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Time course of production of pectolytic enzymes by pathotypes of *choanephora cucurbitarum*

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ABSTRACT

The *in vitro* and *in vivo* production of pectolytic enzymes by three pathotypess of *Choanephora cucurbitrarum* isolated from *Amaranthus hybridus, Vigna uguiculata* and *Abelmoschus esculentus* were investigated. The amount of enzymes produced by the three pathotypes of *Choanephora cucurbitarum* varied with the age of culture. Also, the optimum pH of enzymes activity in each pathotype varied with the length of the incubation period. For *Abelmoschus* pathotype, highest enzymes level of 25RVU was produced on the six day old culture filtrate while for the nine day old filtrate, maximum enzyme level was 14RVU. With filtrate from 12, 15 and 18 days old cultures, optimum enzymes level were 17, 15 and 22RVU respectively. Result on *Amaranthus* pathotype showed that on three day old culture, activity was 5RVU at pH 3.0, 14RVU at pH 4.0, 14RVU at pH 5.0 and 0 at pH 9.0. Six day old culture was observed to have activity of 12RVU at pH 3.0, 75RVU at 4.0, 72RVU at pH 5.0 and 14RVU at pH 9.0 .There was a steady decline on the ninth day. Increase in activity was observed in 12, 15, and 18 day old cultures at pH 4.0 and pH 9.0. With the *Vigna* pathotypes, the enzymes production was optimal on the ninth day with 67 RVU and least in the 18 day old culture with 5RVU. Maximum activity was at pH 4.0 and least at pH 8.0.

INTRODUCTION

Choanephora cucurbitarum is implicated in pathogenesis of many plants especially vegetables, such as *Amarantus hybridus, Vigna unguiculata, Abelmoschus esculentus* and in cucurbits such as *Telfaira occidentalis* (Odebunmi – Osikantu, 1977, Ikediugwu, 1981, Umana and Ikotun, 2000). It is also known to attack cereals such as: millet, rice, and sorghum. It has been observed to cause wet rot disease in castor plant (*Ricinus communis*).

Choanephora cucurbitarum is facultative saprobe that belongs to the sub-division/Zygomacotina, order ; Mucorales and family, Choanephoraceae (Alexopoulous and Mins 1989). This opportunistic fungus is more successful under humid conditions (Oladiran 1988), thrives best at temperature of 25^0C and relative humidity of about 100 percent. A temperature of about 31^0C stimulates the production of large sporangia, but unfavorable for conidial formation.(Umana and Ikotun 2000).

Recently the fungus is known to produce enzymes like other fungi, which are implicated in the degradation of plant cell wall polysaccharides and cytoplasmic materials. (Guillen *et al* 1987; Umana and Ikotun, 2000).

Although some works have been done on enzymatic degradation of plant cell wall by fungi, (Umana and Ikotun, 2000). the time course of production of pectolytic enzymes by pathotypes of *Choanephora cucurbitarum* has not been reported. This paper reports on the time course of production of pectolytic enzymes by pathotypes of *Choanephora cucurbitarum.*

MATERIALS AND METHODS

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

The isolates were inoculated on potato dextrose agar (PDA) and allowed to grow at room temperature of 28^OC $\pm$ 2^OC. Sub-culturing was carried out until axenic cultures were obtained.

The standard basal medium, with pectin as carbon source was prepared using the method described by Punja and Jenkin(1984). Thirty milliliters of the medium was dispensed into 100ml conical flasks. The flasks were plugged with non-absorbent cotton wool wrapped with aluminum foil. This was autoclaved at 121^{o}C (1.05 kg/cm^3) for 20 min and allowed to cool.

The pure culture of the isolates (4mm in diameter) taken from the margin of seven day old culture, were inoculated into the basal medium. The inoculated flasks were incubated at room temperature (28^OC $\pm$ 2^OC) as stock culture and harvested every three days for 21 days. Isolates from each of the crops were treated separately. The cultures were filtered through eight layer cheese cloth and the filtrate used for the bioassay.

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

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Effect of pH on enzyme activity.

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

Enzyme assay

The enzymes preparations were assayed for their activity by the use of Cannon- Fanske Viscometer size 200 Techniques 1320. The Viscometer was filled with 10ml of distilled water and the time of run through the Viscometer by water recorded. The mean value was then calculated after five appropriate buffer solutions were pipetted into the Viscometer and 1ml 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 recoded. 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 all the culture filtrates obtained after every three days of harvest. This was carried out periodically for twenty one days.

The filtrates 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 formulae adopted by Ikotun and Balogun (1987) as shown below:

$$\frac{Iv-Vx}{Iv-Vw} \times \frac{100}{1} \qquad (1)$$

where Iv = first run of reagent at time 0
Vx = run of reagents after 5 min
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 100 was calculated in viscometric units:

$$\text{Relative Activity (RA)} = 1/t \times 10^3 \text{ RVU.} \qquad (2)$$

RESULTS

Effect of age of culture on enzyme

The amount of enzymes produced by all three pathotypes of *Choanephora cucurbitarum* varied with the age of culture. Also, the optimum pH of enzyme activity in each pathotype varied with the length of the incubation period as shown in Figs. 1-3.

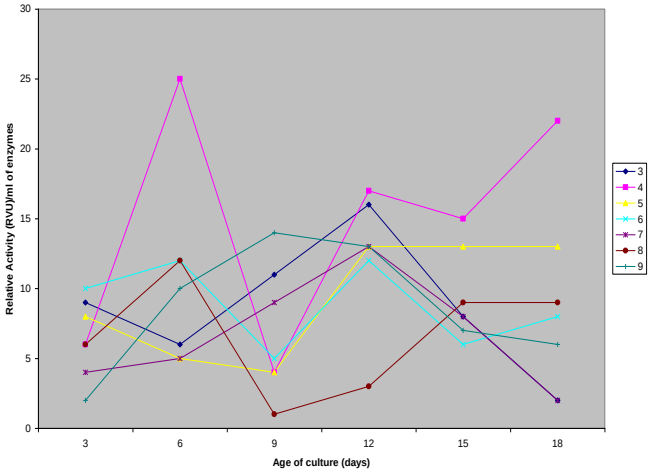

Fig. 1. Production of pectolytic enzymes by *Abelmoschus esculentus*

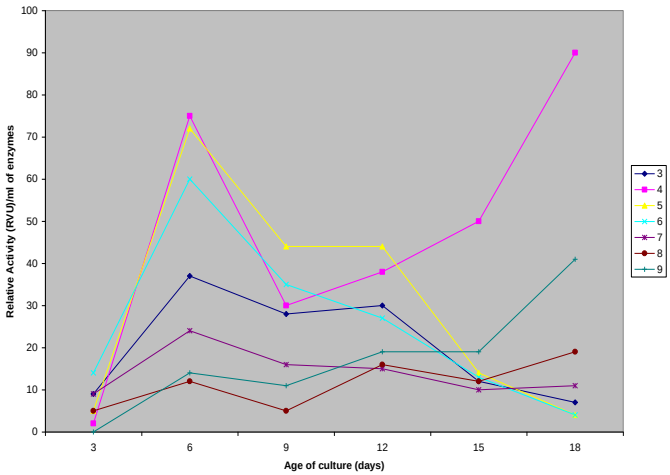

Fig. 2 . Production of pectolytic enzymes by *Choanophora cucurbitarum* from *Amaranthus spp.* in culture.

For the *Abelmoschus* pathotype, the highest enzyme level of 25 RVU was produced on the six day old culture filtrate while for the nine day old, maximum enzyme level was 14RVU. With filtrate from 12, 15 and 18 days cultures, optimum levels were 17, 15, 22RVU respectively. The optimum pH for enzyme activity was 4.0 for 6, 9, 12, 15, and 18 days old culture filtrate.

Filtrate from pathogypes of Vigna and *Amaranthus* showed maximum activity at pH 4.0 and 5.0 and had the least at pH 3.0, 7.0 and 8.0. Results from *Amaranthus* pathotypes show that on 3 day culture, the activity was 5 RVU at pH 3.0, 14RVU at pH 4.0, 14RVU at pH 5.0 and 0 at pH 9.0. Six day old culture was observed to have an activity of 12RVU at pH 3.0, 75RVU at pH 4.0, 72 RVU at pH 5.0 and 12RVU at pH 9.0. There was a steady decline on day 9 for all the pH values. Increase in the activity was observed on 12, 15 and 18 day old culture at pH 4.0 and 9.0 as shown in figure 2.

With the pathotype of *Vigna anguiculata*, enzyme production was optimal on the ninth day with 67 RVU and least for 18 day old culture with 5 RVU as shown in figure 3

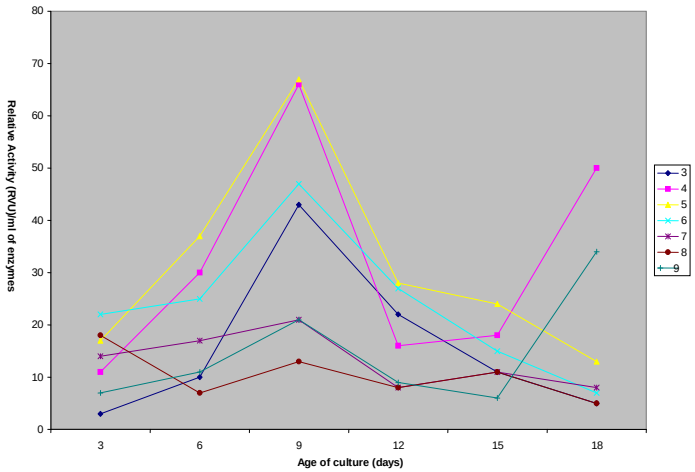

Fig 3. Production of pectolytic enzymes by *Vigna unguiculata*

Maximum enzyme activity was at pH 5.0 and least at pH 8.0. On day 3,6,12 and 15, optimum enzyme levels were 22, 37, 28 and 24 RVU respectively. Optimum pH for enzyme activity on these days was pH 6.0, for day 4, 3 and 5.0 for days, 6, 12 and 15.

DISCUSSION

Choanephora cucurbitarum was observed to produce enzymes with relative activity at lower pH of 3.0 – 5.0 and some had high at higher pH of 8.0-9.0. The observation agrees with Ikotun (1984) and Umana and Ikotun (2000), who reported the production of endo-polygalacturonase by *Penicillum oxalicum* with optimum pH of 5.0 and endo-pectotelyase with optimum pH of 8.0 but that, the exo-polyglactonase was unstable. This instability may account in parts for the fluctuation in culture of different ages as recorded in this work. Ikotun and Balogun (1984) reported that polygalaturonase (hydrolases) produced by *Alternaria solani, Collectricum truncatum, Collectricum capsici* and *Curvularia pallescans* act maximally in acidic medium of 3.0-5.0 and this was supported by Umana and Ikotun 2000. Basham and Bateman (1975) also reported that lyases have high activity in alkaline medium. Arinze (1976), reported that polygalacturonase production was highest when pectin was supplied as carbon source, which is also the type of carbon source used in work.

Some of the enzymes used in this work reduced viscosity of pectin within a very short time and these act maximally at a lower pH of 4.0 and 5.0. The one that took a longer period of time, did so at a higher pH of 8.0 -9.0. This is in conformity with Ikotun (1984) who reported that the enzyme that reduced viscosity of pectin within a short period was endo – polygalaturonase and the ones that degrade at higher pH of 8.0 was endo-pectatelyase.

The type of enzyme produced also varied with the age of the cultures. The enzymes that had activity at lower pH of 4.0 -6.0 occurred abundantly from the sixth day of incubation in all the pathotypes. This supported the opinion of sequential production of pectoloytic enzymes.

This could be the major season why disease symptoms caused by *Choanephora cucurbitarum* are sporadic at the early stages of infection in the field and decrease as the plants age. The younger plants are more susceptible to this opportunistic pathogen than the older plant. From the information obtained from this work, there are sufficient reasons to implicate polygalacturonase as the most important factor in diseases of *Choanephora cucurbitarum.*

The lowering of pH could be due to the presence of oxalic acid which in most rot diseases (Bateman and Beer, 1965). Pectin lyase was observed to be the most prominent enzyme produced in 3 day old culture of *Vigna* isolate. The activity of polygalaturonase must have been affected or inhibited by some environmental factors such as catabolic repression (Copper 1981). Misaghi (1982) reported that proteins bound to plants cells walls inhibit the activity of endo-polygalacturonase at the initial stage.

The work of Umana and Ikotun (2000) implicated polygalacturonase in pathogencity of *Choanephora cucurbitarum.* The ability of this fungus to produce rot *in vivo* and *in vitro* during the course of this work and the amount produced, varied according to the age of culture. Endo pectate lyase was also identified. This is in conformity with the works of Koleosho (1987) that reported the implication of this enzyme in pathogenesis of many fungi.

The production of pectolytic enzymes on different varieties and hosts by the pathotypes suggests the non host specific nature of the fungus hence, a probable wider host range.

CONCLUSION

The result shows that the type of enzyme produced varied with the age of culture. More galacturonase was produced from the sixth day of incubation to the eighteenth day whereas more endo pectate lyase was produced on the eighteenth day. The healthy tissues also produced a little enzyme which points to the fact that some of these enzymes were endogeneously produced by plants.

The work also shows that the pathotypes were able to produce pectolytic enzymes that degrade the plant cell wall in crops other than those from which they were respectively isolated. This indicates that the pathotypes were not host specific. It would therefore be reasonable to suggest that crops in close association should not be planted together or follow each other during rotation.

Further works involving longer period is however recommended as this will shed more light on the actual life span and life cycle of this interesting fungus.

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