

ENZYMES ACTIVITY OF *BOTRYODIPLODIA THEOBROMAE* PAT.
AND *FUSARIUM MONILIFORME* AND THEIR ROLE IN
INDUCING MANGO FRUIT ROTS

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Three isolates of *Botryodiplodia theobromae* isolated from mango, peach and pear and one isolate of *Fusarium moniliforme* isolated from mango were tested in vitro. *B. theobromae* and *F. moniliforme* had the ability to produce many cell wall degrading enzymes in media containing different carbon sources. Maximum activity of cellulase was recorded at pH 4.5 on media containing CMC, xylan and cellulose as a carbon sources. It was also found that *B. theobromae* and *F. moniliforme* revealed a high activity of xylanase in liquid media containing xylan, CMC, cellulose, polygalacturonic acid and pectin at pH 4.5-6.8. It was shown also that *B. theobromae* isolates and *F. moniliforme* could produce high quantities of polygalacturonase (PG). The relationship between incubation periods and a secretion of degrading enzymes cellulase, xylanase and PG in filtrates of culture medium containing xylan as a carbon source at pH 4.5 was studied. The degrading enzymes cellulase, xylanase and PG were determined in infected mango fruits 5 days after inoculation with *B. theobromae* and *F. moniliforme* isolates. It was found that *B. theobromae* isolated from mango was the highest isolate in producing degrading enzymes at pH 4.5. The relationship between the time and secretion of degrading enzymes in infected mango fruits, inoculated with *B. theobromae* (mango isolate) showed that the highest secretion of cellulase was after 2 days of inoculation while secretion of xylanase and PG was after 4 days of inoculation

Mango (*Mangifera indica* L.) is one of the most highly prized of tropical fruits. At the same time, the 'King of fruits' is so abundant that it has also been called 'the apple of the Orient'. Mango fruits have now in Egypt the second in commercial importance where, the crop area is about 55.000 fedd. (Ministry of Agriculture, Egypt). Many fungal diseases attack mango fruits at different growth and ripening stages and post harvesting like *Botryodiplodia* rot, stem-end rots caused by *Botryodiplodia theobromae* Pat. and other fungi and many other diseases. During shipment and storage deterioration due to such diseases might happened due to a previous infection specially in fresh mango fruits. Such spoilage reduce the quality and quantity and cause great losses. During rotting process of mango fruits, many chemical, physical and physiological changes occurred.

B. theobromae Pat. is a wide spread pathogen in different parts of the world as well as in Egypt. It is a progressive and vigorous pathogen that causes several diseases to a large number of economic crops such as orange and grapefruit (LEONARD, 1936), banana (El-Helaly, *et al.* 1968 and Ogundero, 1987), apple (Ali & Morsy, 1972 and Latham & Dozier, 1989), pear and quince fruits (El-Arosi & Wasfy, 1972), sycamores (Lewis and van Arsdel, 1978), avocado (Darvas & Kotze, 1987) grapevines (Mathews & Dodds, 1986) and mango (Fernando, 1937, Srivastava, 1968 and Ragab *et al.*, 1971).

B. theobromae Pat. has been found to produce extra cellular cellulolytic and pectolytic enzymes in culture and polygalacturonase (PG) was high in media containing citrus pectin, sodium polypectate and in sweet potato slices (Arinze *et al.* 1976). Polygalacturonase, polygalacturonic acid lyase, xylanase and cellulase were produced in infected tuber tissue of yam (Turner & Ogundana 1983). The activity of polygalacturonase, polymethylgalacturonase, polygalacturonase transeliminase and polymethylgalacturonase transeliminase were greater for both *B. theobromae* and *Macrophoma mangiferae* in vitro than in mango fruits and the reverse was true for pectin methyl esterase. The enzyme activity reached its maximum after 8-12 days from incubation at 25°C for *B. theobromae* than *M. mangiferae* (Prasad & Sinha 1987). For these reasons this study was done to explain some associated processes of diseased mango fruits during different growth stages. This study was carried out at Institute of Phytopathology, (JLU) Giessen, Germany.

MATERIALS AND METHODS

Fungal isolates

Three isolates of *B. theobromae* Pat., each of them were isolated from rotted mango fruits, and from die-backed peach branches as well as from die-backed cankered pear branches, were used in this study, El-Habba (1995).

Sample preparation

A-From fungal cultures

Different carbon sources (citrus pectin (Roth), polygalacturonic acid (Serva), xylan (Baker), Carboxy methyl cellulose CMC and cellulose (Serva), 1.5% glucose and 3% sucrose (Serva), were added to Czapek's medium for enzyme production (2 g NaNO₃, 1 g K₂HPO₄, 0.5 g MgSO₄·7H₂O, 0.5 g KCl, 0.01 g FeSO₄·7H₂O, 1 ml 1% (w/v) ZnSO₄, 1 ml 0.5% (w/v) CuSO₄, and 0.01% (w/v) and supplemented with 1% (w/v) yeast extract, made up to 1 liter with distilled water, (Valsangiacomo & Gessler 1992).

One hundred ml conical flasks containing 25 ml culture media with the different carbon sources, were inoculated with an 6 mm disks from the tested fungi and incubated at 27°C for 7 days in the dark. Then culture media were filtered and

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centrifuged at 12,000 r.p.m for 10 min. at 4°C. The supernatant was dialyzed against distilled water for 18 hr. at 4°C. The dialyzed samples were centrifuged at 12,000 r.p.m for 10 min. and stored at -18°C. (Turner & Ogundana 1983).

B- From infected mango's fruit

Imported mango fruits from Brazil were brought from market and surface sterilized with ethanol 70%. Plugs (12 mm diameter) were cut into discs about 5 mm thick. Mango discs were transferred to pre-weighed sterile Petri dishes and inoculated with standard fungal discs (2 mm diameter) and incubated at 27°C for 5 days. Un-inoculated mango plugs were used as control. Inoculated and control samples were homogenized separately in 20 ml distilled water containing 0.6 g Poly Clar AT (BDH) to adsorb phenols, using a Virtis blender (Ogundana et al. 1971). The homogenate was centrifuged, dialyzed and stored as mentioned before.

Enzyme assays

1-Determination of polygalacturonase (PG) activity

For assaying PG activity, agar diffusion assay was used according to the method described by Dingel *et al.*, (1953). The enzyme activity in units was calculated using a standard curve with commercial polygalacturonase (CPG) (Pectinase PS from *Aspergillus niger* 0.15-0.3 units/mg, Serva) where a unit is the amount of enzyme forming 1 μ mole product in 1 h. at 30°C.

2-Estimation of cellulase and xylanase activity

This quantitative colorimetric assay is based on the preceptibility of the non-degraded, highly polymerized, substrate of CM-Cellulose-RBB and CM-Xylan-RBB 4 mg/ml (Loewe-Biochem., Blue Substrates Co. Germany 1994) by hydrogen chloride, after incubating the substrates together with the enzyme. As a result non-degraded substrate is precipitated and can be removed by centrifugation. The absorbancy of the supernatant was blotted as a function of incubation time to measure enzyme activity. Reaction mixture contained 0.1 ml aqueous substrate solution 4 mg/ml, 0.2 ml 0.1 M Citrate buffer (Mc-Lavine buffer) and 0.1 ml enzyme solution, incubate within Eppendorf tube. Incubation temperature and time, type and pH of incubation buffer, and final substrate concentration depended on the specific properties of the enzyme under investigation. Reaction was stopped by 0.1 ml 1 N HCl and the Eppendorf tubes were cooled on ice for 10 min., centrifuged for 5 min. at 12,000 r.p.m. Incubation mixtures were measured colorimetrically at 620 nm. (Loewe-Biochem. Blue Substrates Co. Germany 1994). The resulting data were changed to units (u/ml) with the use of a standard curve of xylanase (xylanase Xs from *Trichoderma viride* EC 3. 2. 1. 8 Sigma Chem. Co. U.S.A.) and cellulase TC (Cellulase Cs 0,7 u/mg from *Trichoderma reesi* EC 3. 2. 1. 4. Serva Biochem. Co., Germany).

RESULTS

1. In vitro Enzymes assay

1.1. Cellulase produced in culture filtrate.

Data presented in Fig (1A) show that *B. theobromae* (mango isolate MB) has the ability to produce cellulase in liquid media containing different carbon sources. The highest quantity was produced in media contained CMC, followed by xylan and cellulose as carbon sources to be 0.09, 0.06 and 0.048 u/ml, respectively. No secretion of cellulase enzyme was recorded in medium containing pectin, PGA, glucose and sucrose as a carbon sources at pH 6.8. The highest activity of cellulase of all pathogenic isolates was 0.099 u/ml produced by *F. moniliforme* in medium containing xylan as a carbon source at pH 6.8 after 7 days incubation at 30°C

Data presented in Fig. (1B) show that the activity of the cellulase enzyme produced at pH 4.5 were higher than at pH 6.8. The highest activity of the enzyme was 0.23 u/ml secreted by *B. theobromae* (mango isolate MB) in medium containing cellulose as a carbon source followed by 0.22 u/ml by *F. moniliforme* in the same medium containing xylan. While media containing PGA and glucose were not favourable for producing cellulase by all isolates.

2.1. Xylanase produced in culture filtrates

Data presented in Fig. (2A) show that all *B. theobromae* isolates have the ability to secrete xylanase on different carbon sources at pH 6.8. However the highest activity of the enzyme was recorded by *B. theobromae* (mango isolate MB) in the media containing xylan, CMC and PGA respectively. No secretion of xylanase was recorded in different carbon sources after 7 days from inoculation by *F. moniliforme* except in liquid medium containing xylan as a carbon source.

Data presented in Fig. (2B) show that the activity of xylanase produced by *B. theobromae* isolates in the liquid medium containing xylan, CMC and cellulose as carbon sources at pH 4.5 were less than others at pH 6.8. Only the quantities of xylanase secreted by *B. theobromae* (pear isolate PrB) isolate were increased at pH 4.5 in media containing xylan, CMC, cellulose PGA and pectin. Low quantity of the enzyme was secreted by *F. moniliforme* in media containing xylan and CMC as carbon sources at pH 4.5.

3.1. PG enzyme produced in culture filtrates

Results presented in Fig. (3 A) show that very low quantities of PG enzyme were secreted at pH 6.8 by *B. theobromae* isolates and *F. moniliforme* isolates in the liquid medium containing different carbon sources

Results presented in Fig. (3 B) show that pH 4.5 was more favourable for producing PG by both pathogenic fungi. The quantities of the enzyme increased to reach 1 u/ml by *B. theobromae* (pear isolate PrB) in liquid medium contained CMC

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as a carbon source and 0.66 u/ml by *B. theobromae* (MB) isolate in the same medium at pH 4.5 after 7 days from inoculation.

4.1. Relationship between the incubation periods and secretion of enzymes by *B. theobromae* Pat.

Data presented in Fig. (4 A) show a continued increasing in cellulase and xylanase activity in relation to the incubation time of *B. theobromae* (MB) in medium containing xylan as a carbon source at pH 4.5 to reach maximum after 7 days incubation (0.111 and 1.993 u/ml). On the other hand, the maximum activity of PG was obtained after 2 days incubation and it decreased again by increasing the incubation period.

Data presented in Fig. (4 B) show a continued increasing quantities of cellulase, xylanase and PG enzymes in relation to incubation time of *B. theobromae* (MB) in liquid medium containing CMC as a carbon source at pH 4.5 to reach maximum after 7 days (0.076, 1.221 and 0.3 u/ml respectively).

2. In vivo studies

2.1. Production of degrading enzymes Cellulase, Xylanase and PG in infected mango fruits.

Data presented in Fig. (5) show that the maximum secretion of PG, xylanase and cellulase was recorded by *B. theobromae* (MB) in infected mango fruits 5 days after inoculation where the quantities of the enzymes were 0.36, 0.188 and 0.007 u/ml respectively. *B. theobromae* (peach and pear isolates) secreted also PG in infected mango fruits but less than those secreted by mango isolate of *B. theobromae*.

2.2. Relationship between the incubation period and secretion of degrading enzymes.

Data in Fig. (6) show that the maximum quantity of cellulase was recorded 2 days after incubation of infected mango fruits with *B. theobromae* (MB) to be 0.07 u/ml. Maximum quantities of xylanase and PG were recorded after 4 days from incubation of the same infected mango fruits to be 0.22 and 0.23 u/ml respectively. No detection of the same enzymes was recorded in non-infected control ones.

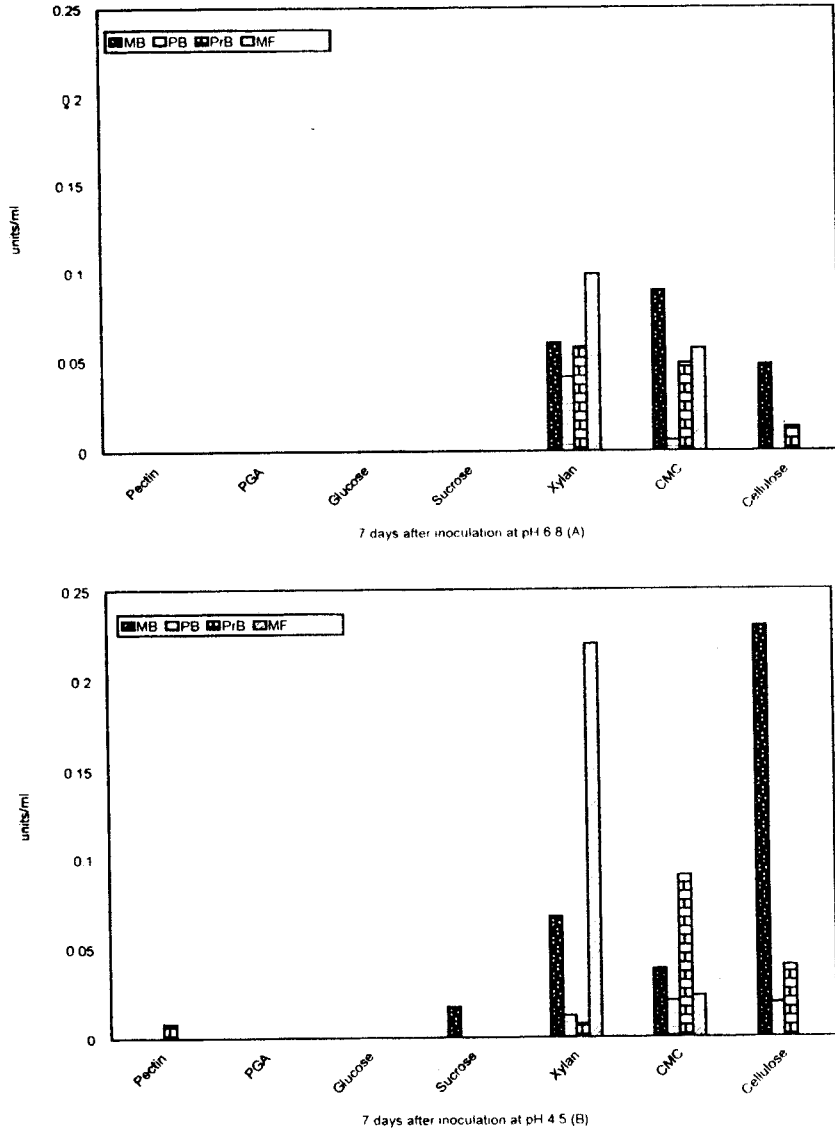


Fig. (1 A&B) : Cellulase enzymes produced on different carbon sources by *B. theobromae* and *F. moniliforme* in culture filtrates at pH 6.8 and 4.5

- MB - *B. theobromae* (Mango isolate)
- PB - *B. theobromae* (Peach isolate)
- PrB - *B. theobromae* (Pear isolate)
- MF - *F. moniliforme* (Mango isolate)

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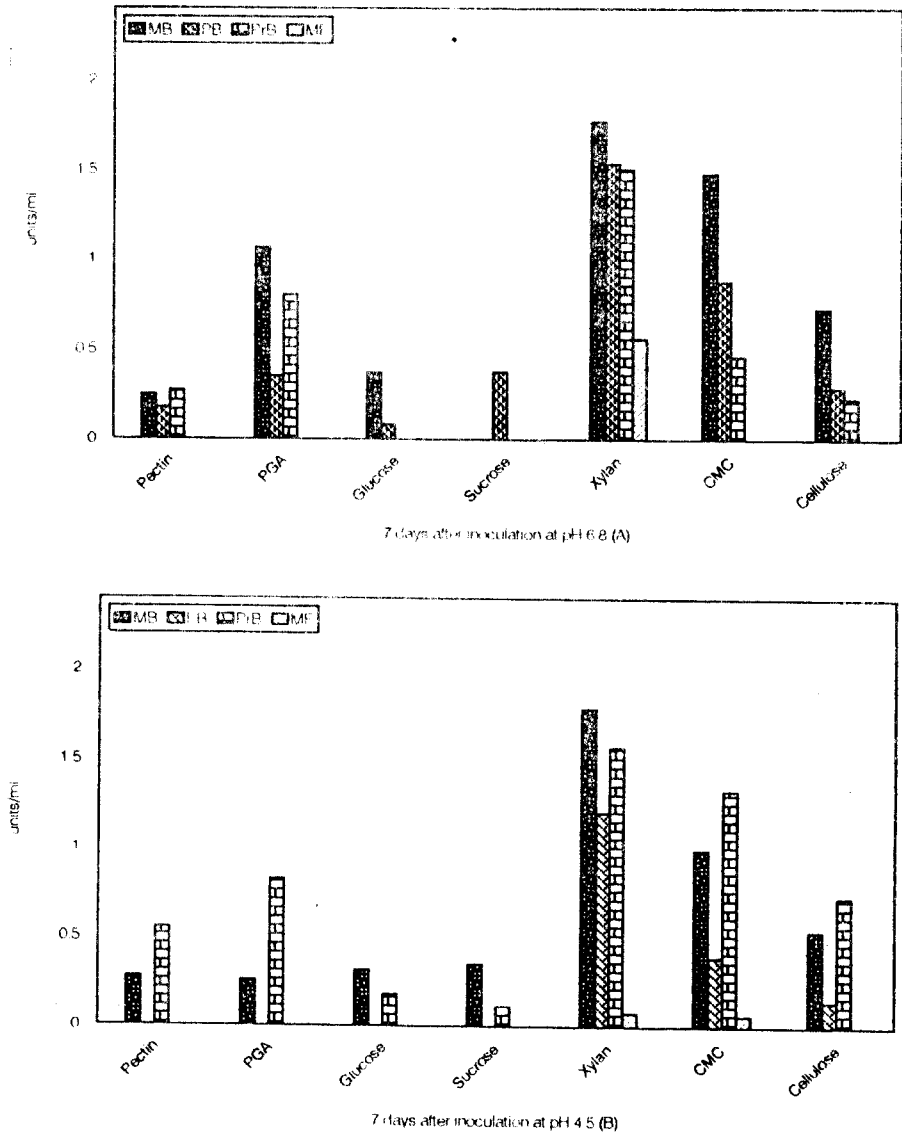


Fig. (2 A&B) : Xylanase enzyme produced on different carbon sources by *B. theobromae* isolates and *F. moniliforme* in culture filtrate at pH 6.8 and 4.5.

MB = *B. theobromae* (Mango isolate)
 PB = *B. theobromae* (Peach isolate)
 PrB = *B. theobromae* (Pear isolate)
 MF = *F. moniliforme* (Mango isolate)

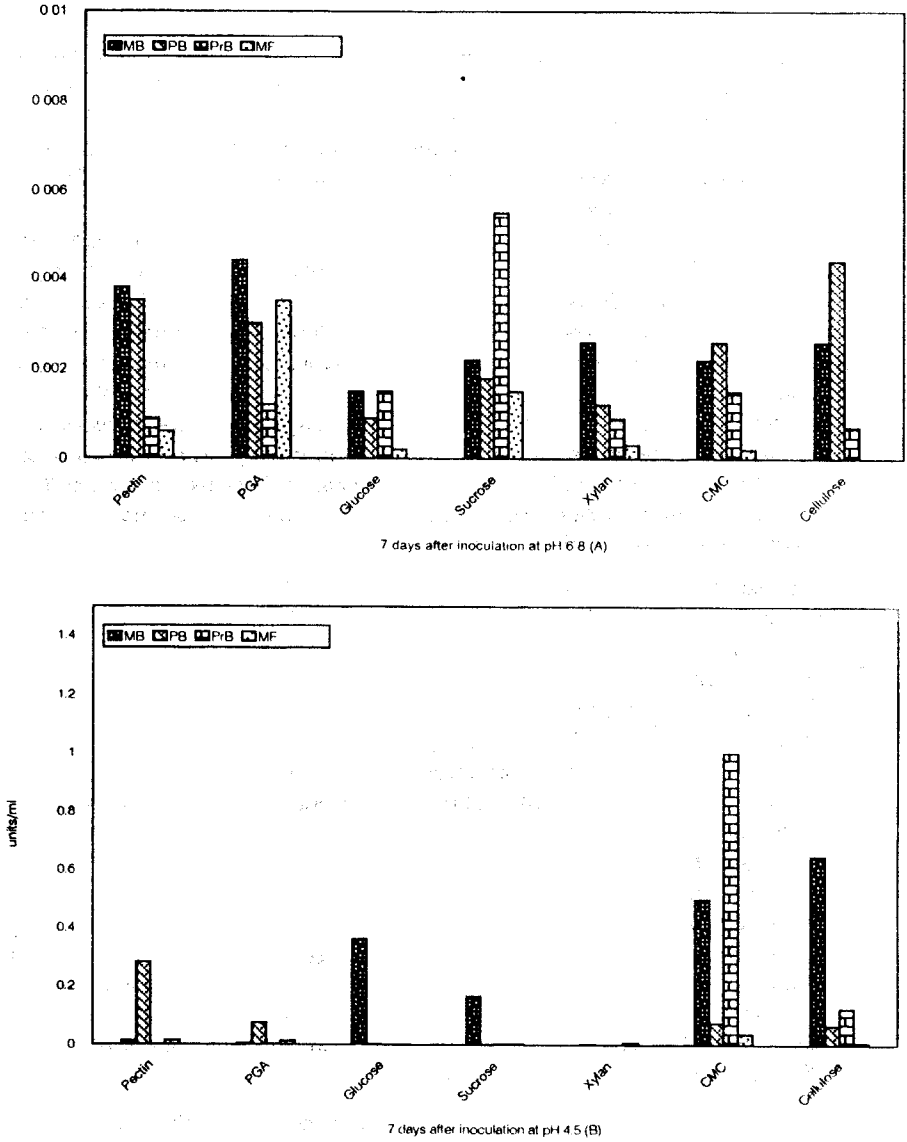


Fig (3 A&B) : PG enzyme produced on different carbon sources by *B. theobromae* isolates and *F. moniliforme* in culture filtrate at pH 6.8 and 4.5.

MB - *B. theobromae* (Mango isolate)
 PB - *B. theobromae* (Peach isolate)
 PrB - *B. theobromae* (Pear isolate)
 MF - *F. moniliforme* (Mango isolate)

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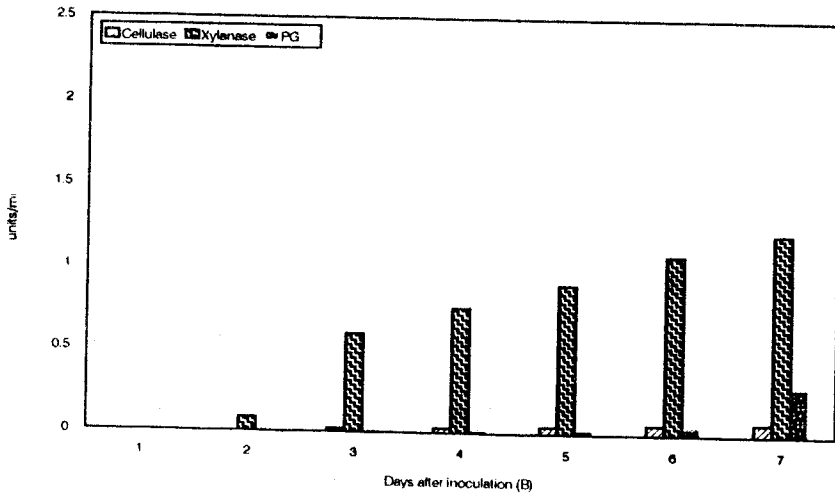
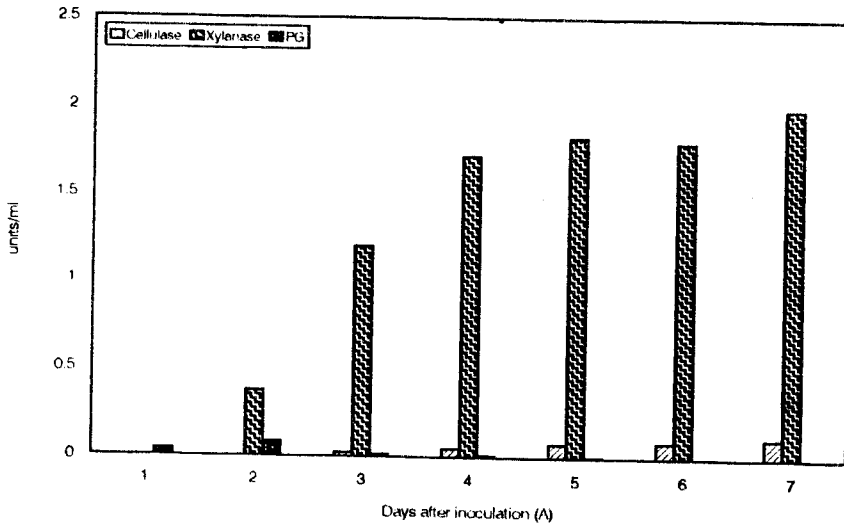


Fig (4 A&B) : Relationship between the incubation periods and the tested enzymes produced by *B. theobromae* (MB) in liquid medium containing Xylan and CMC as a carbon sources at pH 4.5.

A: Xylan

B: CMC

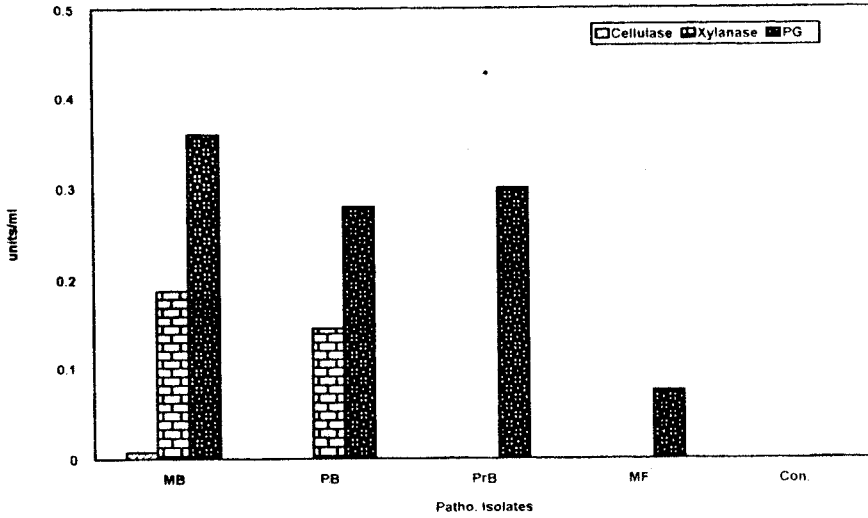


Fig (5) Production of degrading enzymes *Cellulase*, *Xylanase* and *PG* in infected mango fruits 5 days after inoculation with different pathogenic isolates

MB = *B. theobromae* (Mango isolate)
 PB = *B. theobromae* (Peach isolate)
 PrB = *B. theobromae* (Pear isolate)
 MF = *F. moniliforme* (Mango isolate)

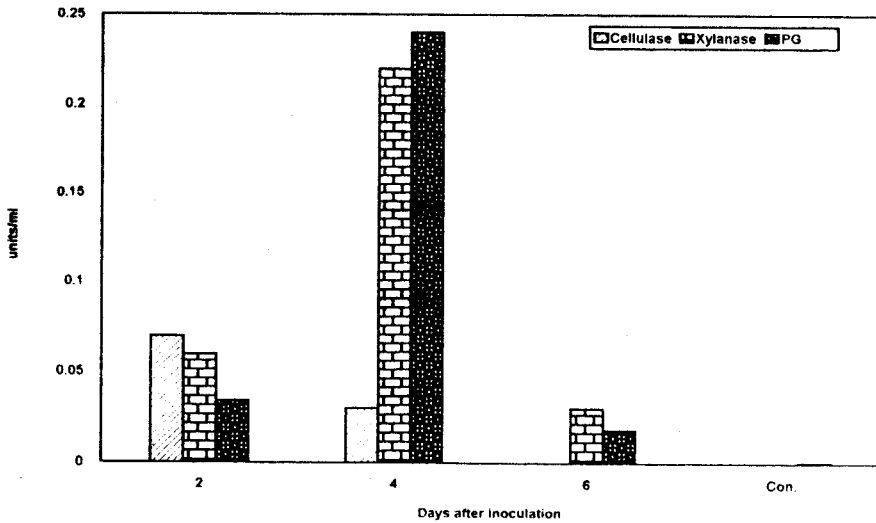


Fig (6) Relationship between incubation periods and activity of degrading enzymes in mango fruits infected with *B. theobromae* (mango isolate)

DISCUSSION

Botryodiplodia theobromae Pat. was found to be the main causal pathogen of stem end rot, Botryodiplodia rot of mango fruits and other orchard fruits. It was necessary to know the mode of infection and the ability of the pathogenic fungi to produce enzymes involved in the development of infection. This study was concentrated on three enzymes that may play an important role in the infection process i. e. cellulase, xylanase and polygalacturonase, cellulose is one of the main substances in the peel and tissues of mango and xylan is one of the components formed through the degradation of cell walls and also during the formation of callus in the presence of wounds (Burke et al. 1974 & Aspinall 1980). The obtained results indicated that *B. theobromae* and *F. moniliforme* could produce in vitro cellulase in media containing xylan, CMC and cellulose as carbon sources and the production of the enzyme at pH 4.5 was higher than at pH 6.8. While the same isolates failed to produce cellulase in medium containing pectin, PGA, glucose and sucrose. This result indicated that cellulase is an induced enzyme in the presence of certain substances in vitro and/or in vivo. It is important here also to say that this enzyme play preliminary role in cell maceration and starting infection and it is clear that *F. moniliforme* is more active in this respect.

It was also recorded that *B. theobromae* and *F. moniliforme* are active producers of xylanase on different carbon sources at pH 6.8 and 4.5 with the same activity. Production of enzyme was higher in media containing xylan, CMC and PGA and inoculated with *B. theobromae* isolates, whereas *F. moniliforme* was able to produce xylanase in the presence of xylan only. This result show that the secretion of xylanase enzyme degraded xylan during callus formation, thus no healing of the wounds occurs and infection progresses. Xylans, 1,4- linked polymers of xylopyranose are the most abundant hemicellulose in angiosperms occurring mainly in the secondary wall. (Burke et al. 1974 & Aspinall 1980). Several lines of evidence suggest that xylanases might be a significant biochemical component in the interaction between plants and pathogens. First, the abundance of xylan in the cell walls of plants makes it a significant potential source of nutrition as well as a potential major mechanical barrier to penetration. Second, xylanases from a variety of pathogenic and non-pathogenic fungi induce plant defense responses including necrosis, electrolyte leakage and synthesis of superoxide.ethylene, phytoalexins and pathogenesis-related proteins (Ishii 1988, Fechs et al. 1989, Bailey et al. 1990, Lotan & Flchr 1990 and Dean & Anderson 1991).

The obtained results indicated that PG produced by *B. theobromae* isolates and *F. moniliforme* play the important role in the progress of infection in mango fruits. Data show that pH 6.8 was not favourable for production of this enzyme whereas pH 4.5 was more suitable for production of the enzyme especially in media containing CMC and cellulose and this pH 4.5 is similar to that obtained in mango fruits. This result helps to understand the nature of PG enzyme in inducing rapid maceration and rotting symptoms of mango fruits. It was also noticed that all isolates

of *B. theobromae* have the ability to produce PG at low pH values and that seems serious because it means that the different isolates of *B. theobromae* isolated from different hosts were pathogenic to mango fruits. Also the clear reduction in the production of enzymes in media containing glucose and sucrose interpret the decreasing of infection at later growth stage of fruits where the free sugars were more than in less mature stages and this was clear in this study. Similar results were obtained by **Arinze and Smith (1979)**, they found that *B. theobromae* isolate which was isolated from sweet potato produced four polygalacturonase isoenzymes, PG 1, 2, 3 and 4 in liquid culture. PG 2, 3 and 4 macerated and killed tissue discs of sweet potato and potato tubers. **Turner and Ogundana (1983)** found also that PG could be produced as the first in the sequence of wall-degrading enzymes secreted in infected yam tissue by *B. theobromae*. **Arinze (1985)** found also that PG enzyme completely macerated sweet potato roots, potato tubers and tomato fruits within 5 hours. **Ogundero (1987)** found that *B. theobromae* and *Colletotrichum musae* produced extra cellular amylases, pectin methyl esterase and CM-cellulase. **Wasfy *et al.*, (1985)** stated that three isolates of *B. theobromae* isolated from apple, pear, and passion flowers produced pectin methyl esterase, polymethylgalacturonase and polygalacturonase in vitro and also in rotted fruits. Also, they found that enzyme activity in rotted tissues was higher than in healthy fruits

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النشاط الأنزيمي لفطر البوترويدوبلوديا ثيوبرومي وفطر الفيوزاريوم مونيليفورم
ودورهم في احداث أعفان ثمار المانجو

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- * بارند اشتاينهور ** جهاد الهباء
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أختبرت ثلاث عزلات من البوترويدوبلوديا ثيوبرومي المعزولة من المانجو والخوخ والكمثرى بالإضافة لعزلة من الفيوزاريوم مونيليفورم المعزولة من المانجو معمليا وطبيعيًا . وقد وجد أن كلا الفطرين له القدرة على افراز العديد من الأنزيمات المحللة في البيئات المحتوية على مصادر الكربون المختلفة وكانت أعلى درجة نشاط لأفراز انزيم السليوليز عند درجة الحموضة ٤,٥ عندما كانت مصادر الكربون هي الكربوكسي ميثايل سليولوز والزيلان والسليولوز . وقد وجد أن عزلات البوترويدوبلوديا والفيوزاريوم يمكنهم افراز كميات عالية من انزيم الزيلانيز في البيئات المحتوية علي الزيلان والكربوكسي ميثايل سليولوز والسليولوز وحامض البولي جالاكتورونيك والبكتين عند مستوى درجة حموضة ٤,٥-٦,٨ . وقد أتضح أيضا أن عزلات البوترويدوبلوديا والفيوزاريوم يمكنهم انتاج كميات عالية من انزيم البولي جالاكتورونيز . وقد درست العلاقة بين فترات التحضين وافراز الأنزيمات المحللة من السليولوز والزيلانيز والبولي جالاكتورونيز في روائح البيئة المزروعة المحتوية على الزيلان كمصدر كربوني عند مستوى درجة حموضة ٤,٥ . وقد قدرت أيضا في هذه الدراسة الأنزيمات المحللة من السليولوز والزيلانيز والبولي جالاكتورونيز في ثمار المانجو المصابة بعد خمسة أيام من التلقيح بعزلات البوترويدوبلوديا والفيوزاريوم ووجد أن البوترويدوبلوديا ثيوبرومي المعزولة من المانجو كانت أعلى العزلات في انتاج الأنزيمات المحللة عند مستوى درجة حموضة ٤,٥ . وعندما درست العلاقة بين فترة التحضين وافراز الأنزيمات المحللة في ثمار المانجو المصابة والملقحة بالبوترويدوبلوديا ثيوبرومي المعزولة من المانجو , اتضح أن أعلى درجة افراز لأنزيم السليوليز كانت بعد يومين من التلقيح بينما كان ذلك بعد أربعة أيام من التلقيح لأنزيمي الزيلانيز والبولي جالاكتورونيز .