris and spores. The supernatant was carefully removed, subjected to 95% ammonium sulphate precipitation and centrifuged again at 5000 x g for 20 min, after which the precipitates were dissolved in 10 ml of sterile distilled water. This crude enzyme preparations were stored in cold air incubator at -10°C until required.

The medium used to assay pectolytic enzyme activity was prepared by dissolving 5 g of pectin in 500 ml of distilled water by heating. Fifty millilitres of the substrate medium of the substrate were mixed with 50 ml of the appropriate buffer solutions with the following pH values: 3, 4, 5, 6, 7, 7, 8, 9 10. 11. 12 and 14.

The enzyme preparations were assayed for their activity by the use of Cannon-Fanske Viscometer size 200 Techniques 1320. The Viscometer was filled with 10 ml distilled water and the time of run through the Viscometer by water was recorded. The mean value was then calculated after five runs. Nine mililitres of 1% pectin in appropriate buffer were pipetted into the Viscometer and 1 ml of the enzyme preparation added.

The reagents were thoroughly mixed by blowing through the Viscometer several times. The reagents were sucked into the measuring chamber of the Viscometer and the initial run at time 0 noted and recorded. The subsequent runs were carried out at intervals of 5 minutes until a constant reading in seconds were obtained. The same procedure was employed for all the pH values and for all the culture filtrates obtained after every three days of harvest.

The filtrate from different crop isolates of the fungus were treated separately.

The percentage loss in viscosity by pectin due to enzyme activity was calculated using the formula adopted by Ikotun and Balogun (1987) as shown below.

$$\frac{1v - Vx}{1v - Vw} \times \frac{100}{1}$$

where 1v = First run of reagent at time 0
Vx = Run of reagents after 5 mins
Vw = Time of run for water (all in seconds).

From the above formula, the relative activity of the enzyme which is defined as the reciprocal of the viscosity of pectin multiplied by 1000 was calculated in Viscometer units.

Relative Activity (RA) = $1/t \times 10^3$ RVu

RESULTS

The activity of the pectolytic enzymes against pH on the third day is shown in Fig. 1. The three pathotypes show generally low activity at pH 4.0. The pathotype from *V. unguiculata* had the highest activity of 28 RVu at pH 8.0 and the lowest activity of 7 RVu at pH 9.0. *A. hybridus* and *A. esculentus* pathotypes had their highest activities at pH 6.0 with 14 RVu and 9 RVu respectively. The lowest activities in all pathotypes were observed at pH 9.0.

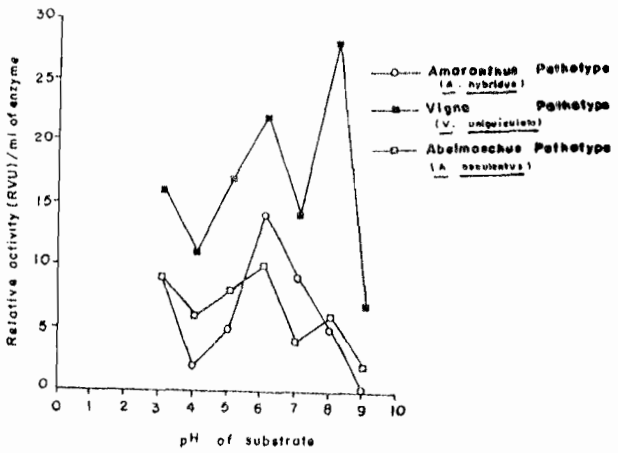

Fig. 1 In Vitro production of pectolytic enzyme on three day-old cultures by pathotypes of Choanephora cucurbitarum from Okro

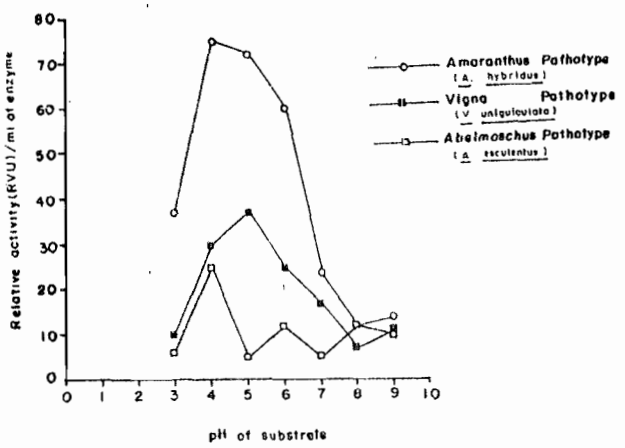

Fig. 2 In Vitro production of pectolytic enzyme on six day-old cultures by pathotypes of Choanephora cucurbitarum from Okro.

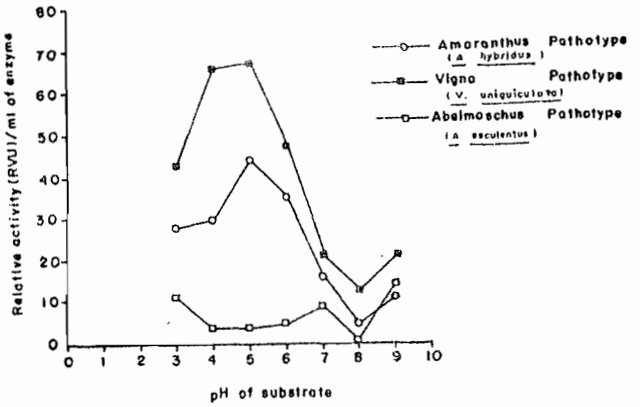

Fig. 3 In Vitro production of pectolytic enzyme on nine day-old cultures by pathotypes of Choanephora cucurbitarum from Okro

In the six day-old cultures *A. hybridus* had a remarkably higher activity of 75 RVu at pH 4.0 and the lowest of 14 RVu at pH 9.0. whereas the *V. unguiculata* pathotype showed highest activity of 32 RVu at pH 5.0 and the activity of 12 RVu at pH 8.0. Also, the *A. esculentus* pathotype had its peak of activity at pH 5.0 as shown in Fig. 2.

Figure 3 shows the results obtained from nine day-old cultures. *V. unguiculata* pathotype produced the highest amount of pectolytic enzymes with the peak at pH 6.0 and the lowest at pH 8.0. For the *A. hybridus* pathotype optimum enzyme activity was at pH 5.0. There was a steady decline from pH 6.0 - pH 8.0 and a slight increase at pH 9.0. The highest activity of *A. esculentus* pathotype was recorded at pH 7.0 with relative activity of only 9 RVu. This was followed by a sharp decline at pH 8.0 and a slight increase at pH 9.0 .

The result for twelve day-old cultures given in Fig. 4 shows that the *A. hybridus* pathotype produced the highest amount of activity at pH 5.0 and the lowest at pH 7.0. Slight increase at pH 8.0 and 9.0 was also observed. *V. unguiculata* pathotype was observed to be next in enzyme production with the peak of activity at pH 5.0 and the lowest at pH 8.0. The *A. esculentus* pathotype produced the lowest amount of enzyme. The highest activity observed for this pathotype was at pH 4.0 and the lowest at 8.0. On the fifteenth day, the *A. hybridus* pathotype produced the highest amount of pectolytic enzymes reaching a peak activity of 50 RVu at pH 4.0 whereas the least activity was at pH 7.0 followed by a steady rise at pH 8.0 and 9.0 as shown in Fig 5. This was followed by the *A. hybridus* pathotype with the highest relative activity at pH 5.0 with which a steady decrease was observed thereafter till the lowest was obtained at pH 9.0.

The *A. esculentus* pathotype produced the least amount of pectolytic enzymes with its highest relative activity at pH 4.0 and the lowest at 6.0. The effect of pH on the

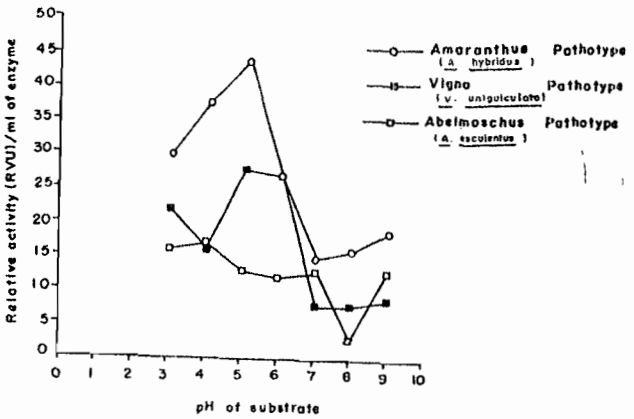

Fig. 4 In Vitro production of pectolytic enzyme on twelve day-old cultures by pathotypes of Choanephora cucurbitarum from Okro.

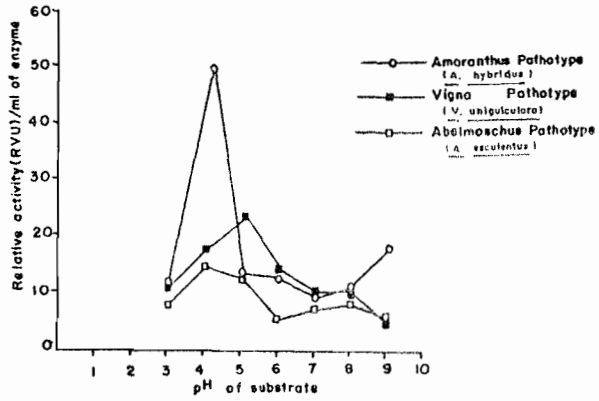

Fig. 5 In Vitro production of pectolytic enzyme on fifteen day-old cultures by pathotypes of Choanephora cucurbitarum from Okro.

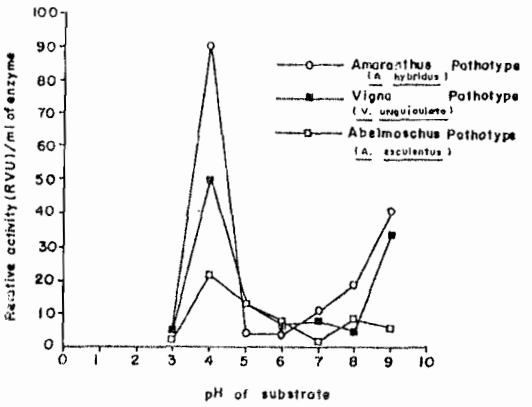

Fig. 6 In Vitro production of pectolytic enzyme on eighteen day-old cultures by pathotypes of Choanephora cucurbitorum from Okro.

activity of the three pathotypes after 18 days of incubation showed that optimum enzyme activity of all the pathotypes occurred at pH 4.0 as shown in Fig. 6. However, whereas the activity of *A. hybridus* and *V. unguiculata* pathotypes flunctuated before pH 5.0 and 9.0 that of *A. esculentus* pathotype declined steadily reaching its lowest at pH 9.0.

DISCUSSION

Choanephora cucurbitarum had been shown to produce pectolytic enzymes *in vitro* when it was supplied with pectin as the carbon source. It has also been observed to produce enzymes *in vivo*.

It was observed to produce enzymes with high relative activity at lower pH of 3.0 - 5.0 and some had high activity at a higher pH of 8.0 9.0. This observation agrees with Ikotun (1984) who reported the production of endo-polygalacturonase by *Penicillium oxalicum* with optimum pH of 5.0 and endo-pectate lyase with optimum pH of 8.0 and that the exo-galacturonase was unstable. This instability may account, in part, for the flunctuation in culture of different ages as recorded in this work. Ikotun and Balogun (1987) reported that polygalacturonase (hyrolases) produced by *Alternaria solani*, *Collectotricum truncatum*, *C. capsici* and *Curvularia pallescens* act maximally in acidic medium of pH 3.0 - 5.0. Basham and Bateman (1975) also reported that lyases have high activity in alkaline medium. The result agrees with the work of Basham and Bateman (1975) that reported high activity for lyases in alkaline medium.

Arinze *et al* (1976) reported that polygalacturonase production was highest when pectin was utilized as the carbon source which was exactly the same carbon source used in this work.

Some of the enzymes reduced viscosity of pectin within a very short period of time, and these enzymes acted maximally at a lower pH of 4.0 and 5.0, the one that took a longer time to degrade pectin did so often at a higher pH of 8.0 and 9.0. This is consistent with Ikotun (1984) who reported that the enzyme endo-polygalacturonase reduces the viscosity of pectin within a short period and endo-pectate lyase degrades at a higher pH of 8.0 - 9.0.

The type of enzyme produced varied with the age of cultures. The enzymes that had high activity at lower pH (4.0 - 6.0) occurred abundantly from the sixth day of incubation. This could be the probable reason why disease symptom caused by this fungus is sporadic at the early stage of infection in the field and decreases as the plant ages.

From the result obtained there are sufficient reason to implicate polygalacturonases as the most important factor in disease causation by *C. cucurbitarum*.

The lowering of pH could be due to the presence of oxalic acid which in most rot diseases enhances the activity of polygalacturonases (Bateman and Beer, 1965).

Pectic lyase was observed to be most prominent enzymes produced in 3-day old culture *V. unguiculata* isolate . The activity of galacturonase must have been inhibited by some environmental factors such as catabolic repression (Cooper, 1981). According to Misaghi (1982) proteins bound to plant cells walls inhibit the activity of endopolygalacturonase at the initial stage.

Although, there is dearth of literature concerning enzyme production by *C. cucurbitarum* work on enzymes of other micro-organisms implicate polygalacturonase in pathogenicity of many fungi.

Bateman and Beer (1965) reported that the ability of *Sclerotium rolfsii* to produce large amount of polygalacturonase in host tissues appears to be an important factor in tissue maceration. The ability of *C. cucurbitarum* to produce rot condition can also be attributed to the presence of polygalacturonase which was produced both *in vitro* and *in vivo* during the course of this work. Endo-pectate lyase which is also implicated in pathogenesis of many fungi was also identified in this work. This is also in line with the work of Ikotun (1984) and Koleosho *et al.* (1987) who reported the production of pectolytic enzymes by *Penicillium oxalicum* and the involvement of

polygalacturonase in the pathogenicity of *Pythium aphanidermatum* on different cowpea varieties.

Pectolytic enzymes which are also produced by *C. cucurbitarum* have been reported to produce soft rot disease in many plants (Oladiran, 1980). Other diseases caused by this fungus include wet rot of castor plant (Karmal and Singh, 1975), pod rot of *V. unguiculata* (Oladiran, 1980; Osofisan, 1989) and shoot disease of *Amaranthus* (Odebunmi-Osikanlu, 1977; Ikeduigwu, 1981)

The ability of the pathotypes from different sources to induce production of pectolytic enzymes on different host suggests the non-specific nature of the fungus hence a probable wider host range.

CONCLUSION

This study revealed that *C. cucurbitarum* was capable of producing pectolytic enzymes both *in vitro* and *in vivo*. The enzymes produced were polygalacturonase which was active at pH 4.0 - 6.0 and endo-pectate lysate which was active at pH 8.0 - 9.0. The results also showed that the types of enzymes produced varied with the age of the cultures. More galacturonase was produced from the sixth day of incubation to the eighteenth day, whereas endo-pectate lyase was produced only on the eighteenth day.

It was observed that the effects of pH on all the pathotypes were the same. This indicates that the *C. cucurbitarum* has a wider host range and that the pathotypes are not host specific and can attack other susceptible crops planted in close association with their host. It would therefore be appropriate to suggest that susceptible crops such as *Amaranthus* spp, *V. unguiculata* and *A. esculentus* employed in this work, should not be planted together in mixed cropping or should not follow each other.

Future work involving cross infection of many host plants is however, envisaged as this work shed more light on the actual host range and the fungal host interaction of *C. cucurbitarum*.

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