

## CHARACTERIZATION OF ENDOPOLYGALACTURONASE PRODUCED BY COLLETOTRICHUM DEMATIUM F.SP. TRUNCATA IN CULTURE AND INFECTED SOYBEAN SEEDLINGS

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### Abstract

An isolate of *Colletotrichum dematium* (Pers. ex Fr.) Grove f. sp. *truncata* (Schw.) produced endopolygalacturonase (endo PG) in culture in response to soybean seed exudates and in infected soybean seedlings 20hr after inoculation. No other depolymerizing type of pectic enzyme was detected during fungal growth under these conditions. Endo PG from these sources had pH optimum of 5.3 and molecular weights of 42,000 Kda. Enzyme preparation from culture and infected seedlings readily macerated soybean epicotyl sections. However, by using isoelectric focusing technique, it was found that the endo PG produced by *Colletotrichum dematium* f. sp. *truncata* in response to seed exudates had a single isoelectric point (PI) of 7.3, whereas the endo PG from infected epicotyls had a major peak at PI 7.9 and a minor peak at PI 7.3. Differences in elution patterns were observed when preparations from the two sources were purified by ion-exchange chromatography. Results of this study suggest that *Colletotrichum dematium* f.sp. *truncata* produces different forms of endo PG and that the ionic properties of the predominant form produced during pathogenesis differ from those of the single-peak form produced in culture.

### INTRODUCTION

Soybean (*Glycine max* (L.) Merr) is considered one of the main oil crops all over the world. It occupies special importance in Egypt because it contains oil at 20% of the dry seed weight, and being an important source of protein which reaches 40% of the dry seed weight (Kassem, 1982)

Soybean plants are severely attacked by many pathogenic fungi which infect

different parts of the plant causing different amounts of losses. Anthracnose disease causes appreciable economic loss and could be a threat to soybean plants in many countries of the world including Egypt (Sinclair, 1982, El-Wakil, 1982; Morrall 1988; Koch *et al.*, 1989; Warther and Elrod, 1990; Ahmed - Saieda, 1994 and Attia *et al.*, 1995). *Colletotrichum dematium f.sp. truncata* is the main fungal pathogen on soybean, causing anthracnose disease (Jasnic, 1983 and Wong, *et al.*, 1983).

Pectin enzymes are among the first cell wall degrading enzymes excreted by plant pathogens *in vivo*. They cause changes in cell membrane permeability, resulting in electrolyte leakage and cell death. Studies of soybean infected by the anthracnose fungus (Lorenzo *et al.*, 1990) suggested that pectic enzyme production by this fungus is regulated by substrate induction and catabolite repression by free sugar present in the soybean apoplast.

Histological studies revealed rapid disintegration of plant tissues after successful infection. During this process the fungal hyphae penetrate the plant cell wall and that during this process cell wall degrading enzymes are being produced by the penetrating pathogen. Pectin degrading enzymes seem to be widely involved in this process as has been shown for several plant-pathogen interactions. Digestion of pectin appears to be essential to establish fungal infection in the plant (Perez Artes and Tena, 1994).

Cytological and biochemical evidence suggests that pectolytic enzymes contribute to fungus penetration and to the initial processes associated with infection of epicotyls of soybean seedlings (Sinclair, 1991). *Collectotrichum* spp. is reported to produce a variety of pectolytic enzymes in culture and during pathogenesis (English *et al.*, 1971 and Fisher *et al.*, 1973).

Most microbial plant pathogens have the ability to secrete enzymes that degrade the polysaccharides of plant cell walls. Among these enzymes the polygalacturonases are of key importance in the infection processes, (Anderson, 1978).

Plant cells are surrounded by a rigid wall in which several polysaccharides, each of defined structure are specifically interconnected, (English *et al.*, 1971). Sequential secretion of cell wall degrading enzymes has been demonstrated during growth of several fungal pathogens in isolated host cell walls (Brookhouser and Weinhold, 1979 and Page, 1961).

This study compares the properties of the pectolytic enzymes produced by

*Collectotrichum dematium* f.sp. *truncata* during the early stages of pathogenesis on soybean with those produced in culture on soybean seed exudates.

## MATERIALS AND METHODS

### Inoculation of soybean seedlings :

The isolate of *Collectotrichum dematium* f.sp. *truncata* used in this study was isolated from infected soybean plants from Giza Governorate during 1994 growing season. In greenhouse tests, the fungus was virulent to soybean seedlings (Ahmed-Saieda, 1994). Pure cultures were obtained using the hyphal tip technique and kept on a Glucose Peptone Agar (GPA) slants for further studies. For the production of inoculum, the pathogen was grown in still culture in Petri dishes that contained (GPA) medium and incubated at 22°C for 7 days (Attia *et al.*, 1995).

Fifteen 4-day old seedlings of soybean (*Glycine max.* L.Merr.) Crawford cv. were arranged in horizontal positions on glass plates (16x21 cm) and the seedlings were covered with moistened quartz sand. A thin layer of moistened sand was placed between the glass plates and epicotyls. A 2-mm diameter disk, cut from the mycelial mat of 4-day-old cultures, was washed and placed on the moist sand adjacent to each epicotyl. Aluminum foil was wrapped around each glass plate, enclosing seedling and epicotyls. After holes were punched in the aluminum foil and the plates (and plants) were placed in a vertical position in plastic pans containing a layer of water 1 cm deep, and the pans were covered with clear plastic. Inoculated plants were incubated under 3.229 lux of continuous light and at temperature of 22°C (Wrather and Elrod, 1990).

### Extraction of pectic enzymes from inoculated seedlings:

Soybean (*Glycine max* L. Merr). Crawford cv. epicotyls were sampled 20 hr after inoculation and segments, 2 cm long, were cut at the inoculation site from 15 seedlings on each glass plate. Extracts were prepared by grinding the epicotyl segments from each of three replicates glass plates for 1 min at 4°C in 5 ml of 0.5 M NaCl (pH 6) in a VirTis homogenizer. Each of the three extracts was strained through three layers of cheesecloth and centrifuged at 400 xg for 20 min. at 4°C. The supernatants were dialyzed overnight against distilled water at 4°C and stored at -18°C. Uninoculated seedlings incubated under these conditions did not contain measurable pectolytic activity (Brookhouser and Weinhold, 1979).

**Collection of soybean seed exudates:**

Seed exudates were obtained by placing 12.6g of soybean seed ( $100 \pm 3$  seeds) in each of 50 Petri dishes each containing 15 ml of sterilized distilled water for 3 hours at 22°C. The liquid in the dishes was filtered through a 0.22  $\mu\text{m}$  Millipore filter and the volume was adjusted to 25 ml per 100 seeds. This solution represented the nonfractionated seed exudates. One hundred milliliters of this exudate solution was dialyzed overnight against 2 liter of distilled water at 4°C and is hereafter referred to as the dialyzed seed exudate.

**Production of pectolytic enzymes by *Collectotrichum dematium* f. sp. *truncata*:**

Seed exudates were used most frequently to produce pectolytic enzymes in culture. In some comparisons, sodium polypectate (1 mg/ml) or cell walls isolated from soybean epicotyls by the the method of English *et al.* (1971) were tested as polygalacturonase inducers in culture.

Twenty mycelial disks, 2 mm in diameter, were cut from 2-days old cultures of *Collectotrichum dematium* f.sp. *truncata* and placed in 25 ml Erlenmeyer flasks containing 10 ml sterile distilled water. The flasks were placed on a water bath shaker and incubated at 22°C at 40 oscillations per minute. After 20 hr, the water was decanted and 10 ml of seed exudates were added to each flask. Samples were removed from flasks at various time intervals, dialyzed overnight against distilled water, and assayed for pectolytic enzyme activity.

**Pectolytic enzyme assay :**

Assaying of pectolytic enzymes produced by *Collectotrichum dematium* f. sp. *truncata* in both seed exudates culture and extracts of infected soybean seedlings 72 hrs after inoculation was carried out according to the method described by Ayers *et al.* (1966).

**Paper chromatography :**

One-dimensional paper chromatography was used to determine the reaction products released from polygalacturonic acid by the pectic enzymes produced by *Collectotrichum dematium* f.sp. *truncata* on soybean epicotyls and seed exudate culture was carried out according to the method described by Page (1961).

**Gel filtration, isoelectric focusing and ion exchange chromatography :**

Gel filtration chromatography was used to determine the molecular weights of endo PG produced by *Collectotrichum dematium f.sp truncata* on soybean seedlings and in culture (Andrews, 1964). A column (2 cm width x 40 cm high) of Sephadex G-75 was equilibrated with 0.1 M KCl in 0.01 M sodium acetate buffer (pH 5.3) at 4°C. Four milliliters of the enzyme preparation from seedlings or cultures were applied to the column. In other experiment, the two enzymes preparation were mixed and then applied to the column. The endo PG was eluted from the column by passing the above salt-buffer mixture through the column at a flow rate of 1 ml/hr. Fractions (2 ml) were collected. Blue dextran (M.W. 2, 000,000), bovine serum albumin (M.W. 67, 000), ovalbumin (M. W. 45,000) and a chymotrypsinogen (M.W. 12, 500) were used as standards. An LKB 8100 ampholine electrofocusing apparatus equipped with a 110-ml column containing pH 3-10 amboline carries in a sucrose gradient, was used to determine the isoelectric points of the endo PG produced on soybean seedlings and in cultures.

Six milliliters of one of the enzyme preparations was applied to the column. For each determination, the voltage was initially set at 100 v and raised from 100 v/day to 300 v. After 2 days at 300 v, 2 ml fractions were collected at a flow rate of 1 ml per min. The pH and endo PG activity of each fraction were determined. A column (1.4 cm x 20 cm) of diethylaminoethyl cellulose in the hydrogen form and equilibrated with 0.005 M Tris-HCl buffer (pH 8.4) at 4°C was used for ion exchange chromatography. For each determination 4 ml of enzyme sample containing 0.1 ml of 0.1 M Tris-HCl buffer (pH 8.4) was passed through the column and the sample was then eluted with a linear gradient of NaCl Tris buffer at 4°C. The flow rate was 30 ml/hr. Fractions (2 ml) were collected and assayed for endo PG.

## RESULTS

Physical separation and pH optima for activity of forms of endopolygalacturonase (endo PG) produced in culture and in soybean stems infected by *Collectotrichum dematium f.sp. truncata* were investigated. In Figs. (A-F) activity in each of the 2-ml fractions was determined viscometrically by using 0.9% sodium polypectate in 0.1 M acetate buffer (pH 5.3) as substrate.

### Optimum pH for activity :

The optimum pH for endo PG activity was determined in seed exudate culture or extracted from soybean epicotyl. Both enzyme preparations were assayed visco-

metrically at a range of pH values between 3.8 and 6.6. Both the polygalacturonase extracted from soybean seedlings 48hr after inoculation and that from filtrates of cultures grown on seed exudates had pH optima of 5.3 (Fig. A).

#### Reaction products :

The sequence of reaction products released from polygalacturonic acid by the polygalacturonase produced in culture was determined with paper chromatography. Two culture enzyme preparations were used. One consisted of the dialyzed filtrate obtained 24 hr after 0.1% sodium polyacetate was added to mycelial disks. Pectolytic activity in both culture preparations as found with diseased tissues (Lorenzo *et al.*, 1990), released polymers rather than monomers from polygalacturonic acid as the initial reaction products. As reported previously, approximately 0.5% hydrolysis was sufficient to reduce viscosity by 50% (Brookhouser and Weinfeld, 1979). Together, these data indicate that the polygalacturonase produced by *Collectotrichum dematium* f.sp *truncata* on soybean epicotyls and in the liquid culture both attacked polygalacturonic acid in an endo manner.

#### Gel filtration chromatography :

Gel filtration chromatography with a column of Sephadex G-75 type revealed that the endo PG produced by the fungus in seed exudate-culture and the endo PG extracted from infected epicotyls had similar molecular weights. A single peak of activity was detected in approximately the same fraction when the polygalacturonase from these two sources was applied separately or mixed (Fig. B). From the elution volumes of blue dextran, bovine serum albumin, ovalbumin, chymotrypsinogen and the endo PG, it was estimated that the molecular weight of the endo PG produced in the plant and in culture was about 42,000 daltons.

The partition coefficient ( $K_{av} = (V_e - V_o) / (V_t - V_o)$  in which  $V_e$  = elution volume,  $V_o$  = void volume, and  $V_t$  = total volume of the gel bed] was 0.155 for the polygalacturonase from these two sources.

#### Isoelectric focusing :

Isoelectric focusing was used to compare the endo PG produced by *Collectotrichum dematium* f.sp *truncata* in flasks containing soybean seed exudates as the inducer with the endo PG extracted from inoculated soybean seedling. The endo PG produced by the fungus in response to soybean seed exudates had a single isoelectric

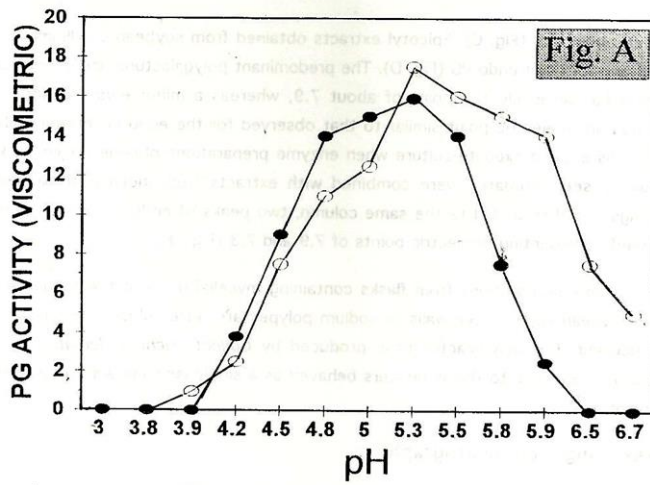


Fig. A. polygalacturonase (PG) activity as a function of pH. Enzyme preparation were dialyzed extracts obtained from soybean seedlings 20 hr after inoculation (●-●), and filtrates obtained from cultures 24 hr after soybean seed exudates were added to mycelial disks of *Collectotricum dematium f. sp. truncata* ○-○

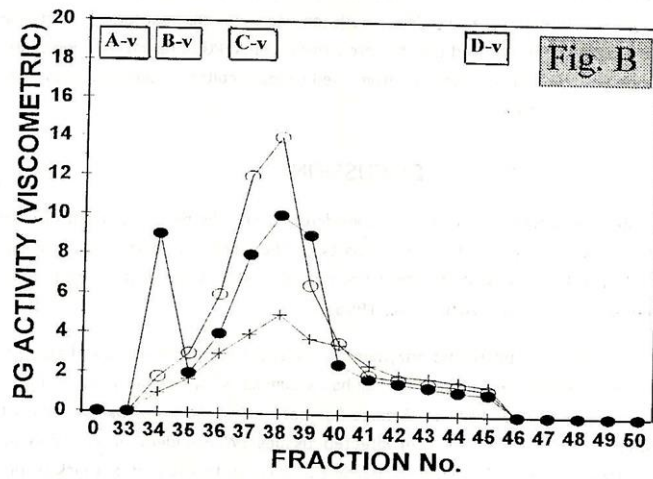


Fig. B. Mobility of endo PG in Sephadex G-75. The enzyme preparation applied to the column were extracts prepared from soybean epicotyls 20 hr after inoculation with *Collectotricum dematium f. sp. truncata* (+--+); filtrate obtained from cultures 24 hr after soybean seed exudates were added to mycelial disks of the fungus (○-○); and a mixture of the above two preparations (●-●). Two milliliter fractions were collected at a flow rate of 7 ml/hr. The markers A,B,C and D represent the elution volumes of blue dextran, bovine serum albumin, ovalbumin, and  $\alpha$ -chymotrypsinogen, respectively; were used as standard.

point of about 7.3 (Fig. C). Epicotyl extracts obtained from soybean seedlings contained two forms of endo PG (Fig. D). The predominant polygalacturonase in epicotyl extracts had an isoelectric point of about 7.9, whereas a minor endo PG activity peak had an isoelectric point similar to that observed for the endo PG produced by the fungus in seed exudate-culture when enzyme preparations obtained from flasks containing seed exudates were combined with extracts from inoculated soybean seedlings and then added to the same column, two peaks of endo PG activity were observed, representing isoelectric points of 7.9 and 7.3 (Fig. E).

Enzyme preparations from flasks containing mycelial disks of the fungus and either soybean epicotyl cell walls or sodium polypectate were subjected to isoelectric focusing. The polygalacturonase produced by *Collectotrichum dematium f.sp truncata* in response to these inducers behaved as a single isozyme with an isoelectric point of 7.3.

#### **Ion-exchange chromatography :**

With ion exchange chromatography, the predominant endo PG in extracts from soybean seedlings eluted well before the endo PG in filtrates from seed exudate cultures. When the enzyme preparations from these two sources were mixed, applied to a column, and eluted with the same NaCl gradient, two distinct peaks of endo PG activity were detected (Fig. F). Thus, both ion exchange chromatography and isoelectric focusing demonstrated that the predominant endo PG obtained from inoculated seedlings and the endo PG obtained from seed exudate-culture apparently possess different ionic properties.

## **DISCUSSION**

Soybean anthracnose disease is considered among the most important diseases which attack soybean plants in many parts of the world including Egypt (Sinclair, 1982, El-Wakil, 1985; Morrall, 1988; Koch *et al.*, 1989; Warther and Elrod, 1990; Ahmed Saieda, 1994 and Attia *et al.*, 1995).

Evidence that pectolytic enzymes are essential for penetration and colonization of plant tissues by pathogenic fungi has accumulated but remains circumstantial. For example, extent of tissues damage has been shown to be of parallel levels to pectolytic enzymes isolated from infected tissues (Wijesundera *et al.*, 1984 and Perez Artes and Tena, 1994). An important problem in this regard is a lack of phy-



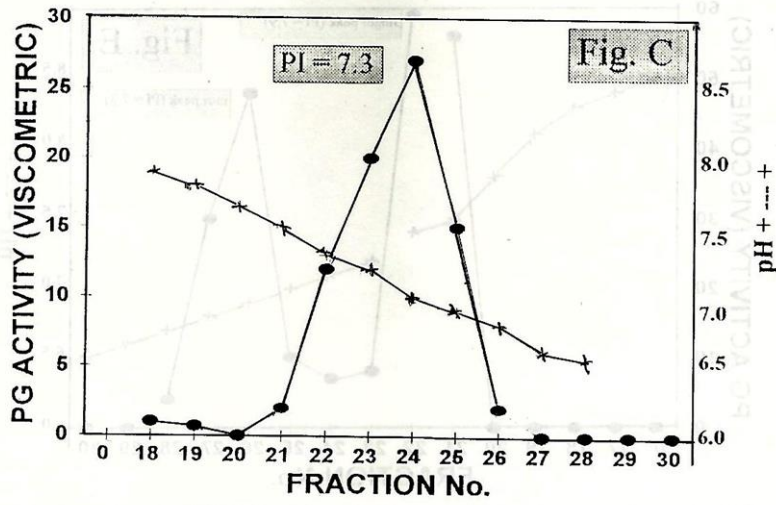


Fig. C. Isoelectric focusing of endo PG produced in culture by *Collectoricuhum dematium f.sp. truncata* in response to soybean seed exudates.

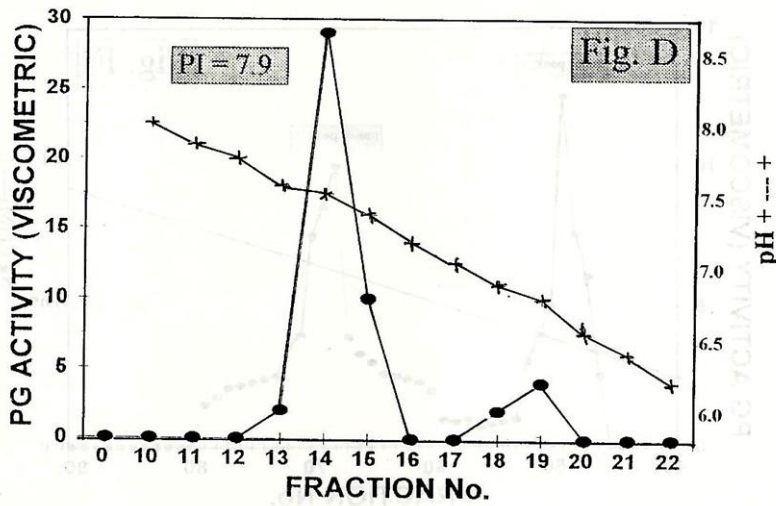


Fig. D. Isoelectric focusing of endo PG extracted from soybean epicotyls 20 hr after inoculation with *Collectoricuhum dematium f.sp. truncata*.

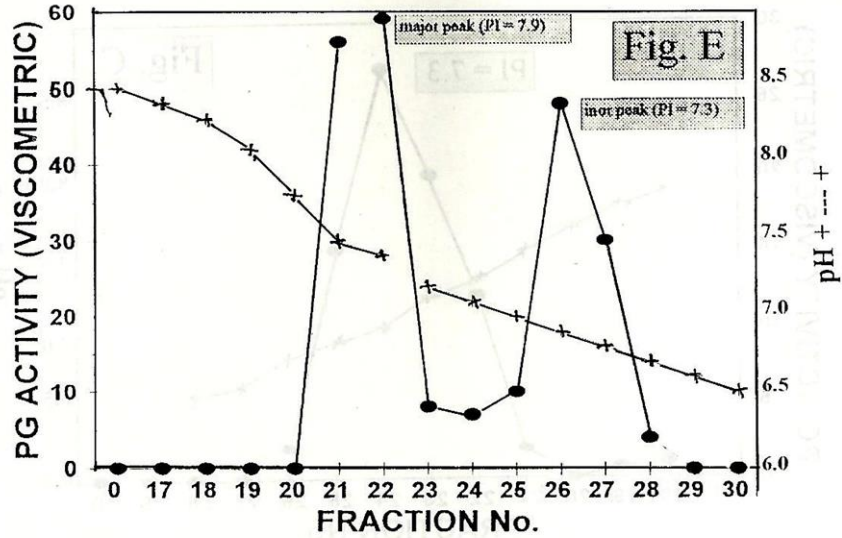


Fig. E. Isoelectric focusing of a mixture of polygalacturonase by *Collectoricuham dematium f.sp. truncata* in response to seed exudates in culture and polygalacturonase extracted from soybean epicotyl 20hr after inoculation with the pathogen.

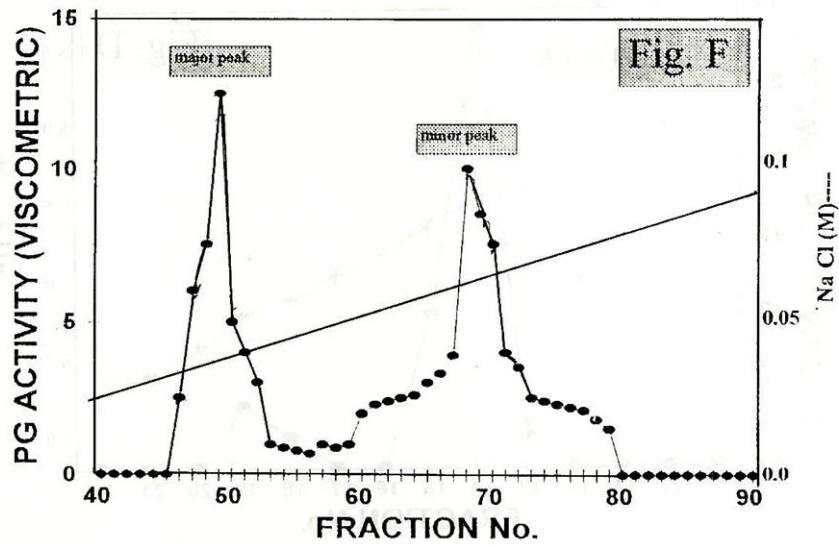


Fig. F. DEAE cellulose chromatography of a mixture of polygalacturonase produced by *Collectoricuham dematium f.sp. truncata* in response to seed exudates in culture and polygalacturonase extracted from soybean epicotyls 20hr after inoculation with the pathogen.

topathogenic fungi that have been intensively studied by using the tools offered by modern genetics and molecular biology. We have demonstrated here that it is possible to study the regulation of pectolytic enzymes in *Collectotrichum dematium f.sp truncata*, a pathogen that is closely related to a number of phytopathogenic fungi.

In order for a fungal pathogen to penetrate into a plant, several structural barriers must be breached. The outermost structural barrier is cutin, consisting of esterified hydroxy fatty acids with the help of cutinase produced by the pathogen (Holz and Knox-Davies, 1985). Immediately beneath the cutin is a pectinaceous layer.

Fungal and bacterial phytopathogens produce a variety of extracellular enzymes that degrade pectin and other complex carbohydrate barriers in the cell wall of plant (Bateman and Basham, 1976). The action of many of these enzymes has been associated with the virulence and the role of such enzymes has been demonstrated for bacterial pathogens (Cooper, 1983). However, there has been, up to this time, no direct evidence that a glycosidase is essential for either successful penetration of the host epidermis or the intercellular growth within the host by fungal pathogens. There has been, however, indirect evidence that pectinase production might be essential for the penetration of *Collectotrichum dematium f.sp truncata* into its host (Lorenzo *et al.*, 1990).

The endo PG produced by *Collectotrichum dematium f.sp truncata* in response to soybean exudates in culture was similar to the endo PG extracted from infected soybean epicotyls on the basis of catalytic properties and molecular weight. However, ion exchange chromatogram and isoelectric focusing techniques revealed that the predominant endo PG produced in the plant had different ionic properties from that produced in culture. These results suggested that *Collectotrichum dematium f.sp truncata* produced two molecular forms of endo PG whose production was dependent upon the conditions of growth. It is particularly important that the same form of endo PG was produced in liquid culture regardless of whether the inducing materials were seed exudates, isolated soybean epicotyl cell walls or pectate.

Dichotomies between the forms of individual types of pectic enzymes produced in culture and during pathogenesis by plant pathogens were observed in several investigations. For example, Pupillo *et al.* (1976) discovered that several strains of *Erwinia chrysanthemi* produced a molecular form of an acid endopectate lyase in infected tissues not produced in culture. Studies with *Fusarium solani f.sp. cucurbitae*

also revealed differences in the isoelectric points and certain other properties between the forms of endopectate lyase produced in culture and infected tissue (Hancock, 1976).

While the present study indicates that the predominant molecular form the endo PG produced by *Collectotrichum dematium f.sp truncata* in infected tissue possesses a significantly different isoelectric point from the molecular forms of endo PG produced in culture it is possible that post synthetic structural modification accounts for these differences. However, since similar purification techniques were applied to enzymes from culture and infected tissue, it is doubtful that these procedures would alter their properties in the sense discussed by other workers (Swinburne and Corden, 1969; Wijesundera *et al.*, 1984 and Abdel Alim, 1996). Definitive answers to these questions will require genetic studies and further detailed work on the molecular nature of the two endo PG variants.

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دراسة خصائص انزيم الاندوبولى جلاكتويورينيز المنتج بواسطة  
الفطر *Colletotrichum dematium f.sp. truncata* المسبب لمرض  
انتراكونوز فول الصويا

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يعتبر فول الصويا من أهم المحاصيل البقولية ليس فقط على المستوى المحلى بل أيضا على المستوى العالمى، ويصاب هذا المحصول بعدد من الأمراض التى تؤثر على إنتاجيته.

تم عزل الفطر *Colletotrichum dematium f.sp. truncata* من أوراق وسيقان وبذور نباتات فول الصويا والتى تم جمعها من منطقة الجيزة، وأمكن عزل الفطر بنسبة عالية من بذور فول الصويا.

تمت الدراسة على مدى إنتاجية هذا الفطر لانزيم الاندوبولى جلاكتويورينيز (endo PG) فى عينتين مختلفتين الأولى عبارة عن مزرعة من مستخلص بذور فول الصويا السليمة والثانية من البادرات المصابة بالفطر وذلك بعد ٣٠ ساعة من العدوى. وقد أوضحت النتائج المتحصل عليها أن درجة ال pH المثلى لانزيم (endo PG) المنتج من العينات كانت ٥,٣ كما أن الوزن الجزئى له كان ٤٢ ألف كيلو دالتون.

أوضحت النتائج المتحصل عليها أن هناك اختلاف واضح فى إنتاجية الفطر للانزيم الاندوبولى جلاكتويورينيز (endo PG) وذلك فى كل من المزرعة السليمة والبادرات المصابة (وبتوظيف طريقة) Isoelectric focusing وجد أن انزيم (endo PG) المنتج بواسطة الفطر فى مستخلص البذور السليمة يعطى نقطة تعادل كهربى (PI) مقدارها ٧,٣ ولكن الانزيم (endo PG) المنتج من الفلقات المصابة يعطى نوعين من الأشكال (الإنزيمية) الأول له نقطة تعادل كهربائى (PI) مقدارها ٧,٩ والأخرى فه نقطة تعادل كهربائى (PI) ٧,٣ وكان الأول هو الأنزيم الرئيسى وقد وضع هذا من الاختلاف بين العينات المصابة والسليمة فى إنتاجية ونشاط الانزيم، وقد ظهر أيضا هذا الاختلاف السابق ذكره فى التجارب الخاصة بالتنقية لمحاليل الانزيم وذلك باستخدام كروماتوجرافى التبادل الأيونى حيث بينت النتائج المتحصل عليها من هذه الدراسة ان الفطر ينتج أشكالا مختلفة من الانزيم (endo PG) وأن الخصائص الأيونية للإنزيم الرئيسى فى العينات المصابة تختلف عن الانزيم المستخلص منه فى العينات السليمة الغير معدية وهذا يوضح أن الفطر *Colletotrichum dematium f.sp. truncata* له القدرة على إنتاج انزيم الاندولولى جلاكتويورينيز وأن هذا الانزيم يلعب دورا هاما فى ميكانيكيات احداث الأصابة وتدهور نباتات فول الصويا المصابة بالفطر.