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PRODUCTION OF CELL WALL DEGRADING ENZYMES BY *CORYNESPORA CASSIICOLA* IN CULTURE AND INFECTED RUBBER TISSUE

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SUMMARY

All isolates of Corynespora cassiicola *used in the investigation secreted pectin lyase when grown in liquid medium with pectin as the main source of carbon. However, none of the isolates secreted polygalacturonase in the same medium. The extracts of rubber tissue inoculated and infected with* C. cassiicola *also showed only pectin lyase activity. When grown in liquid medium with carboxymethyl cellulose as the main source of carbon all isolates secreted cellulases viz. cellobiase and β - glucosidase with a markedly high production in C_1 and C_4 isolates. Infected rubber leaf tissue with* C. cassiicola *also had a high activity of both cellobiase and β - glucosidase. The results of this study suggest that principal pectic enzyme involved in pathogenesis of the rubber isolate of* C. cassiicola *is pectin lyase and in the later stages cellulolytic enzymes possibly play a major role.*

Key words: cellulolytic enzymes, *Corynespora cassiicola, Hevea brasiliensis*, pectic enzymes

INTRODUCTION

Corynespora cassiicola (Berk & Curt) Wei is an ubiquitous fungus which affects more than 60 hosts in all over the world (Ellis & Holliday, 1971). During the late 1980's this pathogen has spread in rubber plantations of South East Asia in epidemic proportions causing die-back of selective clones. Today, nearly a decade after the first epidemic, several outstanding rubber clones in India (Rajalakshmy & Kothandaraman, 1996), Indonesia (Sinulingga *et al.*, 1996), Ivory Coast (Breton, *et al.*, 1996), Malaysia (Shamsul Kamar A. Shukor & Shamsuri Mohd. Hidir, 1996), Sri Lanka (Jayasinghe & Silva, 1996) and Thailand (Rodesuchit & Kajornchaiyakil, 1996) have been severely affected and the fungus *C. cassiicola* has become a potential threat to the natural rubber industry in the world (Jayasinghe, 1997). The biology and the management of the fungus are well documented (Chee, 1987; Liyanage,

1988; Liyanage *et al.*, 1989; Darmono *et al.*, 1996; Breton *et al.*, 1996; Ramli Othman *et al.*, 1996) and involvement of a toxin in pathogenicity has also been studied extensively (Onesirosan *et al.*, 1975; Liyanage & Liyanage, 1986; Breton *et al.*, 1996; Situmorang *et al.*, 1996). However, no literature is available on the role of enzymes in host pathogen interactions except for a short communication on production of pectic enzymes by papaya isolate of *C. cassiicola* (Agarwal & Gupta, 1978).

The production of cell wall degrading enzymes by plant pathogenic fungi has been shown by many workers (English *et al.*, 1971; Agarwal & Gupta, 1978; Wijesundera *et al.*, 1984; Kanakaratne & Adikaram, 1990; Senaratna *et al.*, 1991; Cam *et al.*, 1994) and these enzymes have been shown to play a major role in the penetration and development in several plant diseases (Wood, 1960; Bateman & Miller, 1966; Bateman & Basham, 1976; Byrde & Archer, 1977; Collmer & Keen, 1986; Wijesundera *et al.*, 1989 and Walton, 1994).

The present study reports pectolytic and cellulolytic enzyme production by rubber isolates of *C. cassiicola* both in culture and in infected rubber tissue. Observations for the rubber isolates of *Colletotrichum gloeosporioides* is also reported here for comparison purposes as enzyme production in *C. gloeosporioides* is well documented (Prusky *et al.*, 1989; Sivanathan & Adikaram, 1990; Kanakaratne & Adikaram, 1990; Senaratne *et al.*, 1991 and Wijesundera, 1994).

MATERIALS AND METHODS

The organism

Four *Corynespora cassiicola* isolates; C_1, C_2, C_3 and C_4 used in this study were obtained from diseased *Hevea* leaves. All cultures were derived from single conidia and maintained on potato dextrose agar (PDA) at 28±2°C. *Colletotrichum gloeosporioides* culture was obtained from diseased leaves of *Hevea brasiliensis* clone PB 86.

Enzyme production in culture

All isolates were grown in ammonium tartrate liquid medium described by Byrde & Fielding (1968). Citrus pectin (Sigma) was used as the main source of carbon for pectolytic enzyme studies where as carboxymethyl cellulose (Sigma) was used for cellulolytic enzyme studies. Twenty ml of the liquid medium was dispensed in 250 ml Erlenmeyer flasks and each flask was inoculated with 1 cm^2 block obtained from the peryphery of 6-day old cultures of the isolates growing on PDA at 28±2°C.

All inoculated liquid cultures were harvested by filtering through Whatman No. 1 filter papers. The resulting culture filtrate was stored at 0°C and used to detect enzyme activity.

Enzyme production in host tissue

Young detached leaves of *Hevea brasiliensis* clones RRIC 100 & RRIC 104 were inoculated with either *Corynespora cassiicola* or *Colletotrichum gloeosporioides* spores. *Corynespora* spores were obtained by brushing naturally infected lesions from rubber leaves while *C. gloeosporioides* were from 7-day old cultures grown on PDA. Six drops of an aqueous conidial suspension (.02 ml, 1 x 10^5 spores per ml) of the fungus were placed on either sides of the mid rib of each leaf. The inoculated leaves were incubated up to 4-days at 28 ± 2°C under 100% R.H. Leaves inoculated with sterile distilled water served as controls.

Inoculated leaf tissue were harvested at 24 h intervals. Three grams of leaf tissue including both inoculated sites and the healthy margins were collected and extracted according to Fielding (1981). Tissue was ground in a chilled motar and pestle in 10 volumes (v/w) of .1 M tris HCl - buffer (pH 7.6), Cysteine hydrochloride 10 mgl^{-1} and 1.0 M NaCl and left at 4°C for 1 h. Then it was filtered using a few layers of muslin and the filtrate centrifuged at 5000 rpm for 5 minutes. The supernatent was used to determine the enzyme activity.

Determination of enzyme activity

The agar plate method of Dingle *et al.* (1953) and viscosity reduction method employed by Nema (1992) were used to determine the polygalacturonase (PG) activity. In viscosity reduction method the reaction mixture contained 10 ml of 1% sodium polypectate (Sigma) in 0.1 M sodium acetate buffer (pH 5) and 6 ml of culture supernatant. Percent loss in viscosity was determined using ubbelhode (Technico) viscosity meter. Enzyme activity is expressed as arbitrary units.

Thiobarbituric acid method (Ayers *et al.*, 1966; Wijesundera *et al.*, 1984) was used to assay pectinlyase (PL) activity. (In both PG & PL assays sampling were done upto 23 days in culture media and 4 days on host tissue). Enzyme activity is expressed as arbitrary units.

Cellulolytic enzymes, ß-glucosidase and cellobiase activities were measured by the hydrolysis of the chromatogenic substrates, þ - nitrophenyl - ß - D - glucopyranoside and þ-nitrophenyl - ß - D - cellobioside respectively (Byrde & Fielding, 1968; Wijesundera *et al.*, 1984; Senaratne *et al.*, 1991). The colour of the phenate ion was estimated by measuring the absorbance at 403 nm. (Sampling were

done upto 36 days in liquid medium and 4 days on host tissue respectively). Enzyme activity is expressed as arbitrary units.

RESULTS AND DISCUSSION

Pectolytic enzymes

Time course of enzymes secretion

When grown in ammonium tartrate liquid medium with citrus pectin as the main carbon source, polygalacturonase (PG) was not detected in any of the four *C. cassiicola* isolates (C_1,C_2, C_3 & C_4) whereas the reference culture, *C. gloeosporioides* produced PG from the 2nd day onwards. However, all the four *C. cassiicola* isolates and *C. gloeosporioides* showed a markedly high pectinlyase (PL) activity from the 4th day onwards (Fig. 1). The activity of PL varied among *C. cassiicola* isolates. In the isolates; C_1, C_2, C_3 & C_4, the maximum PL activity was detected on the 7th or 8th day while in *C. gloeosporioides* (the reference culture), two peaks were detected on the 7th and 16th days.

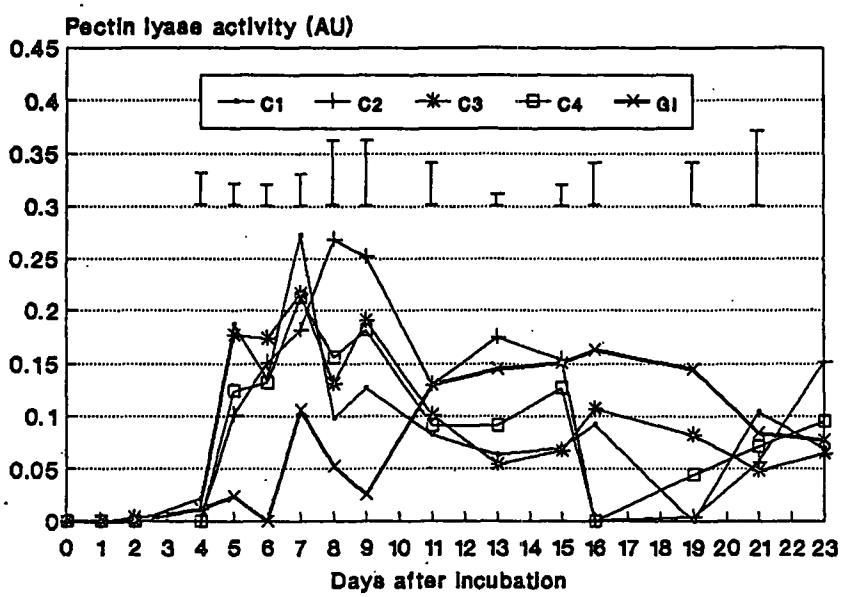

Fig. 1. Pectin lyase production by four isolates of *C. cassiicola*; C_1,C_2,C_3,C_4 and *C. gloeosporioides* (reference culture) in ammonium tartrate liquid medium with citrus pectin as the main source of carbon at room temperature. AU-Arbitrary units

Enzyme production in host tissue

The extracts of the *Hevea* leaves of clone RRIC 100 and 104 inoculated either with *C. cassiicola* or *C. gloeosporioides* obtained at 24h intervals for a period of four days, did not show any PG activitiy at any stage when assayed by both cup-plate method and viscometry. Pectin lyase activity was detected in the leaves inoculated with *C. cassiicola* as well as with *C. gloeosporioides*. Affected tissue with both fungi showed a markedly high PL activity reaching a maximum after 96 h of incubation (Table 1). PG and PL were not detected in extracts of the control leaves.

Table 1. *Pectolytic enzyme activity in extracts of rubber leaf infected with* C. cassiicola *(observations for* C. gloeosporioides *on rubber is also included).*

Incubation period (h)	Pectinlyase activity @ (AU)			
	Hevea infected with *C. cassiicola*		*Hevea* infected with *C. gloeosporioides*	
	RRIC 100	RRIC 104	RRIC 100	RRIC 104
0*	.01 ± 0	.01 ± 0	.01 ± 0	.01 ± 0
24	.013 ± .002	.01 ± 0	.01 ± 0	.01 ± 0
48	.063 ± .002	.01 ± 0	.008 ± .002	.065 ± .003
72	.063 ± .002	.073 ± .003	.055 ± .003	.055 ± .003
96	.075 ± .003	.004 ± .002	.078 ± .002	.048 ± .002

No polygalacturonase activity was detected on *Hevea* infected with any of the isolates of *C. cassiicola*

*, Healthy leaves
@, Absorbance at 550 nm
AU, Arbitrary units

The inability to detect PG secretion in the ammonium tartrate liquid medium and on infected tissue by rubber isolate of *C. cassiicola* probably indicates that the rubber isolates of the pathogen does not have the ability to secrete this enzyme. In a study with the papaya isolate of *C. cassiicola,* Agarwal & Gupta (1978) showed the involvement of PG as the principal pectic enzyme in the pathogenesis. This contradiction may be due to the differences in the types of isolates used in both studies. High levels of PL enzyme activity shown by all *Hevea* isolates of *C. cassiicola* suggest a major role for PL in disease development.

Cellulolytic enzymes

Time course of enzyme production

All the four isolates of *C. cassiicola* & *C. gloeosporioides* produced cellobiase and ß - gllucosidase in ammonium tartrate liquid medium (Figs. 2 & 3). However, in *C. cassiicola*, the activity of both the enzymes varied with different isolates. Isolates C_1 & C_4 of *C. cassiicola* secreted the highest amount of cellobiase & ß - glucosidase. *Colletotrichum gloeosporioides* showed the highest activities on 14th and 30th day for glucosidase and cellobiase. Production of cellulases by *C. gloeosporioides* in culture has been shown by various workers (Kanakaratne & Adikaram, 1990; Senaratne *et al.*, 1991).

C. cassiicola isolates C_1 & C_2 showed two peaks of activity *viz*. 20th and 30th days for both cellobiase and ß - glucosidase. (In the isolate C_2, the maximum activity was on the 30th day for cellobiase & ß - glucosidase as well). In C_3, the peak was detected on 18th and 30th day after incubation for cellobiase and on 10th & 18th day for ß - glucosidase. In C_4, the maximum activity was observed on the 33rd day for both cellobiase and ß - gllucosidase.

It is very likely that the isolates C_1 & C_3 from *C. cassiicola* produce several forms of cellobiase and ß - glucosidase. *C. cassiicola* isolates C_2 & C_4 seems to be producing only one form of each enzyme.

Cellulolytic enzyme production in host tissue

In the *C. cassiicola* infection, considerably high amounts of both cellulolytic enzymes were present compared to *C. gloeosporioides* infection. Enzymes were detected on the inoculated leaves of highly susceptible clone RRIC 104 after 24 h of incubation whereas on the leaves of RRIC 100, a clone regarded as resistant, the enzymes were detected only after 48 h of incubation (Table 2). Therefore, it seems that cellulolytic enzyme production commences early in the susceptible clones.

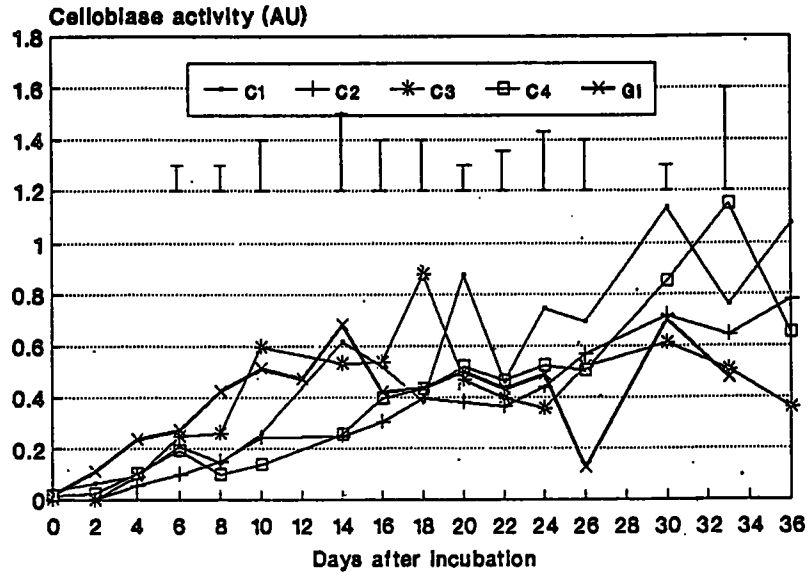

Fig. 2. Cellobiase production by four isolates of *C. cassiicola*; C_1,C_2,C_3,C_4 and *C. gloeosporioides* in ammonium tartrate liquid medium with carboxy-methyl-cellulose as the main source of carbon at room temperature. AU-Arbitrary units

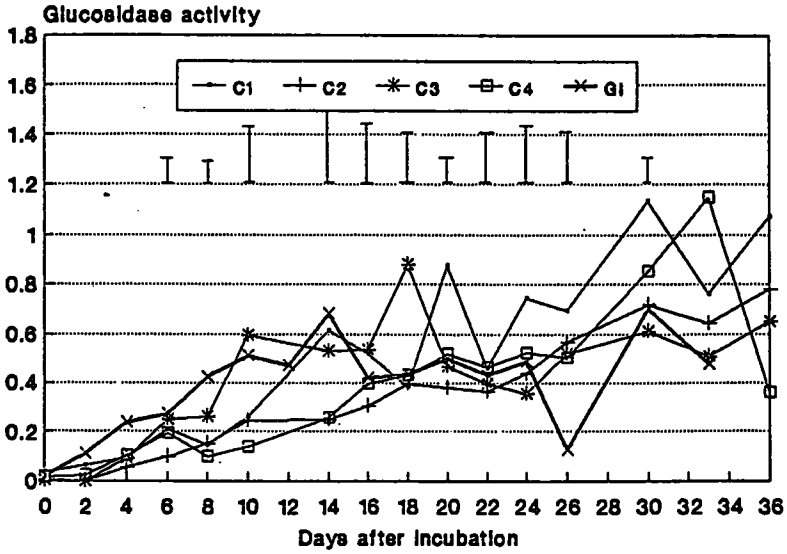

Fig. 3. ß-glucosidase production by four isolates of *C. cassiicola*; C_1,C_2,C_3,C_4 and *C. gloeosporioides* in ammonium tartrate liquid medium with carboxy-methyl-cellulose as the main source of carbon at room temperature. AU-Arbitrary units

Table 2. *Cellobiase activity in extracts of rubber leaf infected with* C. cassiicola *(observations for* C. gloeosporioides *on rubber is also included)*

Incubation period (h)	Cellobiase enzyme activity @ (AU)			
	Hevea infected with *C. cassiicola*		*Hevea* infected with *C. gloeosporioides*	
	RRIC 100	RRIC 104	RRIC 100	RRIC 104
0*	0	0	0	0
24	0	.043 ± .002	0	0
48	.02 ± 0	.013 ± .002	0	.028 ± .004
72	.058 ± .004	.02 ± 0	.02 ± 0	0
96	.073 ± .002	.03 ± .004	0	0

*, Healthy leaves
@, Absorbance at 403 nm
Au, Arbitrary units

As in the table 3, ß - glucosidase was not detected in healthy tissue during our study. However, small quantities of ß - glucosidase, the enzyme responsible for the cleavage of cyanogen has been reported from *Hevea* leaf tissue (Jayasinghe & Wijesundera, unpublished). This disparaty may be due to the differences in the spectrophotometer used in this study. Jayasinghe & Wijesundera (unpublished) have used a highly sensitive spectrophotometer, GBC UV/vis 914 while Corning colorimeter 253 (manual) was used in our study. The activity of both enzymes increased markedly following the infection of both *C. cassiicola* and *C. gloeosporioides*. The detection of the enzymes of ß - glucosidase following infection may be due to a host reaction towards injury or due to the enzyme produced by the pathogen. Breakdown of cell wall components due to cellulolytic enzymes of phytopathogenic fungi have been shown as far back in 1957 (Husain & Kelman, 1957; Kelman & Cowliny, 1965 and Cooper, 1983).

Table 3. *β - glucosidase activity in extracts of rubber leaf infected with* C. cassiicola. *(Observations for* C. gloeosporioides *is also included)*

Incubation period (h)	ß-glucosidase enzyme activity @ (AU)			
	Hevea infected with *C. cassiicola*		*Hevea* infected with *C. gloeosporioides*	
	RRIC 100	RRIC 104	RRIC 100	RRIC 104
0*	0	0	0	0
24	0	.043 ± .002	0	0
48	.02 ± 0	.013 ± .002	0	.028 ± .004
72	0.58 ± .004	.02 ± 0	.02 ± 0	0
96	.073 ± .002	.03 ± .004	0	0

*, Healthy leaves
@, Absorbance at 403 nm
Au, Arbitrary units

It is likely that the principal enzyme involved in pathogenesis in *C. cassiicola* of *Hevea* is PL and in the latter stages of infection, cellulolytic enzymes probably play a major role.

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