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# Role of glucose in production and repression of polygalacturonase and pectate lyase from phytopathogenic fungus *Fusarium moniliforme* NCIM 1276

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**Summary**

A mangrove fungal isolate *Fusarium moniliforme* NCIM 1276 was found to produce the maximum extracellular endo-polygalacturonase and pectate lyase activities in liquid medium containing 1% citrus pectin and 0.2% glucose at pH 5 and pH 8, respectively. The enzyme secretion started after 10 h, when the spores were completely germinated, and maximum production occurred after 72 h in the extracellular medium. The production of polygalacturonase was enhanced by 0.2% glucose and completely repressed by 1% glucose. Pectate lyase secretion was also induced by a similar concentration of glucose in the extracellular medium and significantly decreased at 1% glucose. Quantification of extracellular and intracellular polygalacturonase and pectate lyase protein concentrations by ELISA and immunocytolocalization data suggested that glucose may control the expression of both enzymes at a transcriptional level.

*Abbreviations:* PG – polygalacturonase; PL – pectate lyase; PGA – polygalacturonic acid

## Introduction

Plant pathogenic fungi secrete cell-wall degrading enzymes (CWDEs) such as pectinases, which are important in degradation of plant pectin, a polymer of galacturonic acid found in the middle lamella of the cell wall. Cell-wall degrading enzymes are essential for fungal pathogens that do not have specialized penetration structures as well as for necrotrophic pathogens during the late stages of the invasion process. Pectinases are the first enzymes to be secreted by fungal pathogens when they attack plant cell walls (De Lorenzo *et al*. 1997; Idnurm & Howlett 2001).

Polygalacturonase (PG) (EC 1.2.3.15) and pectate lyase (PL) (EC 4.2.2.1) both act on homo-galacturonic regions of the pectin molecule by hydrolytic and trans-elimination cleavage mechanisms, respectively. Secretion of these enzymes during infection of plants has been reported for various plant–pathogenic fungi (Collmer & Keen 1986; Di Pietro & Roncero 1998; Garcia-Maceira *et al*. 2001; ten Have *et al*. 2001), and they have also been described in the non-pathogenic fungus *Rhizoctonia* AG-G (Machinandiarena *et al*. 2005) and in several yeasts (da Silva *et al*. 2005). In addition, *Sclerotinia sclerotiorum* produced several PG isoenzymes during the saprophytic as well as the parasitic mode of growth (Li *et al*. 2004). PG produced by *Erwinia carotovora* (Lei *et al*. 1985) and *Agrobacterium tumefaciens* Biovar 3 (Rodriguez-Palenzuela *et al*. 1991) has been known to determine virulence and cause necrosis in *Vigna unguiculata* (Cervone *et al*. 1987). Apart from acting as virulence factors during infection, endo-PG and PL may also function as virulence determinants by releasing their end products (oligogalacturonides) which act as inducers of plant defense molecules such as phytoalexins (Davis *et al*. 1984). Similarly, PL has been also implicated in the pathogenicity of *Fusarium solani* var *pisi* and *Colletotrichum gloesosporioides* (Crawford & Kolattukudy 1987; Yakoby *et al*. 2000) thereby suggesting that in nature both PG and PL are agents of fungal pathogenicity.

Environmental conditions and medium constituents have been known to affect the expression of PG and PL genes in various pathogenic organisms. For example, in an earlier study it has been shown that, in *Botrytis cinerea*, PG gene expression is subject to ambient pH and the carbon source available. These genes are found to be repressed by glucose and induced by pectin or polygalacturonides present in the medium (Wubben *et al*. 2000), whereas constitutive PG expression was also reported in *B. cinerea* (Van der Cruyssen *et al*. 1994). Additionally, in *Aspergillus nidulans*, the secretion of PG

occurs at low pH and PL at higher pH of the medium (Dean & Timberlake 1989). Recently, another study showed that during ripening of *Persea americana* cv. Fuerte fruits, the pericarp pH regulates PL secretion and affects the pathogenicity of *Colletotrichum gloeosporioides* (Yakoby *et al*. 2000). These evidences suggest that the concentration of glucose as well as medium pH or host cell sap pH may regulate the expression of pectinolytic enzymes during pathogenesis.

While searching for high activity pectinase producers from mangrove ecosystems, a fungal strain identified as *Fusarium moniliforme* was isolated from decaying leaves of mangrove plants (*Rhizophora apiculata* and *Avicennia officinalis*) in the saline, detritus-rich mud of a mangrove estuary on the west coast of India (18°55′ N, 72°54′ E) (Rao 1996). In liquid medium containing 1% citrus pectin as carbon source, the organism produced a single PL at pH 8 and a single PG at pH 5 (Rao *et al*. 1996; Niture *et al*. 2001). In the present study, we have investigated the effect of glucose on the extracellular and intracellular PG and PL expression under different pH conditions by ELISA based quantification of protein contents. Further, glucose acts as a catabolic repressor for enzyme synthesis at cellular level, which was visualized by immunocytolocalization studies.

## Materials and methods

Pectin (citrus fruit 8% methyl esterified), polygalacturonic acid (PGA), di-, tri-, and saturated D-galacturonic acid, Freund's complete adjuvant, horseradish peroxidase (HRP), anti-rabbit IgG (whole molecule) and paraformaldehyde were purchased from Sigma Chemical Company USA. Tetramethylbenzidine/Hydrogen peroxide (TMB/$H_2O_2$) and anti-rabbits IgG gold conjugate were purchased from Bangalore Genei Pvt Ltd India. Divinyl sulfone (DVS) was purchased from Aldrich Chemical Company USA. ELISA plates were purchased from Greiner Labortechnik Pvt Ltd Chandigarh, India. Araldite, DMP-30 (2,4,6)tri[dimethylaminomethyl]phenol), glutaraldehyde and uranyl acetate were purchased from Pelco International USA. Wheat bran was obtained from the local market. All chemicals and reagents were of analytical grade.

### *Liquid medium conditions*

*Fusarium moniliforme* was isolated from decaying leaf litter from a tidal mangrove estuarine ecosystem on the west coast of India (Rao 1996). The fungus was routinely maintained on Czapek–Dox agar containing 1% (w/v) pectin at 10 °C.

In all the experiments reported herein, a spore suspension was prepared in sterile distilled water from a 5-day-old Czapek–Dox agar slant. An aliquot containing $10^6$ spores per ml was inoculated into fresh medium. The medium contained 37.9 mM $(NH_4)_2SO_4$, 11.5 mM $K_2HPO_4$, 14.7 mM $KH_2PO_4$ and 6.8 mM $CaCl_2$ in 50 ml sterile glass-distilled water with 1% pectin and 0.2% glucose as carbon source except where stated. The pH was adjusted to pH 5 for PG and pH 8 for PL with sterile 0.1 M NaOH or 0.1 M HCl. Culture flasks were incubated for 96 h at 200 rev/min and 30 °C. Pectin was replaced with D-galacturonic acid, PGA, cellulose, starch, xylan, wheat bran and glucose in order to determine production of the two enzymes on these carbon sources. The effect of glucose on the production of PG and PL was studied by supplying various concentrations of glucose (0.1–1%) in the pectin medium. Under optimal culture conditions, the time course of enzyme production was examined by harvesting the culture broth at different time periods. Fungal biomass was harvested by centrifugation, washed with sterile distilled water twice and the pellet dried till constant weight at 80 °C. For determination of intracellular PG or PL activity and immunochemical determination of concentration, the wet mycelial pellet was washed with sterile water and sonicated for 1 min in acetate buffer (50 mM) pH 5 or Tris/HCl (50 mM) pH 8. The cell debris was removed by centrifugation and enzyme activity and protein concentrations were determined by standard assay conditions and using sandwich ELISA as described below.

### *Enzyme assays*

*PG*. The assay measured reducing sugars released from 0.3% PGA by the method of Nelson (1944) and Somogyi (1952). The reaction mixture (1 ml) was 0.1 M sodium acetate buffer pH 5.0 with 0.7% NaCl and 0.25% Na–EDTA. The reaction product absorbance was monitored at 500 nm. One unit of enzyme activity is defined as the amount of enzyme, which releases 1 $\mu$mol of galacturonic acid per minute at 40 °C at pH 5.0 (Collmer *et al*. 1988).

*PL*. The assay measured the increase in absorbance at 232 nm of 0.24% PGA in 60 mM Tris/HCl buffer, pH 8.5 with 0.6 mM $CaCl_2$ at 40 °C. One unit of lyase activity was defined as the amount of enzyme, which produces 1 $\mu$mol of unsaturated galacturonide ($\varepsilon = 4600\ M^{-1}\ cm^{-1}$) per minute (Collmer *et al*. 1988).

Protein concentration was determined by the Lowry method using bovine serum albumin as a standard.

### *PG and PL polyclonal antisera preparation*

Extracellular PG and PL produced by *Fusarium* in shake flask cultures were purified as described earlier (Rao *et al*. 1996; Niture *et al*. 2001). Polyclonal antisera were raised as described previously (Niture & Pant, 2004) and purified in accordance with the protocols given by Dunbar & Schwoebel (1990). Protein specific antisera were purified on an immunoaffinity matrix prepared according to the method of Sairam *et al*. (1974) and the specificity of each of the two antisera was individually checked by western dot blot as described earlier (Towbin *et al*. 1979). Both the protein specific antisera were labeled with HRP and

the sandwich ELISA was standardized according to Perlmann & Perlmann (1994). In all further experiments unknown antigen concentrations were quantified using a standard graph of this ELISA as described previously (Niture & Pant 2004).

### *Transmission electron microscopy (TEM)*

*Fusarium*, grown on 1% pectin or 1% glucose alone in standard liquid culture medium conditions for PG or PL production, was fixed in glutaraldehyde, dehydrated in an alcohol series and embedded in araldite (Mollenhauer 1964). Sections were cut with a glass knife on an LKB Bromma 2088 ultratome V then picked up onto nickel grids. Immunolabeling was carried out as described by Horisberger & Rosset (1977). The ultra thin sections were blocked with 1% BSA for 3 h at 25 °C and incubated with primary antibodies of either PG or PL (1 $\mu$g/ml in PBS/T containing 1% BSA) at 4 °C overnight. Subsequently, the sections were washed and incubated with anti-rabbit IgG gold conjugate (1:300 dilution) for 3 h at 25 °C and then stained with uranyl acetate for 10 min in the dark, washed with 5 mM lead citrate and viewed on a Zeiss EM 109 TEM.

### *Statistical analyses*

Each experiment was performed at least 3 times and the data are shown as mean ± SD where applicable. Statistically significant differences between groups in each assay were determined using a Student's *t*-test.

## Results and discussion

### *Western dot blot analysis*

The present fungal strain *Fusarium moniliforme* produced a single form of PG and PL in liquid medium containing 1% pectin at pH 5 and at pH 8, respectively. The biochemical properties of these two enzymes were totally different from each other (Rao *et al.* 1996; Niture *et al.* 2001). Polyclonal antisera were raised against purified PG and PL in two different New Zealand white rabbits and purified as described earlier (Niture & Pant 2004). To examine the specificity and cross-reactivity of antibodies with antigens, western dot blotting was performed. As shown in Figure 1a and b, PG antiserum did not cross-react with PL and the PL antiserum did not cross-react with PG antigen, thereby suggesting that the antibodies were specific for the protein against which they were raised. In an earlier study, we have shown that the antibodies did not cross-react with any other intra- or extra-cellular protein of *F. moniliforme* (Niture & Pant 2004). After labeling with HRP, these protein-specific antibodies were used for ELISA-based quantification intra-or extra-cellular PG and PL concentrations.

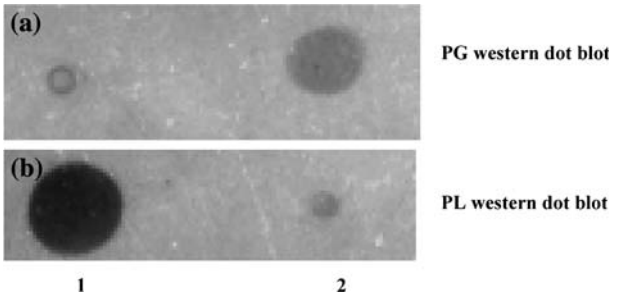


*Figure 1.* Western dot blots of PG and PL (a) PG western dot blot. (b) PL western dot blot. Spot 1: PL antigen (0.2 $\mu$g)-coated. Spot 2: PG antigen (0.2 $\mu$g)-coated

### *Effect of different carbon sources on the production of PG and PL activity*

The present fungus was isolated from decaying leaves of mangrove plant where its mode of nutrition is saprophytic rather than pathogenic. Under laboratory conditions when *F. moniliforme* was grown in medium containing different carbon sources, it produced both the pectin-degrading enzymes, *viz*., PL and PG at pH 5 and at pH 8 (Table 1), irrespective of whether the medium contained D-galacturonic acid, PGA, pectin, cellulose, starch, xylan or wheat bran (1%, w/v each). In the presence of 1% pectin, the production of both enzymes was substantially enhanced (0.23 U/ml for PG and 3.6 U/ml for PL) as compared to other carbon sources. In contrast, in the presence of 1% (w/v) glucose, no measurable PG activity was detected in the extracellular medium at pH 5. However, a low level of PL activity (0.05 U/ml) was detected even in the presence of 1% glucose only at pH 8 under the standard assay conditions.

### *Effect of glucose on extracellular enzyme production*

To examined the role of glucose on the regulation of PG and PL production, the isolate was grown in the presence of different concentrations of glucose (0.1–1%)

*Table 1.* Effect of different carbon sources on PG and PL production at pH 5 and 8, respectively.

| Substrate (1% w/v) | PG (U/ml) | PL (U/ml) |
|---|---|---|
| D-galacturonic acid | 0.12 ± 0.01 | 0.16 ± 0.12 |
| Polygalacturonic acid | 0.21 ± 0.03 | 2.2 ± 0.23 |
| Pectin | 0.23 ± 0.08 | 3.6 ± 0.51 |
| Cellulose | 0.013 ± 0.012 | 1.0 ± 0.23 |
| Starch | 0.08 ± 0.02 | 0.3 ± 0.08 |
| Xylan | 0.06 ± 0.01 | 0.6 ± 0.03 |
| Wheat bran | 0.04 ± 0.001 | 1.74 ± 0.01 |
| Glucose | ND | 0.05 ± 0.01 |

Each value represents the mean of replicated values ± SE. Enzyme activities were determined after 96 h of fungal growth.
ND: not detected.

*Table 2.* Effect of glucose on extracellular PG and PL production at pH 5 and 8 in 1% pectin medium.

| Glucose % (w/v) | Biomass (mg/ml) | PG (U/ml) | PG (μg/ml) | PL (U/ml) | PL (μg/ml) |
|---|---|---|---|---|---|
| 0 | 2.1 ± 0.02 | 0.23 ± 0.1 | 5.1 ± 0.1 | 3.6 ± 0.12 | 18.2 ± 0.03 |
| 0.1 | 4.1 ± 0.5 | 0.25 ± 0.3 | 5.7 ± 0.21 | 4.0 ± 0.2 | 22 ± 1.3 |
| 0.2 | 5.8 ± 0.3 | 0.28 ± 0.01 | 7.7 ± 0.3 | 8.1 ± 0.4 | 27.2 ± 2.4 |
| 0.4 | 6.1 ± 0.2 | 0.1 ± 0.01 | 3.2 ± 0.65 | 6.2 ± 0.81 | 20.8 ± 1.5 |
| 0.6 | 6.3 ± 0.1 | 0.1 ± 0.01 | 1.5 ± 0.1 | 4.3 ± 0.44 | 12.7 ± 0.6 |
| 0.8 | 6.3 ± 0.2 | 0.02 ± 0.001 | 0.28 ± 0.1 | 2.0 ± 0.01 | 8.1 ± 0.3 |
| 1.0 | 6.4 ± 0.2 | ND | ND | 1.6 ± 0.1 | 5.0 ± 0.1 |

All values are 96 h after inoculation. Each value represents the mean of replicated values ± SE. Protein concentrations were determined by Sandwich ELISA.
ND: not detected.

with the same concentration of pectin (1%) at different pH values and fungal biomass, extracellular activities and protein concentrations were measured. On increasing the glucose concentration in the combined medium (pectin + glucose), an increase in the biomass from 4.1 mg/ml at 0.1% to 6.4 mg/ml at 1% was observed (Table 2). PG activity and protein concentration was also increased with increasing glucose concentration of the medium from 0.1 to 0.2%. However, at high glucose concentrations (0.4–1%), a gradual decreased in enzyme activity and PG protein concentration was observed, which was undetectable at 1% glucose concentration under the given assay conditions. These data indicate that at higher glucose concentrations, PG production was repressed, whereas an initial concentration of 0.2%, glucose (w/v) was required for induction of the enzyme. On the other hand, measurable PL activity was observed irrespective of the glucose concentration up to 1% at pH 8 (Table 2). Maximal PL production (27.2 μg/ml) as well as enzyme activity (8.1 U/ml) was noted in the presence of 0.2% glucose, which decreased at higher glucose concentrations (Table 2). It was also noted that, the organism also produced a higher amount of PL activity and protein concentrations compared with PG in the corresponding media. While no detectable PG protein was observed in 1% glucose medium, measurable amounts of PL activity (1.6 U/ml) and PL specific protein (5 μg/ml) were detected. This suggests that although the overall production of PL protein was repressed on increasing the medium glucose concentrations complete repression was not observed as with PG. Hence, it may be suggested that, the production of these enzymes in *Fusarium* is dependent on the balance between the availability of substrate (pectin) and catabolite repressor i.e., glucose. A similar case was found in the production of pectinolytic enzymes from *Aspergillus* sp (Aguilar & Huitron 1987). The effect of varying the pectin concentration on PG and PL production showed that 1% pectin along with 0.2% glucose was essential for maximum production of both enzymes (0.28 U/ml of PG, and 8.1 U/ml of PL) whereas, lower or more than 1% (w/v) concentration of pectin decreased the production of both enzymes in the extracellular medium (data not shown).

*Effect of glucose on intracellular synthesis of PG and PL*

Next we studied the effect of glucose on the intracellular synthesis of PG and PL in the presence of pectin. When the fungus was supplied with pectin (1%) + glucose (0.2%), the organism synthesized maximum intracellular PG and PL (PG 77 ± 4.5 ng/mg of biomass, PL 82 ± 5.3 ng/mg of biomass) at pH 5 and at pH 8, respectively compared with pectin alone (Figure 2). However, in the presence of 0.5–1% glucose along with 1% pectin, there was a 95% decrease in intracellular PG production. The effect of glucose on PL synthesis was slightly different than with PG. In the presence of 0.5% glucose only ~20% reduction in the intracellular concentration of PL was observed whereas, at 1% glucose concentration there was an 80% decrease in PL synthesis compared with 0.2% glucose and 1% pectin. Interestingly, when the fungus was grown in the presence of glucose alone, the organism synthesized significant amount of PL (15 ng/mg biomass) however, intracellular PG concentration was not detectable under these conditions. These data clearly indicate that glucose acts as a catabolic repressor for PG synthesis. Similar results were previously reported by De Lorenzo *et al.* (1987) using their fungal strain of *F. moniliforme*, which produced

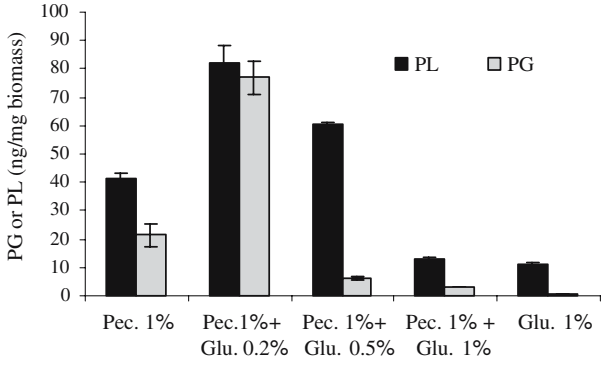


*Figure 2.* Effect of glucose on intracellular protein concentration of PG and PL at pH 5 and 8. The intracellular protein contents of PG and PL were determined by sandwich-ELISA as described in Materials and methods section. Pec. = Pectin. Glu. = glucose.

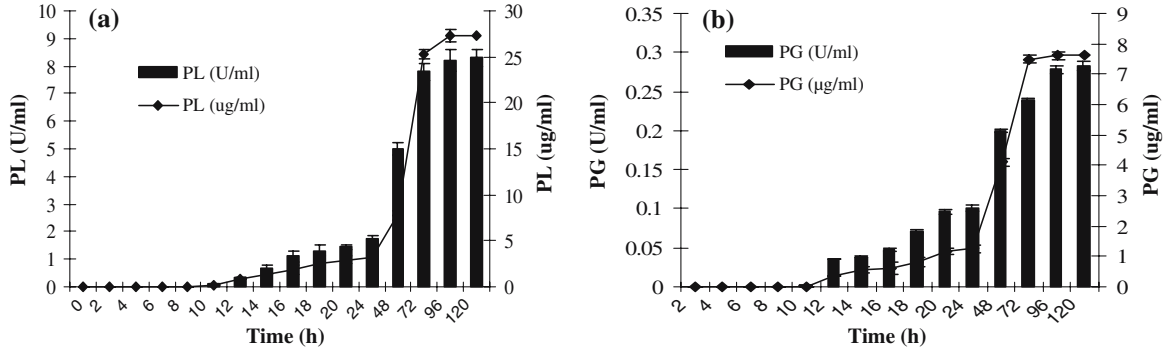


*Figure 3*. Time course of PG and PL production in liquid medium by *F. moniliforme* at pH 5 and at pH 8. (a) PL. (b) PG.

several PG isoenzymes and showed that glucose repressed the PG mRNA and protein production.

### *Time course of PG and PL production*

The time course of PG and PL production at pH 5 and at pH 8 during spore germination was studied. *Fusarium* spores were inoculated into fresh medium containing 1% pectin, and spore germination was observed under the light microscope. After 8 h of incubation at 30 °C, germ tube formation was observed and 80% of the spores were germinated within 10 h. Septum formation and mycelium development began at 10 h after inoculation (data not shown). Both PG and PL protein contents as well as activities were detected in the extracelluar medium by 10 h after inoculation (Figure 3a and b). In contrast, Barashi (1968) has studied endo-PG secretion during spore germination of *Geotrichum candidium* and showed that the release of the enzyme into the extracellular medium occurred even prior to germtube formation. The present isolate did not secrete either PG or PL during the early stages of spore germination. The maximum increase in PG and PL occurred between 24 and 72 h after inoculation and in both cases, the production reached a plateau after 72 h with no further increase in either protein production or enzyme activity being observed (Figure 3a and b).

### *Immunocytolocalization of PG and PL*

Transmission electron micrographs of *Fusarium* microconidia showed that, when the fungus was grown in the presence of 1% (w/v) glucose alone at pH 5, no intracellular PG protein signal was detected in the microconidia or mycelium (Figure 4a). In contrast, in the microconidia or mycelium of *Fusarium* when grown on 1% pectin-containing medium an intracellular PG signal was detected by immunostaining. Intracellular PG protein was localized at the vacuolar region as well as near to the inner side of the cell membrane (Figure 4b, arrow). This data again supported the earlier results where in the presence of 1% glucose no extra- or intracellular PG protein was detected by ELISA (Table 1, Figure 2). Generally in fungi, vacuolar protein transport systems for extracellular secretion of protein have been reported (Takegawa *et al*. 2003). In the current study, we observed the accumulation of PG protein in the vacuole attached to the inner cell membrane (Figure 4b) possibly prior to extracellular secretion**.** Thus the TEM and ELISA data suggest that, in *Fusarium* the synthesis of intracellular PG and its eventual secretion were induced by pectin but not by glucose, which acts as catabolite repressor. Catabolite repression of PG and PL synthesis by glucose during saprophytic growth conditions and during pathogenesis has been reported in a number of fungi (Dean & Timberlake 1989; Di Pietro & Roncero, 1998; Wubben *et al*. 2000; de las Heras *et al*. 2003) including *F. moniliforme* (De Lorenzo *et al*. 1987) and a similar situation was observed in the present study. On the other hand this fungus produced about 1.2% of PL activity in the presence of glucose as compared with pectin (Table 1) suggested that, even 1% of glucose did not repress PL synthesis completely. Similarly, when the fungus was grown in the presence of glucose, a lower signal of PL was detected by immunostaining compared with pectin-grown mycelium (Figure 4c and d). Intracellular PL was also found to be localized in the vacuole attached to the inner cell membrane (Figure 4c). However, its synthesis was found to be substantially induced by pectin and the majority of PL molecules were localized near to the cell wall components possibly prior to extracellular secretion. (Figure 4d, arrow). These data suggests that intracellularly, although the production of PL in the presence of glucose was reduced as compared to pectin, total repression of PL did not occur at a concentration of 1% glucose. Thus, ELISA data and immunocytolocalization results confirmed glucose as a catabolic repressor for PG synthesis in *F. moniliforme*. The detailed molecular mechanism to support this hypothesis and the role of other sugars or oligogalacturonides in the regulation of PG and PL during plant pathogenesis is underway in our laboratory.

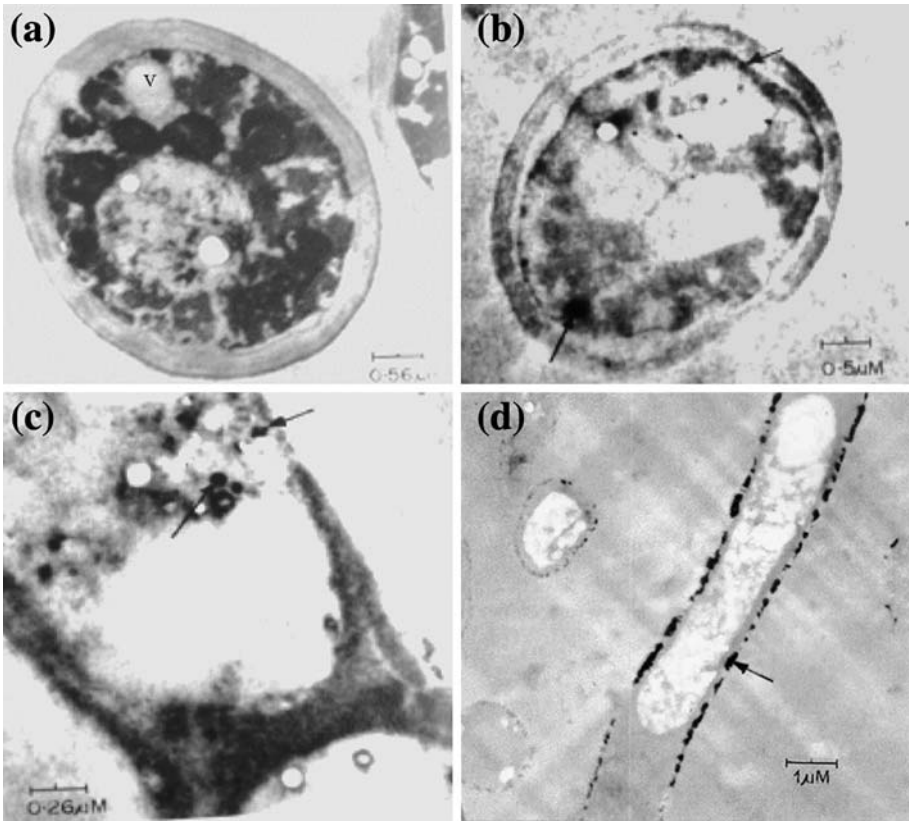


*Figure 4*. Immunocytolocalizaton of PG and PL. PG antibody used. (a) Glucose-grown microconidia of *Fusarium*, (V, Vacuole) (b) pectin-grown microconidia of *Fusarium* (arrow indicates the gold particles localized at the inner side of the cell membrane). PL antibody used. (c) Glucose-grown mycelium segment of *Fusarium*, (d) pectin-grown mycelium segment of *Fusarium* (arrow indicates the gold particles localized near to the cell wall components).

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## References

Aguilar, G. & Huitron, C. 1987 Stimulation of the production of extracellular pectinolytic activities of *Aspergillus* sp. by galacturonic acid and glucose addition. *Enzyme and Microbial Technology* **9**, 690–696.

Barashi, I. 1968 Liberation of polygalacturonase during spore germination by *Geotrichum candidum*. *Phytopathology* **58**, 1364–1371.

Cervone, F., De Lorenzo, G., Degra, L. & Salvi, G. 1987 Elicitation of necrosis in *Vigna unguiculata* walp by homogeneous *Aspergillus niger* endo-polygalacturonase and by α-D-galacturonate oligomers. *Plant Physiology* **85**, 626–630.

Collmer, A. & Keen, N. 1986 The role of pectic enzymes in plant pathogenesis. *Annual Review of Phytopathology* **24**, 383–409.

Collmer, A., Reid, J. & Mount, M. 1988 Assay methods for pectic enzymes. *Methods in Enzymology* **161**, 329–335.

Crawford, M.S. & Kolattukudy, P.E. 1987 Pectate lyase from *Fusarium solani* f. sp. *Pisi*: purification characterization, *in vitro* translation of the mRNA, and involved in pathogenicity. *Archives of Biochemistry and Biophysics* **258**, 196–205.

da Silva, E.G., de Fatima Borges, M., Medina, C., Piccoli, R.H. & Schwan, R.F. 2005 Pectinolytic enzymes secreted by yeasts from tropical fruits. *FEMS Yeast Research* **5**, 859–865.

Davis, K., Lyon, G., Darvill, A. & Albersheim, P. 1984 Host–pathogen interaction XXV, Endo-polygalacturonic acid lyase from *Erwinia carotovora* elicits phytoalexin accumulation by releasing plant cell wall fragments. *Plant Physiology* **74**, 52–60.

de las Heras, A., Patino, B., Posada, M.L., Martinez, M.J., Vazquez, C. & Gonzalez Jean, M.T. 2003 Characterization and *in vitro* expression patterns of an exo-polygalacturonase encoding gene from *Fusarium oxysporum* f.sp. *radicis lycopersici*. *Journal of Applied Microbiology* **94**, 856–864.

De Lorenzo, G., Salvi, G., Degra, L., D'Ovodia, R. & Cervone, F. 1987 Induction of extracellular polygalacturonase and its mRNA in the phytopathogenic fungus *Fusarium moniliforme* . *Journal of General Microbiology* **133**, 3365–3373.

De Lorenzo, G., Castoria, R., Bellincampi, D. & Cervone, F. 1997 Fungal invasion enzymes and their inhibition. In *The Mycota V, Part B: Plant Relationship*, ed. Carroll G.C.-P. Tudzynski, pp. 61–83. Berlin. Springer-Verlag: ISBN 3-540-58006-9.

Dean, R.A. & Timberlake, W.E. 1989 Production of cell wall-degrading enzymes by *Aspergillus nidulans*: A model system for fungal pathogenesis of plant. *The Plant Cell* **1**, 265–273.

Di Pietro, A. & Roncero, M.I. 1998 Cloning expression and role in pathogeniciry of *pgI* encoding the major extracellular endopolygalacturonase of the vascular wilt pathogen *Fusarium oxysporum*. *Molecular Plant Microbial Interactions* **11**, 91–98.

Dunbar, B.S. & Schwoebel, E.D. 1990 Preparation of polyclonal antibodies. *Methods in Enzymology* **182**, 663–670.

Garcia-Maceira, F.I., Di Pietro, A., Huertas-Gonzalez, M.D., Ruiz-Roldan, M.C. & Roncero, M.I. 2001 Molecular characterization of an endo-polygalacturonase from *Fusarium oxysporum* expressed during early stages of infection. *Applied and Environmental Microbiology* **67**, 2191–2196.

Horisberger, M. & Rosset, J. 1977 Colloidal gold, a useful marker for transmission and scanning electron microscopy. *Journal of Histochemistry and Cytochemistry* **25**, 295–305.

Idnurm, A. & Howlett, B.J. 2001 Pathogenicity genes of phyto-pathogenic fungi. *Molecular Plant Pathology* **2**, 241–255.

Lei, S.P., Lin, H.C., Heffernan, L. & Wilcox, G. 1985 Evidence that polygalacturonase is a virulence determinant in *Erwinia carotovora*. *Journal of Bacteriology* **164**, 831–835.

Li, R, Rimmer, R., Buchwaldt, L., Sharpe, A.G., Seguin-Swartz, G. & Hegedus, D.D. 2004 Interaction of *Sclerotinia sclerotiorum* with *Brassica napus*: cloning and characterization of endo- and exo-polygalacturonases expressed during saprophytic and parasitic modes. *Fungal Genetics and Biology* **41**, 754–765.

Machinandiarena, M.F., Wolski, E.A., Barrera, V., Daleo, G.R. & Andreu, A.B. 2005 Characterization and *in vitro* expression patterns of extracellular degradative enzymes from non-pathogenic binucleate *Rhizoctonia* AG-G. *Mycopathologia* **159**, 441–448.

Mollenhauer, H.H. 1964 Plastic embedding mixtures for use in electron microscopy. *Stain Technology* **39**, 111–114.

Nelson, N. 1944 A photometric adaptation of the Somogyi method for the determination of glucose. *Journal of Biological Chemistry* **153**, 375–380.

Niture, S.K., Pant, A. & Kumar, A.R. 2001 Active site characterization of the single endo-polygalacturonase produced by *Fusarium moniliforme* NCIM 1276. *European Journal of Biochemistry* **268**, 832–840.

Niture, S.K. & Pant, A. 2004 Purification and biochemical characterization of polygalacturonase II produced in semi-solid medium by a strain of *Fusarium moniliforme*. *Microbiological Research* **159**, 305–314.

Perlmann, H. & Perlmann, P. 1994 Enzyme-linked immunosorbent assay. In *Cell Biology*. *A Laboratory Handbook*, ed. Celis J. E., Vol. 2, pp. 322–327. San Diego, CA. Academic Press: ICBN 0-121-64727-7.

Rao, M.N. 1996 *Pectinases from Molds: Their production, purification, characterization and applications*, India: University of Pune, Ph.D. Thesis.

Rao, M.N., Kembhavi, A. & Pant, A. 1996 Role of lysine, tryptophan and calcium in the $\beta$-elimination activity of a low molecular mass pectate lyase from *Fusarium moniliforme*. *Biochemical Journal* **319**, 159–164.

Rodriguez-Palenzuela, P., Burr, T.J. & Collmer, A. 1991 Polygalacturonase is a virulence factor in *Agrobacterium tumefaciens* biovar-3. *Journal of Bacteriology* **173**, 6547–6552.

Sairam, M.R., Clarke, W.C., Chung, D., Porath, J. & Li, C.H. 1974 Purification of antibodies to protein hormones by affinity chromatography on divinylsulfonyl sepharose. *Biochemical and Biophysical Research Communication* **61**, 355–359.

Somogyi, M. 1952 Notes on sugar determination. *Journal of Biological Chemistry* **195**, 19–23.

Takegawa, K., Hosomi, A., Iwaki, T., Fujita, Y., Morita, T. & Tanaka, N. 2003 Identification of a SNARE protein required for vacuolar protein transport in Schizosaccharomyces pombe. *Biochemical and Biophysical Research Communication* **311**, 77–82.

ten Have, A., Breuil, W.O., Wubben, J.P., Visser, J. & van Kan, J.A. 2001 *Botrytis cinerea* endopolygalacturonase genes are differentially expressed in various plant tissues. *Fungal Genetics and Biology* **33**, 97–105.

Towbin, H., Staehelin, T. & Gordon, J. 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitro-cellulose sheets: procedure and some applications. *Proceedings of the National Academy Sciences of the USA* **76**, 4350–4354.

Van der Cruyssen, G., De Meester, E. & Kamoen, O. 1994 Expression of polygalacturonases of *Botrytis cinerea in vitro* and *in vivo*. *Mededelingen van de Faculteit Landbouwkunde en Toegepaste Biologische Wetenschappen Universiteit Gent* **59**, 895–905.

Wubben, J., ten Have, A., van Kan, J. & Visser, J. 2000 Regulation of endopolygalacturonase gene expression in *Botrytis cinerea* by galacturonic acid, ambient pH and carbon catabolite repression. *Current Genetics* **37**, 152–157.

Yakoby, N., Kobiler, I., Dinoor, A. & Prusky, D. 2000 pH regulation of pectate lyase secretion modulates the attack of *Colletotrichum gloeosporioides* on avocado fruits. *Applied and Environmental Microbiology* **66**, 1026–1030.