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# CELLULASE ACTIVITY IN FUNGAL INFECTED BANANA FRUITS: EFFECT OF DIFFERENT SYNTHETIC MEDIA ON CELLULASE PRODUCTION

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## ABSTRACT

Banana fruits infected with *Macrophomina phaseolina* R-4242, *Fusarium oxysporum* sp.QJC-1403 and *Nigrospora oryzae* NRRL-54030 had deteriorated within eight days of incubation at room temperature (27±2$^{o}$C). Extracts from the infected fruits exhibited cellulase activity, however uninfected fruits lacked cellulase activity. The cellobiohydrolase ($C_1$) activity could be traced on 2$^{nd}$ day of inoculation in all the four varieties of banana which continued to increase till the end of the observation period. However, the $C_1$ activity showed decreasing trend by the end of 8$^{th}$ day in Cavendish and Curry varieties infected with *N. oryzae* and *F. oxysporum* respectively. Endoglucanase ($C_X$) activity was also witnessed in the diseased tissues of all the four varieties of banana which, however, varied with the pathogens inoculated. $C_X$ activity decreased after 6$^{th}$ day of infection in all the varieties of banana under investigation. The occurrence of cellulase in banana fruits infected with the three fruit-rot fungi and its absence in uninfected fruits suggest the role of this enzyme in pathogenicity of the fungi understudy. Cellulolytic components of the fruits are degraded; the fruits are deteriorated and are lost to the post-harvest pathogens.

The production of cellulase ($C_1$ and $C_X$) by the three pathogenic fungi isolated from banana on seven different media was investigated. All the three pathogens were able to produce cellulase in one or the other media tried. Medium G supported good growth and maximum enzyme production by the three fruit-rot fungi. Supplementation of medium A with carboxymethyl cellulose (medium C) as the substrate did not make much difference in the degree of cellulase production, suggesting the constitutive nature of the enzyme produced by the fungi understudy.

**KEY WORDS**: Cellulase, fruit-rot, banana varieties, cellulose, media, deterioration

## INTRODUCTION

Banana, one of the most important fruit in many countries has a worldwide economic and nutritive importance. It constitutes second largest fruit in production in India and globally bananas rank fourth after rice, wheat and maize in human consumption [1]. It contains an energy value of 116 K cal per 100 gms of edible product. Banana is also rich in vitamin –A, B, C, Mg, Ca, K, Zn and phosphorous. There are approximately 1200 seedless banana fruit varieties grown abundantly in many developing countries. Fungi are responsible for spoilage of these fruits during storage [2, 3].

A wide range of cell wall degrading enzymes are produced by most phytopathogenic fungi, they are the most important biological weapons, which helps the pathogen in the penetration and colonization within the host [4]. Successful plant infection largely depends on the ability of the phytopathogen to gain access into the internal tissue components. A central role in this regard is played by the chain splitting cellulase enzymes [5]. The breakdown of cellulose to soluble sugars by phytopathogens involves the action of a multi-enzyme system [6]. A proposal made by Zhang and Lynd [7] suggests that cellulose is degraded by the synergistic action of three types of enzymes namely endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). According to this view endoglucanase ($C_X$) hydrolyze accessible intramolecular β-1, 4- glucosidic bonds of cellulose chains randomly to produce new chain ends. Exoglucanase or cellobiohyrolase ($C_1$) progressively cleaves cellulose chains from one end to release soluble cellobiose or glucose. β-glucosidase further degrades cellobiose to release glucose.

Several pathologists have implicated the role of cell wall degrading enzymes in various diseases of vegetables and fruit-rots [5, 8-13]. Hence, an attempt has been made to assess the role of cellulolytic enzymes in the disease cause of banana fruits infected with *M. phaseolina*, *F.oxysporum* and *N.oryzae* . Further cellulase producing capacity of the three pathogens was also investigated on different synthetic media to find out the influence of different substrates on cellulase production.

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## MATERIALS AND METHODS

### Organism and culture conditions

The three fruit-rot fungi employed for this research work were isolated from banana fruits in the Department of Microbiology, Kakatiya University,Warangal. The fungi were routinely grown and maintained on Asthana and Hawker's A agar slants. They were subcultured from the old culture onto fresh agar slants. Five day old culture of the organism served as inoculum.

**Inoculation of banana fruits**

Healthy semi-ripe fruits of four varieties of banana; Poovan (Mysore AAB), Rasthali (Silk AAB), Cavendish (AAA) and Curry variety (ABB) of almost same age were surface sterilized with 0.1% mercuric chloride and inoculated with respective pathogens after inflicting scalpel injury [14]. Both the experimental and control sets of fruits were placed in sterile humid chamber. The rims of the chambers were sealed and incubated at room temperature (27± 2°C) for eight days. Atleast five replicates were maintained.

**Extraction of enzyme**

At the end of 2, 4, 6 and 8 days of incubation, the fruit tissue was taken out from infected and uninoculated fruits. Three grams of fruit tissue was homogenized in 15 ml of distilled water and filtered through the filter paper (Whatman No.1).The filtrate was centrifuged at 1800 xg for about 30 minutes . The supernatant was dialyzed and taken as enzyme sample. Heat killed enzyme served as control.

**Enzyme assay**

**Endoglucanase ($C_X$)**

Endoglucanase ($C_X$) (EC 3.2.1.4) activity was assayed viscometrically as suggested by Reese *et al*. [15]. Ostwald-Fenske viscometer made up of corning glass was used for the experiment. The reaction mixture consisted of 15 ml of 0.5% Carboxymethyl Cellulose (CMC), 5 ml of enzyme and 1 ml of citrate buffer (pH 5.5). The loss in viscosity was measured for every 10 minutes over a period of 30 minutes. The reaction mixture with heat killed (inactivated) enzyme and water served as control. The percentage of loss of viscosity was calculated by using the following formula:

$$A_n \text{ min} = \frac{t_1 - t_a}{t_1 - t_o} \times 100$$

where,

$A_n$ min =Percentage of loss of viscosity

$t_1$=Flow time of reaction mixture + inactive enzyme.

$t_a$=Flow time of reaction mixture + active enzyme.

$t_o$= Flow time of water + active enzyme at '0' time

The activity of endoglucanase ($C_X$) was expressed in relative viscometric unit (RVU).

$$RVU = \frac{1000}{t_v\ 50}$$

where,

$t_v$ 50=time required in min to reduce the viscosity of CMC to 50% of the initial viscosity.

**Cellobiohydrolase ($C_1$)**

Cellobiohydrolase ($C_1$) (EC 3.2.1.91) activity was determined by DNS method as suggested by Miller [16].

The reaction mixture consisting of 3.5 ml of 0.5% cellulose powder solution, 1 ml of citrate buffer (pH 5.5), 0.5 ml of enzyme and a few drops of toluene was incubated at 30 ± 1°C for 6 hours. At the end of the incubation period 1 ml of aliquot of the reaction mixture was withdrawn into a test tube and 3 ml of DNS reagent was added and heated in a boiling water bath for 15 minutes. 2 ml of 20% potassium sodium tartarate was added while the contents were hot and then cooled under running tap water. For blank preparation, 1 ml of enzyme was replaced by 1 ml of distilled water. Intensity of the colour developed was read at 575 nm. Released reducing sugars was expressed in glucose equivalent. One unit (IU) of cellulase activity was defined as the amount of enzyme required to liberate 1 µ mole of glucose per minute under the assay conditions.

**Growth and enzyme production on different media**

The fungi (*M.phaseolina*, *F.oxsporum* and *N.oryzae*) were grown in 25 ml of sterilized medium taken in 100ml Erlenmeyer flasks and incubated at room temperature (27± 2°C). The pH of the medium was adjusted to 6 with the help of 0.1M HCl before sterilizing in an autoclave at 121°C for 20 minutes. At the end of 5, 10 and 15 days of incubation period, a set of flasks were harvested on previously dried and weighed Whatman filter paper No.42. The filter papers with the mycelium were dried at 65-75°C for 48 hrs. Fungal growth was expressed in terms of dry weight of mycelia mat (mg/flask).The pH of the culture filtrate was determined either with the help of BDH filter paper or Elico pH meter.

Protein content of the culture filtrates was determined by Lowry's method [17] using Bovine Serum Albumin (BSA) as standard.

The following synthetic media were selected to study the production of cellulolytic enzymes

1)Asthana and Hawker's medium A - A
(Glucose 5.0 g, potassium nitrate 3.5 g, potassium dihydrogen orthophosphate 1.75 g, magnesium sulphate 0.75 g & distilled water 1000 ml)

2)Medium A + 0.5 % CMC - B

3)Medium A + 1% CMC - C

4)Medium A + 1 % banana fruit extract - D

5)Medium A + 1% banana unripe fruit extract- E

6)Medium A + 1% banana leaf extract - F

7)Glucose-peptone medium - G
(Glucose 20.0 g, peptone 4.5 g, asparagine 4.5 g potassium dihydrogen orthophosphate 3.4 g, magnesium sulphate1.9 g, sodium chloride 0.01g and distilled water 1000 ml)

**Enzyme assay**

The filtrate obtained was centrifuged at 1800 xg for 30 minutes to remove mycelial debris and subjected to dialysis before assaying as described earlier.

The statistical analysis performed on the results obtained was calculated and ANOVA (SPSS 12.0 version) revealed the significance of the experiment (Table 1a and 2a).

**Table - 1 : Cellulases ($C_1$ and $C_x^{(+)}$) activity in four varieties of banana (*M. paradisiaca* L.) fruits infected by three fruit-rot fungi**

| Name of the variety | Days of incubation | *M. phaseolina R-4242* | | | *F. oxysporum sp.QJC-1403* | | | *N. oryzae NRRL: 54030* | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Protein µg/ml | $C_1$ U/ml | $C_x^{(+)}$ | Protein µg/ml | $C_1$ U/ml | $C_x^{(+)}$ | Protein µg/ml | $C_1$ U/ml | $C_x^{(+)}$ |
| ***Cavendish*** | 2 | 92 | 0.52 | 21.0 | 98 | 0.47 | 37.6 | 87 | 0.57 | 22.7 |
| | 4 | 110 | 0.68 | 29.0 | 122 | 0.59 | 25.8 | 102 | 0.71 | 27.0 |
| | 6 | 149 | 1.20 | 18.9 | 226 | 0.70 | 17.9 | 248 | 1.20 | 17.3 |
| | 8 | 185 | 1.30 | 13.3 | 258 | 0.98 | 16.3 | 240 | 0.93 | 14.8 |
| ***Rasthali*** | 2 | 83 | 0.25 | 24.4 | 86 | 0.28 | 24.7 | 98 | 0.39 | 16.6 |
| | 4 | 109 | 0.69 | 26.2 | 235 | 0.93 | 20.9 | 128 | 0.43 | 22.8 |
| | 6 | 136 | 1.25 | 28.9 | 260 | 1.15 | 22.6 | 189 | 0.70 | 28.7 |
| | 8 | 192 | 1.42 | 18.9 | 289 | 1.46 | 11.3 | 237 | 0.97 | 13.2 |
| ***Poovan*** | 2 | 79 | 0.22 | 25.6 | 108 | 0.34 | 24.4 | 84 | 0.24 | 22.5 |
| | 4 | 108 | 0.59 | 31.4 | 132 | 0.63 | 26.5 | 113 | 0.34 | 27.4 |
| | 6 | 258 | 0.97 | 28.9 | 265 | 0.93 | 17.8 | 178 | 0.62 | 32.8 |
| | 8 | 292 | 1.32 | 26.0 | 310 | 1.33 | 12.6 | 279 | 0.86 | 20.4 |
| ***Curry variety*** | 2 | 83 | 0.25 | 26.4 | 129 | 0.43 | 27.5 | 78 | 0.29 | 27.3 |
| | 4 | 96 | 0.39 | 24.2 | 119 | 0.25 | 22.5 | 198 | 0.47 | 18.6 |
| | 6 | 126 | 0.47 | 21.8 | 108 | 0.24 | 22.0 | 256 | 0.94 | 15.8 |
| | 8 | 147 | 0.68 | 16.8 | 94 | 0.18 | 20.9 | 312 | 1.07 | 13.4 |

Values represented in the table are averages of results of two separately conducted experiments

(+) Exprressed in relative viscometric units (RVU).

## RESULTS

Critical perusal of Table 1 reveals that the activity of cellulase varied with the variety and the pathogen. The cellobiohydrolase ($C_1$) activity could be traced on 2nd day of inoculation in four varieties of banana which continued to increase till the end of the observation period. However, $C_1$ activity showed decreasing trend by the end of 8th day in Cavendish and Curry varieties inoculated with *N.oryzae* and *F.oxysporum* respectively. Absence of $C_1$ activity in healthy tissues and presence in diseased tissues suggests its involvement in fruit-rot. Dubey and Sunitha Pandey [18], Pannerselvam and Saravanamuthu [19] and Adekunle *et al.* [5] have also reported the involvememt of $C_1$ in the disease cause in fruits studied by them.

Endoglucanase activity was also witnessed in the diseased tissues of all the four varieties of banana which, however, varied with the pathogens. $C_X$ activity was recorded maximum on 4th day in Cavendish, Poovan and Curry varieties while it was maximum on 6th day in Rasthali variety infected by *M.phaseolina* and *N.oryzae*. However, the $C_X$ activity decreased after 6th day of inoculation in all the four varieties under the influence of all the three fruit-rot fungi. Similarly Dubey and Sunitha Pandey [18], Wahid [20], Chakrabarthy *et al*. [13], Dagade and Shyalaja [21] and Adekunle *et al*. [5] have also reported the supportive role of cellulases in the pathogens establishment.. Table 2 reveals that all the three pathogens under investigation secreted cellulases ($C_1$ and $C_X$). However, they differed significantly in their capacity to secrete cellulases. The cellulase production varied both with the fungus and substratum used.

*M.phaseolina* could record maximum $C_1$ enzyme in medium G. Medium D supported $C_1$ upto 10th day of incubation, while medium A and B induced maximum $C_1$ production during latter part of the incubation. On the other hand, medium E and F supported $C_1$ activity upto 10th day of incubation period. It secreted maximum $C_X$ in medium E followed by medium F and C. $C_X$ production showed increasing trend, till the end of the incubation period in all the media except in medium B and G where activity ceased by the end of incubation period. Though *F.oxysporum* secreted $C_1$ enzyme in all the media tried, the degree of production varied with the medium and incubation period. The $C_1$ activity was maximum in medium G followed by medium B , where $C_1$ activity increased with the advancement of incubation. Medium F and D were of same nutritive value for $C_1$ production. Rest of the media were poor substrates for $C_1$ production. *F.oxysporum*

**Table - 2 : Production of cellulases ($C_1$* and $C_x$**) mycelial growth and pH changes on different synthetic media by three fruit-rot fungi of banana (*M. paradisiaca* L.)**

| Medium | Days of incubation | *M. phaseolina* R-4242 | | | | | *F. oxysporum* sp.QJC-1403 | | | | | *N. oryzae* NRRL: 54030 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Dry wt (in mg) | pH | Protein µg/ml | $C_1$* U/ml | $C_x$** | Dry wt (in mg) | pH | Protein µg/ml | $C_1$* U/ml | $C_x$** | Dry wt (in mg) | pH | Protein µg/ml | $C_1$* U/m | $C_x$** |
| *Asthana & Hawkers* | 5 | 47.9 | 5.6 | 70 | 0.07 | 10.9 | 65.6 | 6.1 | 80 | 0.09 | 11.8 | 63.9 | 5.6 | 92 | 0.39 | 19.2 |
| *medium A [A]* | 10 | 82.8 | 6.5 | 98 | 0.12 | 16.8 | 104.5 | 6.3 | 110 | 0.14 | 21.3 | 108.3 | 5.8 | 210 | 0.43 | 24.9 |
| | 15 | 93.0 | 7.0 | 49 | 0.14 | 24.7 | 131.2 | 7.0 | 40 | 0.20 | 15.9 | 134.3 | 6.7 | 110 | 0.18 | 27.8 |
| *Meidum A +* | 5 | 106.6 | 5.7 | 100 | 0.12 | 15.5 | 77.1 | 5.5 | 140 | 0.11 | 22.5 | 53.7 | 5.6 | 120 | 0.43 | 20.7 |
| *0.5% CMC [B]* | 10 | 117.0 | 5.9 | 310 | 0.18 | 17.6 | 93.0 | 6.7 | 100 | 0.20 | 24.2 | 106.2 | 5.8 | 285 | 0.47 | 33.4 |
| | 15 | 145.2 | 6.3 | 55 | 0.24 | 14.1 | 108.2 | 6.9 | 95 | 0.39 | 28.8 | 130.1 | 5.6 | 116 | 0.35 | 40.0 |
| *Medium A + 1.0%* | 5 | 99.7 | 5.6 | 115 | 0.14 | 17.4 | 92.2 | 5.9 | 210 | 0.12 | 23.4 | 49.2 | 5.6 | 120 | 0.36 | 31.1 |
| *CMC [C]* | 10 | 102.5 | 5.9 | 322 | 0.22 | 20.0 | 109.5 | 6.6 | 200 | 0.18 | 27.5 | 68.0 | 6.4 | 298 | 0.40 | 38.7 |
| | 15 | 113.0 | 6.2 | 60 | 0.23 | 29.4 | 146.2 | 6.8 | 150 | 0.24 | 33.6 | 114.1 | 5.9 | 102 | 0.34 | 20.9 |
| *Medium A + 1.0%* | 5 | 66.9 | 5.7 | 75 | 0.24 | 10.8 | 130.6 | 6.0 | 200 | 0.25 | 18.8 | 103.5 | 5.6 | 110 | 0.44 | 15.7 |
| *banana fruit extract [D]* | 10 | 115.2 | 6.3 | 290 | 0.25 | 16.8 | 176.0 | 6.8 | 115 | 0.34 | 25.2 | 134.2 | 6.2 | 240 | 0.50 | 21.6 |
| | 15 | 130.9 | 6.7 | 55 | 0.18 | 24.7 | 209.9 | 7.1 | 80 | 0.33 | 19.5 | 169.4 | 7.0 | 55 | 0.25 | 15.1 |
| *Medium A + 1.0%* | 5 | 90.6 | 5.8 | 190 | 0.25 | 18.4 | 82.9 | 5.7 | 310 | 0.22 | 20.3 | 94.3 | 6.2 | 108 | 0.40 | 15.9 |
| *banana unripe fruit* | 10 | 106.5 | 6.4 | 150 | 0.20 | 23.6 | 109.3 | 6.5 | 280 | 0.25 | 25.2 | 173.7 | 6.7 | 240 | 0.44 | 27.3 |
| *extract [E]* | 15 | 145.9 | 6.8 | 80 | 0.14 | 39.1 | 194.7 | 7.2 | 107 | 0.22 | 28.0 | 214.3 | 7.0 | 84 | 0.43 | 21.3 |
| *Medium A + 1.0%* | 5 | 68.9 | 5.8 | 148 | 0.28 | 17.3 | 92.7 | 6.2 | 110 | 0.22 | 20.9 | 71.4 | 5.8 | 160 | 0.47 | 12.4 |
| *banana leaf extract [F]* | 10 | 84.2 | 6.6 | 115 | 0.23 | 24.5 | 137.2 | 6.7 | 140 | 0.40 | 27.3 | 102.9 | 6.0 | 310 | 0.39 | 21.9 |
| | 15 | 140.6 | 7.0 | 75 | 0.20 | 30.1 | 150.0 | 7.0 | 80 | 0.35 | 22.6 | 135.2 | 6.9 | 110 | 0.24 | 18.2 |
| *Glucose - peptone* | 5 | 156.3 | 5.6 | 142 | 0.24 | 15.5 | 157.6 | 5.9 | 69 | 0.59 | 16.4 | 149.3 | 5.6 | 78 | 0.59 | 25.1 |
| *medium [G]* | 10 | 174.0 | 6.3 | 110 | 0.39 | 17.6 | 238.4 | 6.3 | 138 | 0.47 | 24.6 | 177.3 | 5.9 | 120 | 0.86 | 42.9 |
| | 15 | 276.0 | 7.2 | 105 | 0.47 | 12.2 | 269.3 | 7.0 | 99 | 0.43 | 21.4 | 318.4 | 6.0 | 113 | 1.28 | 22.2 |

Values represented in the table are averages of the results of two separately conducted experiments

* Expressed in U/ml ;** Expressed in relative viscometric units (RVU).

could produce maximum $C_X$ in medium C and minimum in medium A. Rest of the media supported intermediate amount and almost same $C_X$ activity *N.oryzae* secreted $C_1$ in all the media tried. It secreted maximum $C_1$ during its growth in medium G and D. Rest of the media supported varying $C_1$ activity. The $C_1$ activity showed increasing trend upto $10^{th}$ day of incubation, while a continuous increase in enzyme production till end of the incubation period was recorded in medium G. *N.oryzae* recorded increased $C_X$ enzyme secretion, suggesting its adaptive nature. Bhagavan Reddy [22] also reported the adaptive nature of $C_X$ enzyme by the three isolates of *Myrothecium roridum* isolated from bitter-gourd, water-melon fruits and pearl-millet seeds. The fruit extract of banana did not make much effect on enzyme production by the fungi understudy, suggesting host resistance to *N.oryzae* infection or $C_X$ may not be playing crucial role in pathogenesis. Ugwuanyi and Obeta [12] have reported the

cellulolytic activities of pathogenic fungi and their macerating effects on mango fruits. Increased production of $C_1$ and $C_X$ in medium G may be attributed to the release of different amino acids by the hydrolysis of peptone.

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**Table- 1a: ANOVA of cellulases ($C_1$ and $C_X$) activity in four varieties of banana fruits by three pathogens**

| *Sources of variation* | *Sum of Squares* | *df* | *Mean Square* | *P-value* | *F* | *Result* |
|---|---|---|---|---|---|---|
| *Between Groups* | 118508.369 | 35 | 3385.953 | < 0.001 | 6.166 | S |
| *Within Groups* | 59302.359 | 108 | 549.096 | | | |
| *Total* | 177810.729 | 143 | | | | |

P > 0.05 –Not significant (NS); P < 0.05-Significant (S)
S-significant

**Table -2a : ANOVA of cellulases ($C_1$ and Cx) production on different synthetic media by three fruit-rot fungi**

| *Sources of variation* | *Sum of Squares* | *df* | *Mean Square* | *P value* | *F* | *Result* |
|---|---|---|---|---|---|---|
| *Between Group* | 761252.043 | 104 | 7319.731 | < 0.001 | 18.530 | S |
| *Within Groups* | 82952.700 | 210 | 395.013 | | | |
| *Total* | 844204.743 | 314 | | | | |

P > 0.05 –Not significant (NS) P < 0.05-Significant (S)
S-significant

*M.phaseolina* could achieve maximum growth in medium G followed by medium E and B. Medium A, C, D and F were next preferred substrates. *F.oxysporum* could grow luxuriously in medium G. Medium C, E and F are of same nutritive value. Supplementation of medium A with cellulose (medium C) did not make much difference in the degree of cellulase production. Similarly addition of fruit pulp (medium D) also did not induce much cellulase secretion by *F.oxysporum*. *N.oryzae* could achieve maximum biomass in medium G followed by medium E. Medium A, B and F are of same nutritive value and comparatively inferior in enzyme production. The pH of the medium underwent a significant change with the growth of the fungus. The pH drifted towards alkaline side and in majority of the cases the final pH was near neutral.

**DISCUSSION**

**I**n the present study, it was observed that all the three fruit-rot fungi were able to produce cellulolytic enzymes ($C_1$ and $C_X$). However, they differed significantly in producing the respective enzymes. The occurrence of cellulases in banana fruits infected with the three pathogenic fungi understudy suggests the role of cellulases in the disease cause, deterioration of the fruits and pathogenicity of the fungus.

The results also suggest that the enzyme is produced by the organism in order to hydrolyse the complex cellulolytic portions of the fruit cell

wall. Simpler forms of compounds such as glucose are the result of such hydrolysis. These processes result in structural modifications. Knowledge of conditions of growth of these fruit-rot fungi and the role of cellulolytic enzymes will assist the farmer in optimizing production of these fruits and engaging the best conditions for preservation. This study may serve to increase the understanding of different substrates that effect the production and role of fungal cellulases in phytopathogenicity. Studies on production of different hydrolytic enzymes using several plant materials are in progress in our laboratory

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