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CELL WALL DEGRADING ENZYME SECRETION BY *CYLINDROCLADIUM QUINQUESEPTATUM*

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ABSTRACT

Cylindrocladium quinqueseptatum *causes leaf fall of clove in Sri Lanka and is responsible for the leaf spots of rubber in Malaysia. Only one isolate (Kp) from four pathogenic isolates examined secreted polygalacturanase (PG) when grown in liquid media. None of the isolates secreted pectin lyase (PL). The extracts of clove and rubber leaf tissues, inoculated with the Kp isolate did not show any PG and PL activity. The leaf tissue did not have the ability to inhibit* C. quinqueseptatum *PG produced in culture. All isolates of* C. quinqueseptatum *secreted cellulases* viz. *cellobiase and β-glucosidase in culture. Cellobiase was detected in the inoculated rubber leaves. The levels of β-glucosidase, an inherent enzyme of* Hevea *leaves, also increased rapidly following infection. The results indicate that pectic enzymes play a minimal or no role in disease development of* C. quinqueseptatum *infection in cloves and* Hevea. *Thus cellulolytic enzyme probably play a nutritive role.*

Key words: cellulolytic enzymes, *Cylindrocladium quinqueseptatum* *Eugenia caryophyllata*, *Hevea brasiliensis*, pectolytic enzymes

INTRODUCTION

Cylindrocladium quinqueseptatum Boedijn & Reitsma (telemorph: *Calonectria quinqueseptata* Figueiredo & Namekata) causes seedling blight and extensive defoliation in *Eucalyptus* spp. (Figueiredo & Namekata, 1967; Pitkethley, 1976; Sharma & Mohanan, 1982) and in *Eugenia caryophyllata* (Reitsma & Sloof, 1950; Sarma & Nambiar, 1978; Wilson *et al.*, 1979; Jayasinghe & Liyanage, 1982), in humid tropics. The fungus also causes leaf spots and defoliation in *Hevea brasiliensis* (Anon, 1972; Kaiming, 1987), *Annona squimosa* (Figueiredo & Namekata, 1967), *Terminalia paniculata* (Mohanan & Sharma, 1985) and *Camellia sinensis* (Peerally, 1974).

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Plant pathogenic fungi produce many types of cell wall degrading enzymes *in vitro* (Agarwal & Gupta, 1978; Wijesundera *et al.*, 1984, 1989; Kanakaratne & Adikaram, 1990; Arun-Arya & Arya, 1991; Nema, 1992) and *in vivo* (English *et al.*, 1971; Agarwal & Gupta, 1978; Prusky *et al.*, 1989; Senaratna *et al.*, 1991; Cam *et al.*, 1994). These enzymes have been shown to play a major role in the penetration and development of several plant diseases (Wood, 1960; Bateman & Miller, 1966; Bateman & Basham, 1976; Byrde & Archer, 1977; Collmer & Keen, 1986; Wijesundera *et al.*, 1989; Walton, 1994).

The present study reports pectolytic and cellulolytic enzyme production by clove isolates of *C. quinqueseptatum* both in culture and in infected clove and rubber tissues. Observations on the rubber isolate of *Colletotrichum gloeosporioides* is also reported here for comparison purposes as enzyme production in *C. gloeosporioides* has been subjected to extensive studies (Prusky *et al.*, 1989; Sivanathan & Adikaram, 1989; Kanakaratne & Adikaram, 1990; Senaratna *et al.*, 1991; Wijesundera, 1994).

MATERIALS AND METHODS

The organism

Four single spore isolates of *Cylindrocladium quinqueseptatum*, IMI 342173 (Rt), 359378 (Aw), 359379 (Rw) and 359380 (Kp) obtained from diseased cloves in four different localities in Sri Lanka were used. *Colletotrichum gloeosporioides* culture was obtained from diseased leaves of *Hevea brasiliensis* clone PB 86.

Enzyme production in culture

All the isolates were grown in ammonium tatrate liquid medium described by Byrde and Fielding (1968). Citrus pectin (Sigma) was used as the main source of carbon for pectolytic enzyme studies whereas carboxymethyl cellulose (Sigma) was used for cellulolytic enzyme studies. Twenty ml of the liquid medium was dispensed in to 200 ml Erlenmeyer flasks and each flask was inoculated with 1 cm^2 block obtained from the periphery of 7-day old cultures of the isolates growing on CDA at 28 ± 2°C. All inoculated liquid cultures were incubated at 28 ± 2°C under natural light and dark regimes without shaking. The cultures were harvested by filtering through Whatman No. 1 filter paper. The resulting culture filtrate was stored at 0°C and used to detect enzyme activity.

Enzyme production in host tissue

Young detached leaves of *Eugenia caryophyllata* and *Hevea brasiliensis* clone

RRIC 100 were inoculated with either *C. quinqueseptatum* (Kp isolate) or *C. gloeosporioides*. Six drops of an aqueous conidial suspension (0.02 ml, 1 x 10^5 spores ml^{-1}) prepared from seven day old cultures of the fungus were placed on either side of the midrib of each leaf. The upper surface of the leaf was selected for inoculation. The inoculated leaves were incubated upto 4 days at room temperature under 100% RH. Leaves inoculated with sterile distilled water served as controls.

Inoculated leaf tissues were harvested at 24 h intervals. Three grams of leaf tissue including both inoculated sites and the healthy margins were collected and leaf extracts obtained according to Fielding (1981). Tissues were ground using in a chilled mortar and pestle in 10 volumes (v/w) of 0.1 M tris-HCl buffer (pH 7.6), cysteine hydrochloride 10 mg l^{-1} and 1.0 M NaCl and left at 4°C for 1 h. It was then filtered using few layers of muslin and the filtrate centrifuged at 5000 rpm for 5 minutes. The supernatent was used to determine 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 15 ml of 1% sodium polypectate (Sigma) in 0.1 M sodium acetate buffer (pH 5) and 6 ml of culture supernatent. 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 pectin lyase (PL) activity. In both PG and PL assays sampling was done upto 18 days in culture media and upto 4 days on host tissue. Enzyme activity is expressed as arbitrary units.

Cellulose degrading enzymes, β-glucosidase and cellobiase activities were measured by the hydrolysis of the chromatogenic substances, p-nitrophenyl - β - D - glucopyranoside and p - nitrophenyl - β - D - glucobioside respectively (Byrde & Fielding, 1968; Wijesundera *et al.*, 1984; Senaratna *et al.*, 1991). The colour of the phenate ion was estimated by measuring the absorbance at 403 nm. Samplings were done upto 32 days in liquid medium and 4 days on host tissue respectively. Enzyme activity is expressed as arbitrary units.

Inhibitory activity

Mixtures of the enzyme sample (PG) from *C. quinqueseptatum* Kp isolate or *C. gloeosporioides* culture filtrates and the healthy leaf extracts of *Hevea* prepared as above were made in the proportions of 0:100; 10:90; 20:80; 30:70; 40:60; 50:50; 60:40; 70:30; 80:20; 90:10 and 100:0 and were tested for PG activity by cup plate method to

study any effect of host leaf extract on enzyme activity. Controls were prepared with sterile distilled water made in the same proportions.

RESULTS AND DISCUSSION

Pectolytic enzymes

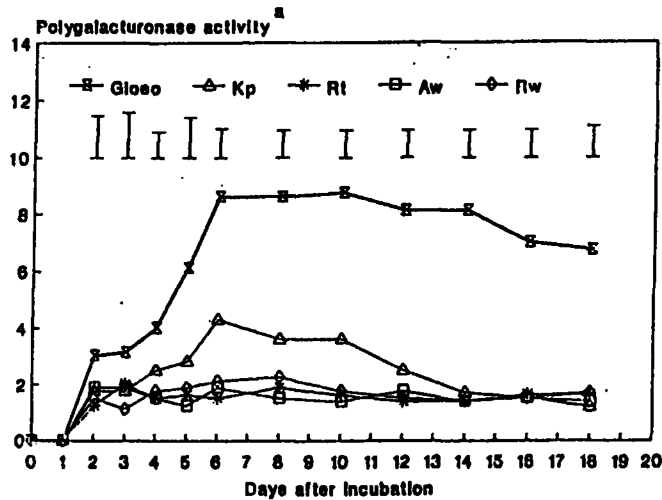
Time course of enzyme secretion : When grown in ammonium tatrate liquid medium with citrus pectin as the main source of carbon, only the Kp isolate of *C. quinqueseptatum* showed significant PG activity (Figs. 1a and 1b). Maximum PG activity of Kp was on 6th day after incubation. However, levels of PG produced were significantly low compared to the PG production by the reference culture, *C. gloeosporioides*. *C. gloeosporioides* maintained a markedly high activity from the 4th day onwards after incubation (Fig. 1). No pectin lyase activity was shown by any *C. quinqueseptatum* isolate whereas *C. gloeosporioides* showed a marked PL activity 5 days after inoculation. It reached a maximum 16 days after incubation (Fig. 2).

Enzyme production in host tissue - The extracts of the clove leaf tissue and rubber leaf tissue inoculated either with *C. quinqueseptatum* (Kp isolate) or *C. gloeosporioides* did not show any PG activity when assayed by both cup plate method and viscometry. PL activity was absent in leaves inoculated with *C. quinqueseptatum*. But PL was detected in leaves inoculated with *Colletotrichum gloeosporioides*. This enzyme activity was observed on the fourth day after inoculation (Table 1).

The inability to detect PG in tissues infected with the Kp isolate of *C. quinqueseptatum* may be due to the presence of inhibitors of PG in plant tissues. A variety of host tissues have been reported to contain proteinaceous inhibitors of PG and these inhibitors have been isolated and at least partially characterized from tissues or cell walls of bean (Albersheim & Anderson, 1971; Fisher *et al.*, 1973; Wijesundera *et al.*, 1989), cucumber (Skare *et al.*, 1975), pea (Hoffman & Turner, 1982), pepper (Brown & Adikaram, 1982), tomato (Brown & Adikaram, 1983), apple (Brown, 1984), pear (Abu-Goukh *et al.*, 1983), peach (Fielding, 1981) and citrus (Barmore & Nguyen, 1985). Since PG was not detected in infected rubber tissue, it was investigated whether this was due to an inhibitory effect. No inhibitory activity was present as both the test mixtures and their respective controls revealed a similar pattern of PG activity (Fig. 3).

Pectolytic enzymes have also been found to remain ionically bound to the host cell walls (Cooper, 1983). This also could not be the reason for the absence of PG activity in infected rubber leaf extracts as a high ionic extractant (having 1.0 M NaCl) was used to extract tissues, thus any wall bound enzymes would have been released.

Therefore, it seems that in *C. quinqueseptatum* infections pectic enzymes do not play a major role.



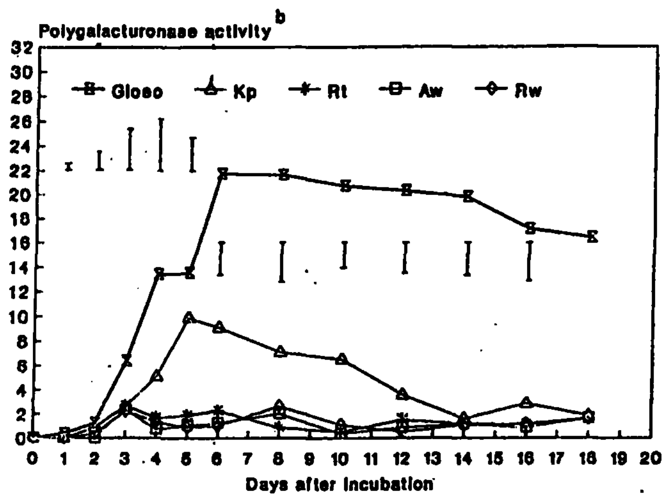


Fig. 1. Polygalacturonase production by four isolates of *Cylindrocladium quinqueseptatum*; Aw, Rw, Rt and Kp and *Colletotrichum gloeosporioides* (reference culture) in ammonium tatrate liquid medium with citrus pectin as the main source of carbon at room temperature as detected by cup plate method (a) and viscometry (b). Activity is given in arbitrary units

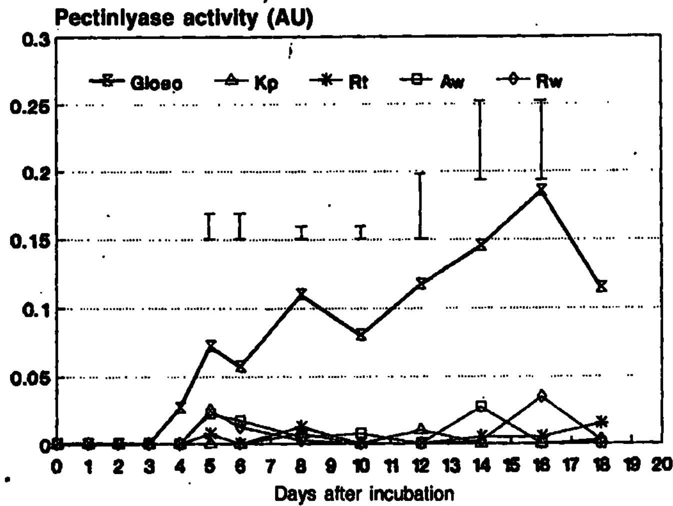


Fig. 2. Pectin lyase production by four isolates of C. *quinqueseptatum*; Aw, Rw, Rt and Kp and *C. gloeosporioides* (reference culture) in ammonium tatrate liquid medium with citrus pectin as the main source of carbon at room temperature. AU - Arbitrary units.

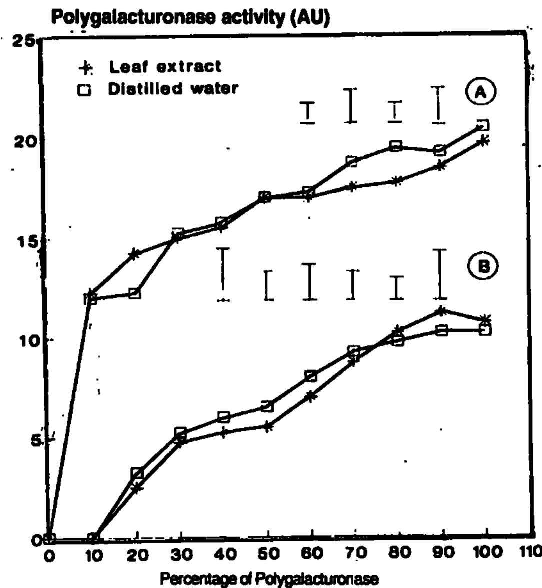


Fig. 3. Effect of extracts of healthy leaf tissue on activity of polygalacturonase enzyme from *Cylindrocladium quinqueseptaum* (B) and reference culture, *Colletotrichum gloeosporioides* (A) AU-Arbitrary units.

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

Incubation period (h)	Pectinlyase enzzyme activity@ (AU)		
	Eugenia infected with *Cylindrocladium*	*Hevea* infected with *Cylindrocladium*	*Hevea* infected with *Colletotrichum*
0*	Nil	0.0296 ± 0.009	0.0296 ± 0.009
24	Nil	0.0213 ± 0.004	0.0066 ± 0.0077
48	Nil	0.0228 ± 0.006	0.0174 ± 0.0055
72	Nil	0.0092 ± 0.002	0.0511 ±0.0207
96	Nil	0.0271 ± 0.003	0.0719 ± 0.0153

No polygalacturonase activity was detected on *Hevea* infected with *C. quinqueseptatum* of *C. gloeosporioides* and *Eugenia* infected with *C. quinqueseptatum*

*, Healthy leaves
@, Absorbance at 550 nm
AU – Arbitary Units

Cellulolytic enzymes

Time course of enzyme production: All isolates of *C. quinqueseptatum* and *C. gloeosporioides* produced cellobiase and β-glucosidase (Figs. 4 & 5). However, in *C. quinqueseptatum* the activity of each enzyme varied with the isolate. *C. gloeosporioides* secreted the highest amounts of cellobiase and β-glucosidase and activity was detected in cultures two days after incubation. In *C. gloeosporioides* maximum activity was detected on 26th day for cellobiase while two peaks on 12th and 30th day were detected for β-glucosidase. Production of cellulases by *C. gloeosporioides* in culture has also been shown by various workers (Kanakaratne and Adikaram, 1990; Senaratna *et al.*, 1991).

In *C. quinqueseptatum* isolates Kp, Rw, Aw the major cellobiase peak was detected on the 26th day. In Rt the major peak was on day 30 (Fig. 4). Isolates Kp and Rw showed a significantly higher cellobiase activity than isolates Rt and Aw. Two major peaks of β-glucosidase activity was seen in all 4 isolates, 14-16 days & 30 days after inoculation (Fig. 5). It is very likely that all isolates of *C. quinqueseptatum* produce one major form of cellobiase and at least two forms of β-glucosidase.

Celluloytic Enzyme production in host tissue: No cellobiase activity was detected in healthy clove or rubber leaf tissue and clove leaves infected with *C. quinqueseptatum*.

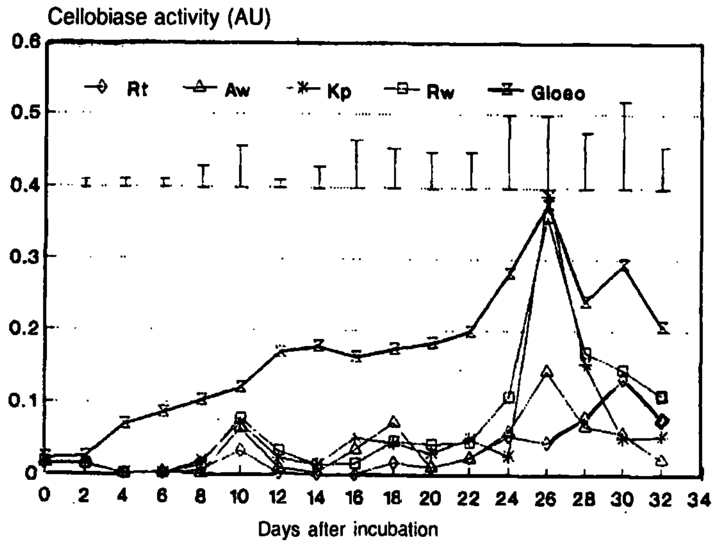


Fig. 4. Cellobiase production by four isolates of C. *quinqueseptatum*; Aw, Rw, Rt and Kp and *C. gloeosporioides* in ammonium tatrate liquid medium with carboxymethyl-cellulose as the main source of carbon at room temperature. AU - Arbitrary units.

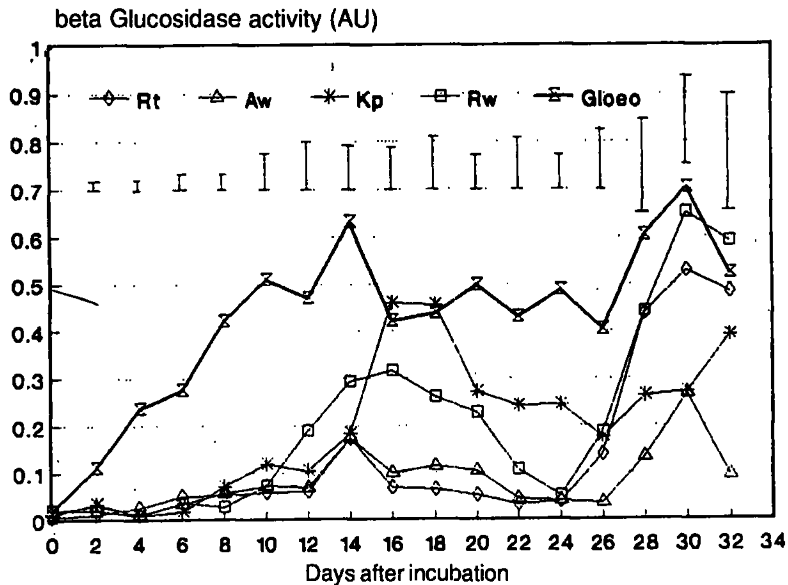


Fig. 5. β-glucosidase production by four isolates of C. quinqueseptatum; Aw, Rw, Rt and Kp and C. gloeosporioides (reference culture) in ammonium tatrate liquid medium with carboxy-methyl-cellulose as the main source of carbon at room temperature. AU-Arbitrary units.

However, a marked increase in activity of cellobiase was detected in rubber leaves on the third day after inoculation with *C. quinqueseptatum* (Table 2). Though there was a detectable cellobiase activity in rubber leaf tissue infected with *C. gloeosporioides* the amounts were lower than in leaf tissue infected with *C. quinqueseptatum* (Table 2).

Table 2. *Cellobiase activity in extracts of clove and rubber leaves infected with* C. quinqueseptatum *(observations for* C. gloeosporioides *on rubber is also included)*

Incubation period (h)	Cellobiase enzyme activity* (AU)		
	Clove infected with *Cylindrocladium*	Rubber infected with *Cylindrocladium*	Rubber infected with *Colletotrichum*
Healthy	Nil	Nil	Nil
24	Nil	0.183 ± 0.005	Nil
48	Nil	0.178 ± 0.031	Nil
72	Nil	0.617 ± 0.025	0.024 ±0.002
96	Nil	0.443 ±0.024	0.008 ±0.003

*, Absorbance at 403 nm
AU, Arbitrary units

β-glucosidase was not detected in healthy clove leaf tissue but was detected in healthy rubber leaf tissue (Table 3). Cyanogenesis is common in the genus *Hevea* (Lieberei *et al.*, 1986) and the enzyme responsible for the cleavage of the cyanogen is, β-glucosidase (Selmar *et al.*, 1987). Hence, the β glucosidase detected in healthy tissue during our study would have been the enzyme responsible for cyanogenesis in *Hevea*. The activity of the enzyme increased markedly following in both *C. gloeosporioides* and *C. quinqueseptatum* infections. The increase in the production of β- glucosidase following infection may be a host reaction towards injury or due to enzymes produced by the pathogen.

With the view of above situation the likelihood of toxins playing a vital role in disease initiation by *Cylindrocladium quinqueseptatum* is worth investigating. The secretion of toxic substances by various isolates of *C. quinqueseptatum* has been shown by Anahosur & others (1976), Kaushi & Gupta (1991) and Jayasinghe (1996). Sharma and Mohanan (1990) has also suggested that necrosis of tissues during pathogenesis of eucalyptus isolates of *Cylindrocladium quinqueseptatum* is due to the effect of toxin produced by the pathogen. It is noteworthy that initiation of the infection and development of symptoms in the diseases caused by *Drechslera* spp. (Meehan & Murphy, 1947; Strobel *et al.*, 1988), *Alternaria* spp. (Tanaka, 1933; Kohmoto *et al.*, 1979) and *Periconia* sp (Scheffer & Pringle, 1961) have been proved to be a result of toxic substances.

Table 3. β *Glucosidase activity in extracts of clove and rubber leaves infected with* C. quinqueeseptatum *(observations for* C. gloeosporioides *is also included)*

Incubation period (h)	β Glucosidase enzyme activity* (AU)		
	Clove infected with *Cylindrocladium*	Rubber infected with *Cylindrocladium*	Rubber infected with *Colletotrichum*
Healthy	Nil	0.784 ± 0.023	0.784± 0.023
24	Nil	1.311 ± 0.034	1.142 ± 0.046
48	Nil	1.314 ± 0.092	1.431 ± 0.074
72	0.152 ± 0.032	1.753 ± 0.026	0.878 ± 0.033
96	0.019 ± 0.007	1.551 ± 0.047	0.901 ± 0.011

*, Absorbance at 403 nm
AU, Arbitrary units

The other question is the mode of penetration of the host by the pathogen in the absence of significant pectic enzyme activity. Bolland *et al.* (1985) pointed out that *C. quinqueseptatum* penetrates foliage and young shoots only via stomata and the toxin increases stomatal openings in both light and dark conditions. This probably facilitates the entry of the pathogen into the host tissue. Our studies suggest that in the latter stages of infection cellulolytic enzymes play a major role in cell wall degradation. Breakdown of cell wall components due to cellulolytic enzymes of phytopathogenic fungi has been shown as far back in 1957 (Husain & Kelman, 1957; Kelman & Cowling, 1965).

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