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# PRODUCTION OF POLYGALACTURONASES IN ISOLATES OF *Cryphonectria cubensis* OF DIFFERING PATHOGENICITY

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## ABSTRACT

*Cryphonectria cubensis* causes a serious stem canker disease on *Eucalyptus* in tropical and subtropical parts of the world. Previously, it was shown that isolates of *C. cubensis* display varying levels of pathogenicity. The aim of this study was to consider whether a relationship exists between the production levels of polygalacturonases and pathogenicity in isolates of *C. cubensis*. Seven isolates of *C. cubensis* known to have differing levels of pathogenicity were assessed for polygalacturonase production using agarose gel diffusion and reducing sugar assays. Our results showed that the levels of polygalacturonase production are not significantly different in natural isolates of *C. cubensis* that have different pathogenicity levels. However, in one hypovirulent isolate, CMW6009, which has been artificially transfected with hypovirus CHV1-713 from *Cryphonectria parasitica*, a delay of six days in the production of polygalacturonases was observed. We conclude that polygalacturonases probably have a minor role in determining the pathogenicity of the *C. cubensis*. Furthermore, the hypovirus CHV1-713 that causes hypovirulence in *C. cubensis*, has a major role in controlling pathogenicity and its mechanism of action may involve disruption of the production of polygalacturonases and other cell wall degrading enzymes.

**Key words:** *Eucalyptus*, *Cryphonectria cubensis*, polygalacturonases, host defence, hypovirulence, *Cryphonectria parasitica*.

## INTRODUCTION

The first contact of microbial plant parasites with their host occurs at the plant cell wall surface. During penetration and colonisation, the pathogen secretes plant cell wall degrading enzymes such as endopolygalacturonases. These enzymes are primarily involved in the nectrotrophic stage of pathogenesis[24]. Many cell wall degrading enzymes have been reported from fungal pathogens[1,2,9,12,15,26]. There are some reports that specifically associate the production of endopolygalacturonases in pathogens with pathogenesis[10,24,15,21].

Endopolygalacturonases (endoPGs) have been reported to have two opposing roles in fungal pathogenesis. Firstly, they are utilised by fungi as agents involved in disease development and secondly, they are thought to act as potential defence signal molecules[3]. The early timing of the production of these endoPGs is consistent with both roles. EndoPGs initiate the production of elicitors for signal transduction. These elicitors are known as oligogalacturonides. They are produced from degradation of homogalacturonan polymer of pectin[4]. Degradation of pectic polymers by endoPGs in the presence of polygalacturonase-inhibiting proteins (PGIPs), gives rise to elicitor-

active oligogalacturonides. It also increases the residence time of such molecules to act as defence signal molecules[3, 4].

Host defence responses include increased production of PGIPs, production of chitinases and glucanases, phytoalexin production, stimulation of the phenylpropanoid pathway, superoxide peroxidations and hypersensitive responses[3]. These events halt the progress of disease in an infected plant. The efficacy of plant defence responses to a pathogen depends strongly on the extent and speed of the onset of the defence signals[9].

*Cryphonectria cubensis* is a well-known and important canker pathogen of *Eucalyptus* spp.[14,27]. This pathogen is most important where susceptible *Eucalyptus* spp. are grown in tropical and subtropical countries[14,27]. Isolates of *C. cubensis* have been shown to display different levels of pathogenicity in both greenhouse and field inoculation trials[23].

In another study, the hypovirus CHV1-713, isolated from the chestnut blight pathogen *Cryphonectria parasitica*[17] was shown to reduce pathogenicity in a highly virulent South African *C. cubensis* isolate, namely CMW2113[22]. Furthermore, the transfected isolate produced a bright yellow orange mycelium compared to the white mycelium of the non-transfected isolate.

The economic importance of *Eucalyptus* has justified studies on pathogens such as *C. cubensis*. Of particular interest is a need for knowledge pertaining to infection. In this regard, we have considered the role of cell-wall degrading enzymes, such as polygalacturonases, in pathogenesis. In this study, we report on the production of polygalacturonases *in vitro* by isolates of *C. cubensis* known to have different levels of pathogenicity.

## MATERIALS AND METHODS

### Fungal Isolates

Seven South African isolates of *Cryphonectria cubensis*, (CMW2113, CMW6103, CMW6106, CMW6097, CMW6087, CMW6111 and CMW6009) were grown at 25 °C on malt extract agar (MEA; 2 % w/v malt extract, 2 % w/v agar) plates for 6 days. The cultures are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. Isolates CMW2113, CMW6103 and CMW6106 have been shown to have high levels of pathogenicity and isolates CMW6087, CMW6097, and CMW6111 are known to have low pathogenicity [23]. Isolate CMW6009 represents the hypovius transfected form of the highly pathogenic isolate CMW2113, which is routinely used in field screening trials to select disease tolerant planting stock. This isolate has been transfected with the hypovirus CHV1-713 from *Cryphonectria parasitica*[22]. The transfected isolate has subsequently also been shown to be hypovirulent.

### Polygalacturonase production in *C. cubensis*

To induce production of polygalacturonases *in vitro*, five mycelial plugs (4 mm²) were taken from the actively growing margins of cultures on 2 % MEA. These plugs were grown in minimum salts liquid medium (100 ml). The medium contained: 0.5 g yeast extract (Merck); 1.0 g NaOH, 3.0 g $_{DL}$-Malic acid; 2.0 g $NH_4NO_3$; 1.0 g $KH_2PO_4$; 0.1 g $MgSO_4$ and supplemented with 0.5 % w/v polygalacturonic acid (PGA) (Sigma Chemical Company) as a carbon source in a litre of sterile distilled water[11]. Cultures were incubated with shaking at 100 rpm at 25 °C in the dark for ten days. Samples from the culture vessels were collected on each of the ten days. Mycelium was separated by suction filtration through Whatman No. 113 filter paper using a Buchner funnel. The filtrates were then filter-sterilised through 0.22-micron disposable syringe filters (Millipore, USA) and stored at 4 °C. Each time a total protein concentration was determined to ensure the equal amounts were used. All samples were assayed for polygalacturonase activity in triplicate.

### Cup-plate agarose diffusion assay

Production of polygalacturonase was assessed by a modified agarose diffusion assay described by Dingle *et al.* (1953)[8]. The assay medium contained 0.5 % ammonium oxalate, 0.2 % sodium azide and 1.0 % Type II agarose (Sigma Chemical Co.) dissolved in 100 ml of 0.2 M potassium phosphate buffer (adjusted to pH 5.3).

PGA (0.01 %) was used as substrate. The medium was transferred to Petri dishes (20 ml per plate). A 4 mm cork borer was used to punch three wells 2.5 cm apart in the solid gel. Each well was filled with 30 µl of either endoPG standard or blank control or the different filtrates to be tested. The plates were incubated overnight at 30 °C. After incubation, the gel was developed by flooding the plates with 10 ml of 0.05 % ruthenium red (Sigma Chemical Co., USA) for 2 h at 25 °C. Excess dye was removed by washing the plates several times with distilled water. A distinct clear zone on the stained agarose gel indicated PG activity. Two diameter readings (at right angles to each other) of the zones were taken from duplicate plates and the average value was calculated. Each isolate was independently tested three times. Production of polygalacturonases was calibrated against a dilution series of *Aspergillus niger* endoPG (418 units$ml^{-1}$, Sigma Chemical Co., USA, one unit equals the amount of enzyme required to catalyse the production of a reducing sugar per minute). Assays without enzyme served as controls. The mean diameter readings were compared for all the isolates and for each day. Differences in the ability of isolates to produce polygalacturonases were analysed using Tukey 's multiple comparison method from SAS software[18].

**Reducing sugar assay**

Polygalacturonase activity in the different filtrates was determined by measuring the reducing-end groups using the p-hydroxybenzoic acid hydrazide (PAHBAH) method[29]. This measurement was done to confirm the outcome of agarose gel diffusion assays. The PAHBAH assays were calibrated against a dilution series of D-galacturonic acid.

The activity of polygalacturonases were determined by incubating 50 µl of the different samples in a 1 ml solution containing 0.25 % w/v PGA and 40 mM sodium acetate (pH 5.0) for 1 h at 30 °C. This reaction was terminated by addition of 1.5 ml freshly prepared 5 % PAHBAH. The sample tubes were boiled for 10 minutes and cooled to room temperature before taking absorbance readings at 410 nm using a spectrophotometer (Pharmacia LKB. Ultrospec III, Sweden). The assays were performed in triplicate. Statistical analyses of data were similar to those for the agarose diffusion assays. One unit of enzyme activity was defined as the amount of enzyme producing 1 µmol of reducing group per minute at 30 °C in 40 mM of sodium acetate (pH 5.0).

**Table -1. Polygalacturonase production in isolates of *C. cubensis* differing in pathogenicity[A]**

| Isolate number | Relative Pathogenicity | No. of days to attain maximum activity | Units of PG activity[a] ($\mu molml^{-1}min^{-1}$) |
|---|---|---|---|
| CMW2113 | high | 8 | 0.19 |
| CMW6103 | high | 4 | 0.18 |
| CMW6106 | high | 7 | 0.20 |
| CMW6087 | low | 8 | 0.19 |
| CMW6097 | low | 5 | 0.21 |
| CMW6111 | low | 5 | 0.19 |
| CMW6009 | low | 8 | 0.20 |

[a] values are an average of three repeats, and not significantly different (F=6.21, DF=179, P<0.0001).

[A] Agarose diffusion method of Dingle *et al.*, 1953 was used to determine these units of acitivity of polygalacturonases[8].

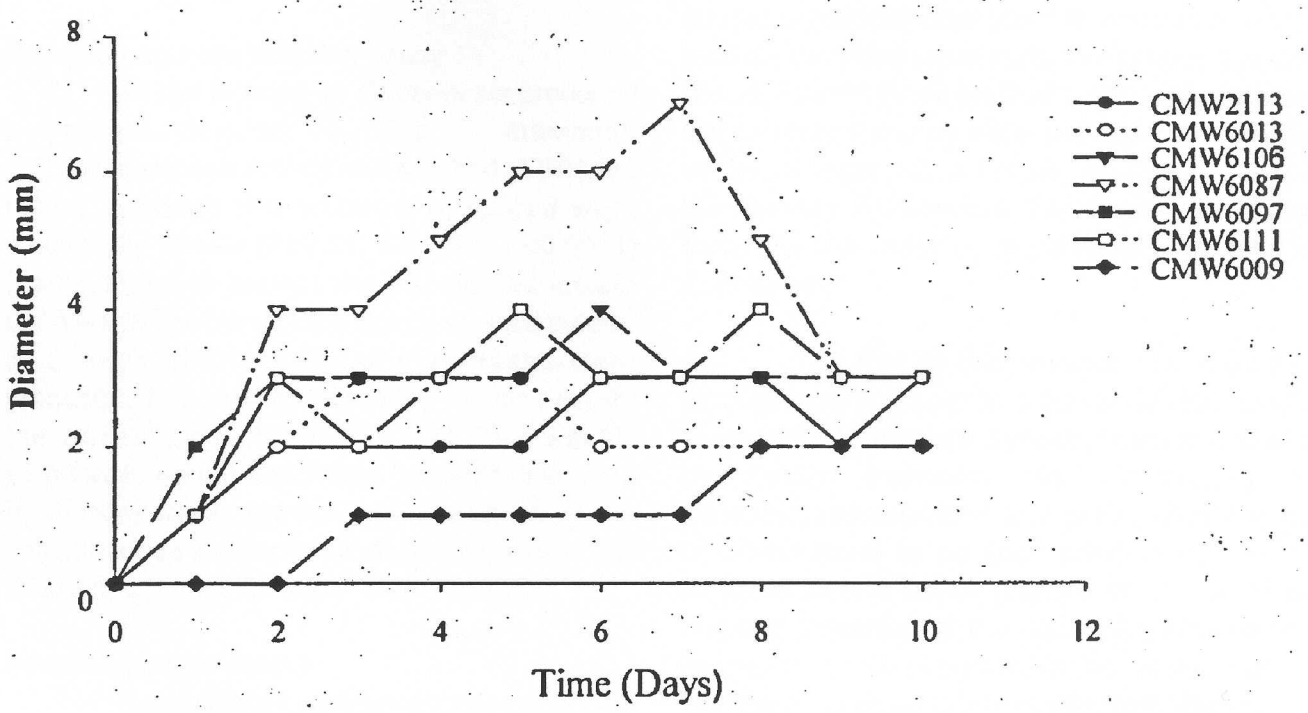


**Fig. - 1. Graphical representation of the trend of polygalacturonase production by seven isolates of C. *cubensis* in minimum salts medium for 10 days. Diameter values are averages of three repeats.**

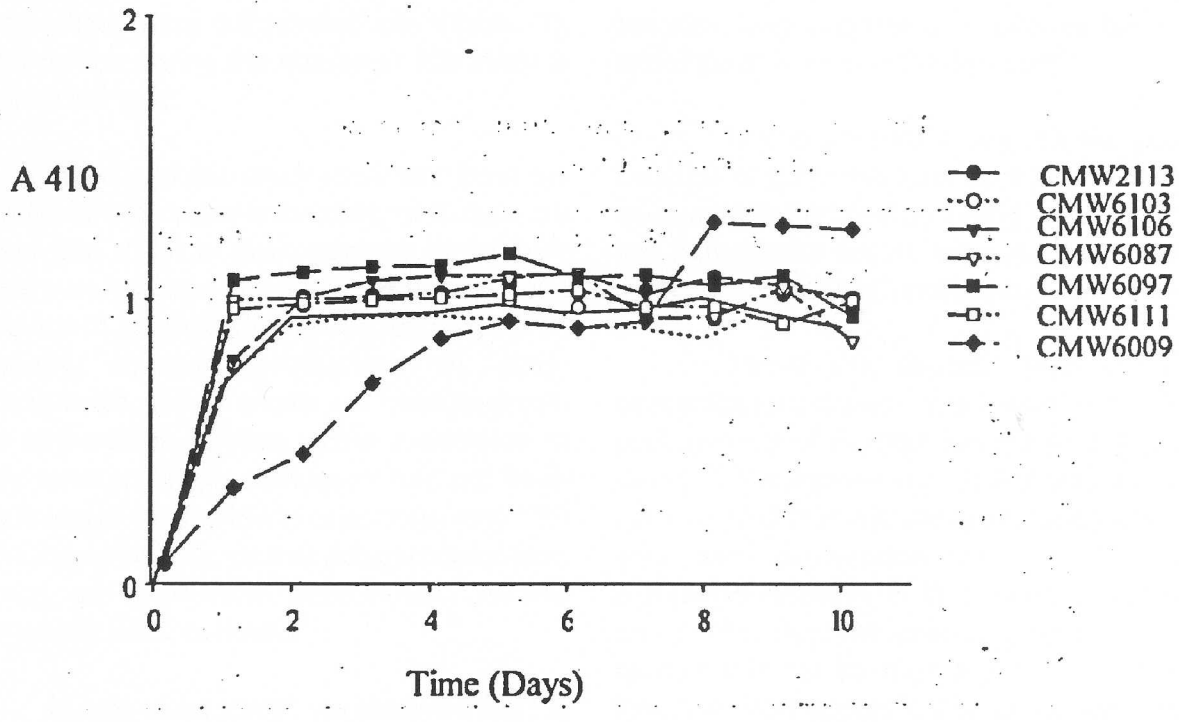


**Fig. - 2. The trend of production of polygalacturonases for seven isolates of *C. cubensis* cultured for 10 days in minimum salts medium. Absorbance values ($A_{410}$) are averages of three repeats. There is a characteristic slow rise in absorbance values for isolate CMW6009. The other six isolates have statistically identical rates of production of polygalacturonases.**

## RESULTS AND DISCUSSION

Cup-plate agarose diffusion assay

All the isolates of *C. cubensis* produced polygalacturonases. The maximum polygalacturonase activity was reached at different times, although the amounts produced were statistically similar (F=6.21, DF=179, P<0.0001) (Table 1) for all except the transfected isolate CMW6009. The transfected isolate showed a delay of 6 days before polygalacturonases were produced. This was considerably longer than for the remaining isolates (Fig. -1). The weakly pathogenic isolate CMW6087 behaved differently. It displayed a gradual increase in poly-galacturonase production until 8 days, which was different to the behaviour of other isolates.

**Reducing sugar Assay**

No significant differences were observed in the production of polygalacturonases for naturally occurring *C. cubensis* using the reducing sugar assay (F=6.21, DF=179, P<0.0001). However, the transfected isolate CMW6009 showed a delayed production of polygalacturonases. A similar trend had been observed with agarose diffusion assays described above. The mean units of PG activity in all the isolates were 0.2 $\mu molml^{-1}min^{-1}$ (Table -1). PG production during the course of this study is illustrated in Fig -2.

Results of this study show that there are no significant differences in the ability of *C. cubensis* isolates that differ in pathogenicity to produce polygalacturonases *in vitro*. However, the hypovirulent isolate CMW6009 displayed an obviously delayed production of poly-galacturonases. These results are consistent with those of previous studies where production of polygalacturonases in pathogens has not been tightly linked to varying levels of pathogenicity[19,13,7]. Our findings also suggest that polygalacturonases are not an important determinant in the pathogenicity of *C. cubensis*.

In a previous study[5], we observed that the DNA sequences of polygalacturonase-inhibiting proteins (PGIPs) in selected *Eucalyptus* species have very similar amino acid sequence. This suggests a low diversity of the PGIPs in the host plant. A high variability and diversity of PGIPs suggests that the host plant is constantly under evolutionary pressure to adapt to a greater diversity of endoPGs[16,20]. Since the PGIPs of *Eucalyptus* have low diversity, it can be expected that the endoPGs of the pathogen, *C. cubensis*, would also have a low diversity. Furthermore, this may suggests that there are few isoforms of polygalacturonases in *C. cubensis*.

The role of endopolygalacturonases in pathogenicity is known to differ for different fungal species[6,13,21,19]. In some species, there is a strong correlation between the production of endopolygalacturonases and pathogenicity, while in others there is no such relationship[6,7,25]. In *C. parasitica*, a close relative of *C. cubensis*, targeted disruption of the endopolygalacturonase (enpg-1) gene resulted in no reduction of pathogenicity on American chestnuts[13]. This implies that the role of endoPGs in the pathogenicity of *C. parasitica* is minor. However, the results of Gao and co-workers (1996)[13] require careful interpretation. Only one isoform of the endoPG was disrupted in their study and this did not result a reduction in pathogenicity. The possibility that other isoforms may still be functional must be considered, because fungi can produce different isoforms of endoPGs at the onset of infection[12,28].

In the present study, all the possible isoforms of polygalac-turonases were collectively assayed. We thus feel confident in our suggestion that polygalacturonases are not a determinant in the pathogenicity of *C. cubensis*.

There are reports that show that polygalacturonases are involved in the pathogenicity of *Botrytis cinerea* and *Aspergillus flavus*[21,6]. The invasiveness of *A. flavus* in wounded cotton bolls was also found to be closely associated with the production of specific fungal polygalacturonases[6]. In *B. cinerea*, production of an endopolygalacturonase (Bcpg1), was responsible for its high level of virulence on tomatoes and apples[21]. Therefore, even though endoPGs appear not to be involved in the pathogenicity of *C. cubensis*, such relationships must be investigated individually from different pathogens.

Results of this study showed that the *C. parasitica* hypovirus (CHV1-713) results in a decrease in the production of polygalacturonases in *C. cubensis.* This provides support for the view that the virus could be useful in biological control of Cryphonectria canker. *In planta*, the isolate has reduced pathogenicity on *Eucalyptus grandis*[22]. In *C. parasitica*, the same hypovirus has been reported to decrease the accumulation of enzymes such as laccases, cutinases, cellobiohydrolases and polygalacturonases and it is associated with hypovirulence[17]. Therefore, the mechanism of action of hypovirulence in *C. cubensis* by the *C. parasitica* hypovirus may involve interaction with polygalacturonase genes or gene products.

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