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The Effect of Calcium on the Expression of Polygalacturonase Activity by *Colletotrichum acutatum* in Apple Fruit

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Abstract: The effect of increasing the calcium concentration of Golden Delicious apple cell walls on polygalacturonase (PG) expression by *Colletotrichum acutatum*, the bitter rot pathogen, was examined. Fungal growth and PG expression were determined by growing *C. acutatum* in Richard's solution supplemented with cell walls extracted from 0 or 4% calcium chloride treated fruit. During the first 18 h PG activity was not detected. However, within 24 to 27 h, the PG activity increased and was approximately 45% greater in medium containing cell walls from 0% calcium treated apples as opposed to medium containing cell walls from 4% calcium treated apples. After 72 h, PG activity in both media was similar. These results correspond with qualitative PG gene expression studies using the Reverse Transcription Polymerase Chain Reaction (RT-PCR) of RNA isolated 24 h post inoculation. Zymograms showed that minor PG isozymes were expressed differently depending on whether the medium was amended with cell walls from calcium treated or non-treated apples, although the four major PG isozymes detected were produced to approximately the same extent under both growth conditions.

Key words: Bitter rot, decay, $CaCl_2$, isozyme, postharvest, pressure infiltration, RT-PCR

INTRODUCTION

Postharvest calcium (Ca) treatment of apple fruit has been shown to reduce decay of several postharvest diseases including blue mold (*Penicillium expansum*), gray mold (*Botrytis cinerea*) and bitter rot (*Colletotrichum acutatum*) (Conway *et al*., 1991). Among these diseases, Ca treatment seems to be the most effective in reducing decay caused by the bitter rot fungus, *C. acutatum*. Bitter rot can be especially severe in orchards during warm and humid weather that provides optimum conditions for spore dissemination and germination. This fungus is also responsible for significant postharvest losses in apple.

The relationship between Ca and the plant cell wall is thought to play a key role in the resistance mechanism. Postharvest treatment of apple fruit results in Ca being incorporated in the cell wall. Conway *et al*. (1988) measured the Ca concentration in cell walls extracted from apple fruit that had been infiltrated postharvest with $CaCl_2$ solutions. They reported that the Ca concentration in apple cell walls increased proportional to the $CaCl_2$ concentration in the treatment. Also, the activity of polygalacturonase (PG, EC 3.2.1.15), produced by *P. expansum*, appeared to be repressed when high Ca cell walls were used as an enzyme substrate (Conway *et al*., 1988).

It has been hypothesized that Ca reinforced cell walls may reduce fruit decay by making cell walls less accessible to PG (Conway *et al*., 1988). Ca alone has shown some direct effects on pectolytic enzyme production by *C. gloeosporioides*. Mason and Jeffries (1993) reported that the Ca concentration in the medium affected the activity of pectate lyase extracted from *C. gloeosporioides* by stimulating enzyme production, while PG production was maintained at the same level or was slightly inhibited.

The expression of pectolytic enzyme genes in bacterial plant pathogens has been shown to be affected by Ca as well. Flego *et al*. (1997) showed that pectate lyase gene expression in *Erwinia carotovora* was induced while PG gene expression was suppressed in

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medium amended with Ca. Additionally, Ca treated tobacco plants showed increased resistance to soft rot caused by *E. carotovora*. Ca is also involved in various plant defense responses, especially where Ca-binding oligogalacturonic acid triggers the synthesis of phytoalexins (Messian *et al*., 1993).

It is difficult to investigate the relationship between the host defense response and the effect of Ca on the host cell wall and the resulting inhibitory activity against pathogens in whole fruit. Therefore, for *in vitro* experiments, we extracted cell walls from Ca treated apples for use as the sole carbon source in fungal growth medium. Since previous work has shown that Ca solutions infiltrated into apple fruit reduced decay caused by the bitter rot fungus (Conway *et al*., 1991), the objective of this study was to determine the effect of Ca on the expression of PG produced by *C. acutatum*.

MATERIALS AND METHODS

Fruit and calcium treatment: Golden Delicious (*Malus domestica* Borkh.) apples were harvested in the pre-climacteric stage from a commercial orchard in Pennsylvania and randomized. The fruit were then infiltrated under a pressure of 68.95 kPa for 2 min at room temperature with 0, 2% (20 g L^{-1}) or 4% (40 g L^{-1}) solutions (w/v) of calcium chloride ($CaCl_2.2H_2O$) in distilled water. Fruit were air-dried for 2 h and stored at 0°C until used.

Pathogen: The *C. acutatum* isolate was obtained from K.D. Hickey at the Penn State Fruit Laboratory and Extension Center, Biglerville, Pennsylvania. A working stock of the fungus was routinely grown on potato dextrose agar (Difco laboratories, Detroit) at 20°C under continuous fluorescent lighting and refreshed every two weeks.

Cell wall extraction: The apple peel and outer flesh were removed to a depth of 2 mm with a mechanical peeler and discarded. The next 3 mm of flesh was similarly removed and stored in 80% ethanol at –80°C until used. The frozen samples were then removed from storage, thawed and homogenized in 20 mM HEPES-NaOH buffer (pH 6.9) containing 0.2% SDS using a Omni mixer homogenizer (Warrenton, VA). The homogenate was filtered through 2 layers of Miracloth. The filtrate was then resuspended in chloroform-methanol (1:1, v/v) for 10 min. The residue was filtered with a sintered glass filter and resuspended in acetone for 10 min. The acetone treated residue was filtered again through the same filter and the sample was placed in a fume hood to evaporate the acetone. The sample was then completely dried in a vacuum oven at 40°C with P_2O_5 for 48 h.

Cell wall calcium content: Dried cell walls (125±5 mg) were ashed at 500°C overnight. The residue was dissolved in 5 mL of 2 N HCl and analyzed for Ca content with a Thermo-Jarrell Ash Model 61 Inductively Coupled Argon Plasma (IACP)-Atomic Emission Spectrometer (AES) (Wilmington, MA). Three samples were measured per treatment.

Pathogenicity test: Fruit inoculation was carried out by wounding apples (surface disinfested with 70% ethanol) at two opposite points on the equator to a depth of 5 mm and a width of 3 mm. Each wound was inoculated with 25 μL of a *C. acutatum* spore suspension (10^5 conidia per mL). After two weeks incubation at 20°C, the apples were rated for severity of decay by measuring the diameter of the decayed area and then computing the total area of decay. The diameter of each lesion was measured on ten fruit per treatment.

Protein and PG activity determination: For *in vitro* studies the decayed tissues were divided into three parts (Fig. 4A). These parts refer to different regions of the lesion; the outer edge, the center and the interior region between the center and outer edge. Apple juice to be analyzed was obtained by squeezing the decayed tissues through an injection syringe. After desalting by using a Centricon YM-10 filter (Millipore, Bedford, MA), PG activity was determined using the reducing sugar assay method (Gross, 1982). This method measures the amount of reducing sugar released from sodium polypectate washed with 80% ethanol to remove residual galacturonic acids. The standard curve for reducing groups was established with D-galacturonic acid. The substrate for the assay was 100 μL of 0.4% sodium polypectate in 100 mM sodium acetate buffer (pH 5.5), which was mixed with a 100 μL aliquot of the sample. The mixture was incubated for 30 min at 37°C and the reaction was terminated by adding 1.2 mL of 100 mM boric acid-borax buffer (pH 9.0) containing 0.1% 2-cyanoacetoamide. Each sample was assayed along with negative controls. Terminating buffer was added to the negative controls prior to incubation. These controls determine the amount of reducing sugar originally present and not the products of the enzyme reactions. Samples were immersed in a boiling water bath for 10 min and cooled on ice. The amount of reducing groups was determined by absorbance at 276 nm. One unit of PG activity was defined as the amount of enzyme required to release 100 pmol of reducing groups per min. Three samples were measured from each of the three parts of the decayed area.

For *in vitro* studies, the fungus was grown at 28°C with shaking at 150 rpm in Richard's solution containing

apple cell walls from fruit treated with 0 or 4% $CaCl_2$ solutions as the sole carbon source. Richard's solution contained 10 g of KNO_3, 5 g of K_2HPO_4, 2.5 g of $MgCl_2$ and 20 mg of $FeCl_3$. Apple cell walls were added at 0.1% w/v in 25 mL of culture solution in 50 mL flasks. Samples were taken daily over a 5 day period to determine PG activity and protein concentration. PG activity was determined as stated above. The amount of protein was determined by the Bradford (1976) method using a protein assay kit (Bio-Rad). Bovine serum albumin was used as the standard. An 800 μL aliquot of each sample was mixed with 200 μL of reaction mixture. The protein was detected at an absorbance of 595 nm. For all PG activity and protein determinations there were three flasks per treatment with three samples being taken from each flask.

Analysis of PG expression: For PG activity expression studies, fungal mycelia (25 mL cultures) were grown at 28°C with shaking at 150 rpm in Richard's solution containing 1% glucose. The cultures were harvested by vacuum infiltration and washed twice with 50 mL of sterile distilled water to remove the glucose. The resulting mycelia were then transferred to Richard's solution with apple cell walls from fruit treated with 0 or 4% $CaCl_2$ solutions as the sole carbon source. The fungal mycelia were harvested every 3 h between 18 and 27 h after inoculation to observe differentially induced PG activity in the amended media. Three samples were assayed for PG activity from each flask and there were three flasks per treatment.

For PG gene expression studies, RNA and reverse transcription (RT-PCR) procedures were carried out following the methods of Gettemy *et al.* (1998) with slight modifications. Fungal mycelia were grown in cell wall medium (25 mL cultures) at 28°C with shaking at 150 rpm, centrifuged and then used for RNA extraction.

DNA was removed by incubation with RQ DNase (Promega, Madison, WI) at 37°C for 30 min prior to synthesis of cDNA. After denaturing the DNase by heating at 65°C for 10 min, cDNA was synthesized using AMV reverse transcriptase (Promega, Madison, WI). One μg of RNA was mixed with 30 units of AMV RTase, 5 μL of the manufacturer's AMV RTase- reaction buffer, 1 μL of 50 μM oligo dT_{20} and 2.5 μL of 20 mM dNTP and incubated at 42°C for 1 h and then at 52°C for 30 min. PCR was performed using 50 pmoles of a forward gene specific primer for CAPG1 5′-CGGAGTTGATGGCTAGGCAGT-3′ and a reverse primer 5′-AAGTTCTTATAGCCCACAGC-CCA-3′. The complement sequence was synthesized at 94°C for 5 min, 61°C for 2 min and 72°C for 5 min, followed by PCR with 30 cycles of 94°C for 2 min, 61°C for 2 min and 72°C for 5 min and a final extension for 15 min at 72°C. PCR products (7-10 μL) were loaded onto a 1.5% agarose gel and separated by electrophoresis at 100 volts/cm for 30 min. After staining the gel in 0.05% ethidium bromide, the intensities of bands were estimated using the AlphaEase™ program after capturing a picture with an Alphaimager 1220 v. 5.5 (Alpha Innotech Co. St. San Leandro, CA). Ribosomal RNA primers were used to normalize for the quantity of RNA in each lane. The sequence of rRNA was obtained from conserved rRNA sequences from various *Colletotrichum* species. The primer sequence for RT-PCR of rRNA was 5′-GTAAAGCTC-CTTCGACGAGT-3′ for the forward and 5-GGTTGACTCCTTGGTCCGTGT-3′ for the reverse complement primer. The fungal mycelia obtained at the 24 h time point was used for RNA extraction and RT-PCR to investigate CAPG1 expression during the early stages of growth. This study was repeated twice.

Isozyme profiles: Zymograms were performed on PGs produced by *C. acutatum* grown in Richard's Solution amended with cell wall extracts from 0 or 4% $CaCl_2$ infiltrated apples. This analysis was conducted following the method of Ried and Collmer (1985). Isoelectric focusing was performed with a pre-cast IEF gel (pH 3.5-9.5, Amersham-Pharmacia, Piscataway, NJ) in a Pharmacia Multiphor II system following the manufacturer's instructions (Amersham-Pharmacia). Prefocusing was conducted at 4 W for 30 min with further focusing at 10 W for 1.5 h. The electrode strip was soaked in 0.04 M phosphoric acid for the cathode and 0.3 M sodium hydroxide for the anode. An IEF standard marker kit (pI range 4.45-9.6) was purchased from Bio-Rad (Hercules, CA) and diluted 5 fold prior to use. Five microliter samples and a marker were loaded on the gel using IEF sample application pieces (Amersham-Pharmacia). The resulting IEF gel was divided into two pieces, one of which contained the pI marker and the other contained the PG isozymes and each piece was stained separately. The pI marker was stained with Commassie blue (0.025%). The agarose gel and the piece of the IEF gel containing the PG isozymes were placed between two glass plates in order to transfer the protein to the agarose gel according to the method of Bertheau *et al.* (1984). This assembly was incubated at 37°C for 3 h. The resulting agarose gel was stained with 0.05% ruthenium red solution overnight and destained with distilled water until the bands were clearly visible.

RESULTS

Cell wall calcium concentration: To investigate the effect of Ca reinforced cell walls on the resistance of apples to

bitter rot, cell walls were extracted and the Ca concentration determined. The Ca concentration of the cell walls increased as the concentration of the $CaCl_2$ with which the fruit were treated increased. Apple cell walls from fruit treated with 4% $CaCl_2$ contained over four times more Ca (1626 μg g^{-1} dry weight) than those from the 0% $CaCl_2$ treated fruit (337 μg g^{-1} dry weight). Cell walls from fruit treated with the 2% $CaCl_2$ had a Ca concentration of 944 μg g^{-1}.

Virulence: There was a negative correlation between fruit decay and the concentration of Ca in the fruit tissue (Fig. 1). However, the difference in decay reduction between 2 and 4% $CaCl_2$ treated apples was not as great as the difference between control apples which were treated with distilled water and the 2% $CaCl_2$ treated apples.

The effect of calcium on PG activity in vitro: PG activity produced by *C. acutatum* during decay of $CaCl_2$ treated and non-treated apples was examined. The purpose was to elucidate whether the reduced lesion size was due to a

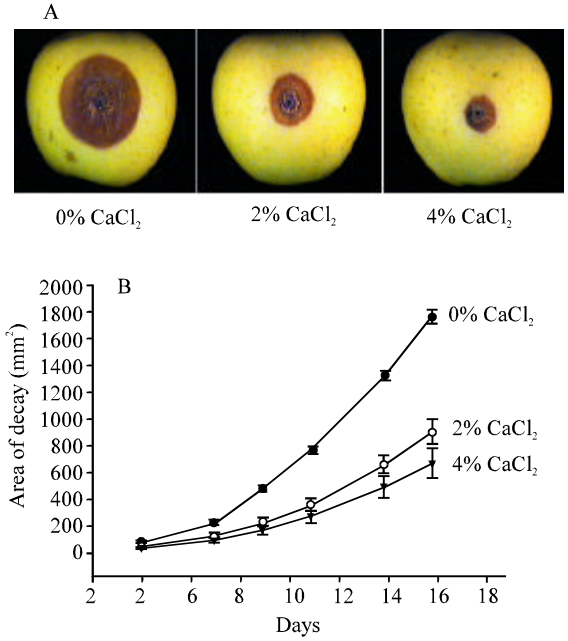

Fig. 1: The relationship between diameter of decay and length of time after inoculation. Apples were infiltrated with 0, 2 or 4% $CaCl_2$ solutions and inoculated with *Colletotrichum acutatum* after two months storage at 0°C. A, The decayed area resulting from 2 weeks of incubation at 20°C. B, The area of decay measured after 2 weeks incubation at 20°C. Error bars indicate standard error of the means

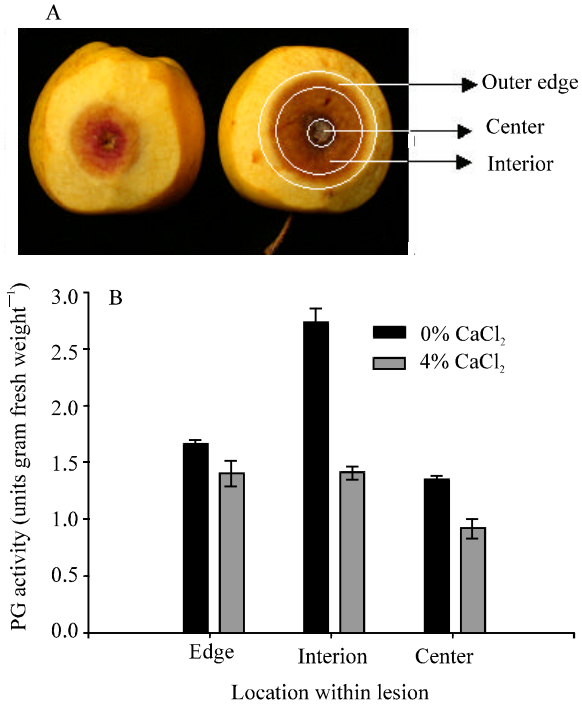

Fig. 2: Polygalacturonase (PG) activity at various locations in the lesion from which the PG activity samples were taken. Fruit were infiltrated with 0 or 4% $CaCl_2$ solutions and inoculated with *Colletotrichum acutatum* after two months storage at 0°C. PG activity within the decayed area was determined after an additional 2 weeks incubation at 20°C. A, Locations within the lesions in which PG activity was determined. B, The relationship between PG activity and location within the lesion. Error bars indicate standard error of the means

change in fungal PG activity. The apple juice to be analyzed was obtained by squeezing areas of the decayed tissues (Fig. 2A) through an injection syringe. PG activities were lower in decayed tissues of the Ca treated apples (Fig. 2B). The most significant difference was seen in the interior region of the decayed area where the most destructive necrotrophic phase of the fungus likely occurs. Twice as much PG activity was noted in non-Ca treated apples than in Ca treated apples. Interestingly, PG activity was higher on the outer edge than in center of the decay, possibly suggesting that at least one PG is expressed in the biotrophic phase during the initial invasion.

The effect of calcium on PG activity *in vitro*: Apple cell walls were used as the sole carbon source in Richard's

solution in which to grow *C. acutatum* to measure PG activity and protein concentration. The protein concentration, representing fungal growth, was greater in the Ca Treated Cell Wall (CTCW) cultures than the Non Ca Treated Cell Wall Cultures (NTCW, Fig. 3A). However, PG activity was higher in the NTCW medium than in the CTCW medium (Fig. 3B), implying that the additional Ca in the cell wall might negatively affect fungal PG expression although Ca apparently stimulates fungal growth (Fig. 3A). The specific activity of PG was higher in the NTCW medium suggesting that the higher PG activity in the NTCW medium was not solely due to increased fungal growth (Fig. 3C).

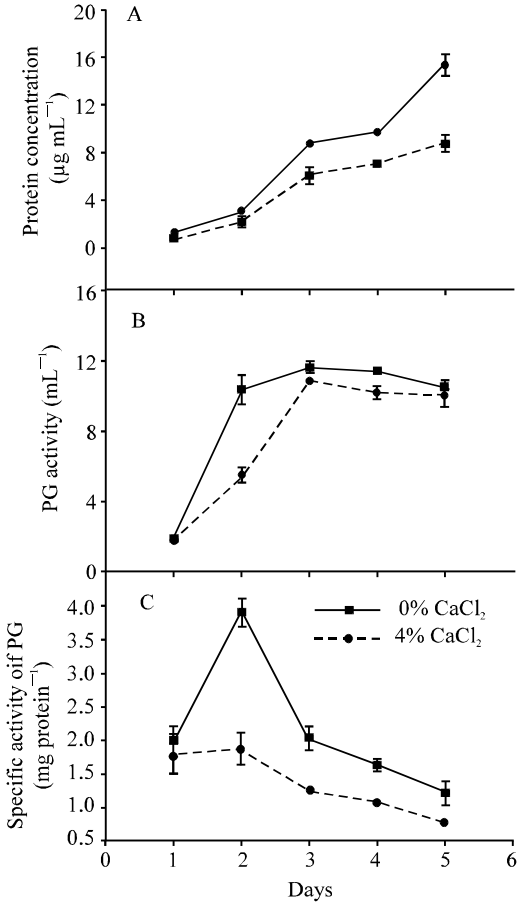

Fig. 3: Effect of high and low calcium cell walls on fungal growth and activity of polygalacturonase (PG) by *Colletotrichum acutatum*. The fungus was grown for 5 days in Richard's solution that was amended with cell walls extracted from apple fruit infiltrated with either 0 or 4% solutions of calcium chloride. Samples were harvested each day to determine A, protein concentration, B, PG activity and C, specific activity. Error bars indicate standard error of the means

The effect of calcium on PG gene expression: The differences in specific activity of PG produced in apple cell wall containing media, especially two days after inoculation, were significant indicating that Ca in the cell wall might have an important influence during the initial stages of fungal growth. To investigate this hypothesis, fungal cultures were harvested every 3 h, from 18 until 27 h after inoculation. PG activity rapidly increased at 27 h in the NTCW medium (Fig. 4A), although the exact time was slightly variable (approx. 24-30 h) from culture-to-culture. In the CTCW medium, PG activity also increased and eventually, reached the same level of PG activity as in the NTCW medium, but the increase was slow compared to the rapid increase of PG activity in the NTCW medium (data not shown). This was supported by the results of the qualitative CAPG1 expression study using RT-PCR on fungal mycelia harvested 24 h after inoculation with conidia using gene specific primers based on the CAPG1 sequence. CAPG1 expression was noticeably higher in the NTCW medium than in the CTCW medium (Fig. 4B). No significant differences were noted in the expression of CAPG2 (E. Park, data not shown).

Isozyme profiles: As another approach to examine the effect of cell wall calcium concentration on PG activity, zymograms were used to determine the isozymes produced by the fungus in the two different media, NTCW and CTCW (Fig. 5). Four strong isozyme bands were observed at pIs of 4.65, 5.9, 6.2 and 9.6 in both cases. The intensities and pIs of these bands were almost identical. However, there were a few differences in several minor bands. When the fungus was grown in NTCW medium, there were two minor bands at pIs of 6.5 and 6.8 that were not apparent when the fungus was grown in CTCW (Fig. 5). Likewise, growth in CTCW medium resulted in a minor band at a pI of 7.7 that was not present in the NTCW growth medium (Fig. 5).

DISCUSSION

The effect of Ca infiltration on apples was investigated at the level of disease severity, enzyme production and PG gene expression. The fruit decay caused by *C. acutatum* was reduced approximately 60% by the 4% $CaCl_2$ treatment. This decrease in decay was inversely related to the Ca concentration of the fruit resulting from the $CaCl_2$ treatment, confirming the results of Conway *et al.* (1991) where Ca treatment of apple fruit inhibited fungal decay caused by *Penicillium expansum*, *Botrytis cinerea* and *Colletotrichum acutatum*. PG activities in samples that were extracted from decayed tissue were lower in Ca treated apples. This negative

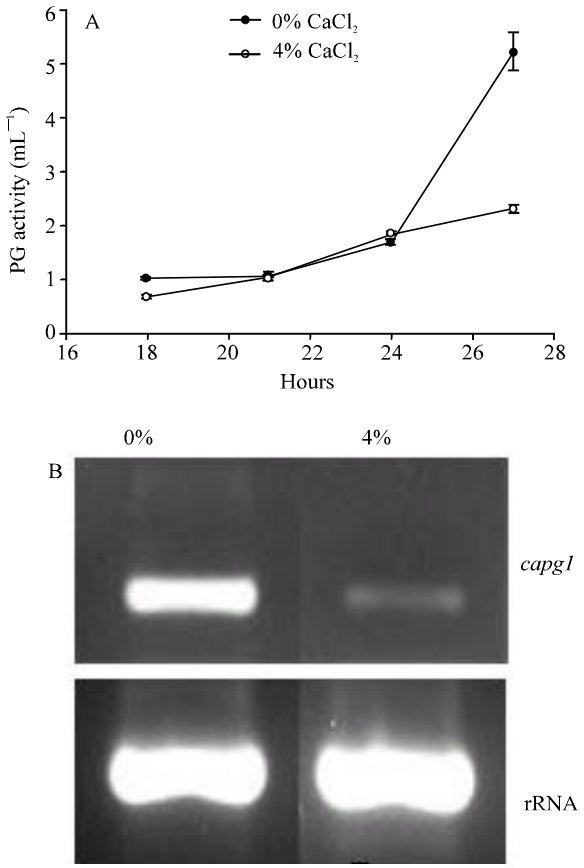

Fig. 4: Polygalacturonase (PG) activity and expression during the early stages of the growth of *Colletotrichum acutatum* in Richard's Solution amended with cell walls from apples infiltrated with 0 or 4% calcium chloride. A, The fungal growth was initiated by conidia and harvested every 3 h between 18 and 27 h after inoculation in order to observe differentially induced PG activity in amended media. Error bars indicate standard error of the means. B, The fungal mycelia obtained at 24 h after inoculation with conidia was used for RNA extraction and RT-PCR to investigate *capg1* expression during the early stages of growth

correlation of PG activity with the severity of decay suggests a role for this enzyme as a virulence factor in disease development.

The accessibility of fungal PG to its target sites in the cell walls may be blocked by Ca binding suggests a possible reason for this Ca-mediated resistance (Conway, 1988). The mechanism by which this may occur has not been clearly elucidated; however, there are at least two possibilities.

Since a role of PG is to degrade plant cell walls to provide nutrients for the fungus, any negative interference in PG production or activity would delay the growth and the resulting advance of the fungus. Another possibility is a negative effect on the induction of PG or other pectic enzymes. Since pectic products derived from host cell walls can regulate the expression of various pectic enzymes (Mason and Jeffries, 1993; Cook *et al*., 1999; Cervone *et al*., 1989), a negative effect on any one pectic enzyme may result in a cascade of effects on the regulation of other pectic enzymes. Ca binding between two adjacent galacturonic acid residues in the pectin backbone is thought to cause the inaccessibility of the host cell wall to PG. This would inhibit the release of mono-galacturonic acid that the fungus utilizes as a signal to turn on PG expression. This inhibition of PG expression would retard fungal invasion. This is consistent with the results of previous research that showed that the decreased amount of water-soluble polyuronides was proportionally related to a reduction of decay (Conway and Sams, 1983).

There are many reports indicating that Ca enhances the plant defense response (Messian *et al*., 1993; Heo *et al*., 1999; Poovaiah, 1988) but the actual mechanism(s) responsible has not been determined. To avoid the complexity of the plant-fungus interaction and to concentrate on the influences of Ca on the interaction between fungal PGs and host cell walls, cell walls were extracted from Ca treated and non-treated apples. Using these cell walls as the sole carbon source, *C. acutatum* was grown *in vitro*. Unexpectedly, the fungus grew better in the presence of the Ca Treated Cell Walls (CTCW) than the presence of Non Treated Cell Walls (NTCW). This was likely caused by the greater availability of Ca in the CTCW medium. However, PG activity was lower in the CTCW medium than in the NTCW medium. This corresponds to the results of Mason and Jeffries (1993) who reported that adding $CaCl_2$ to pectin medium decreased PG production by *C. gloeosporioides*. However, it was not clear whether this reduction in PG production was caused by the inability of PGs to reach their target sites due to Ca binding pectin, or a direct Ca inhibitory effect on PG production.

The reduction of PG activity was particularly significant during the first two days after inoculation. Since this difference in PG activity could significantly influence the outcome of the host-parasite interaction, the events occurring during this time period were closely examined. PG activity rapidly increased in the NTCW medium while it increased at a much slower rate in the CTCW medium. The reduction of PG activity also corresponds to the reduction in CAPG1 gene expression

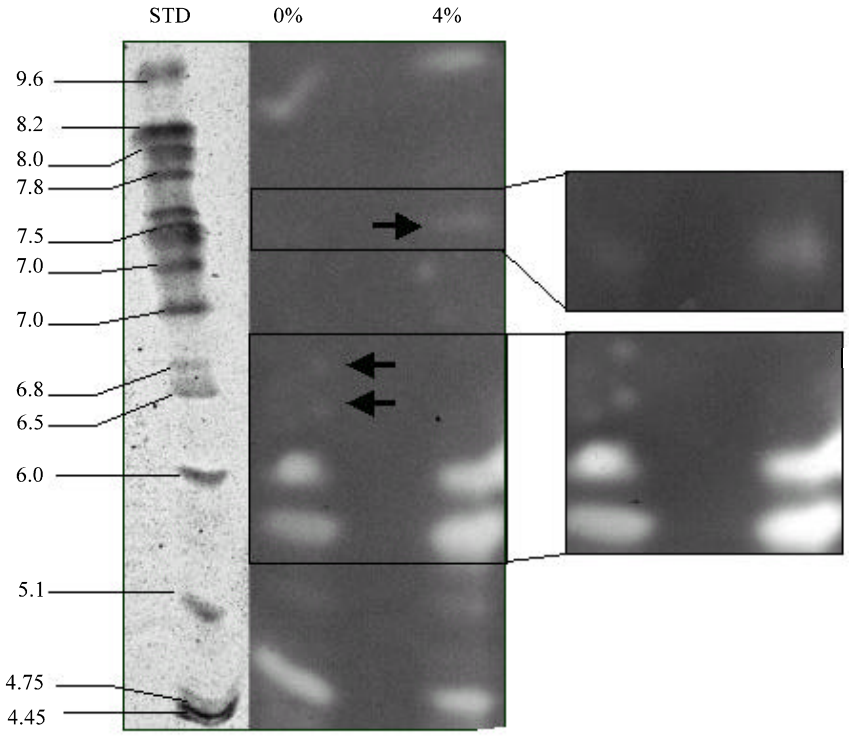

Fig. 5: A zymogram depicting the polygalacturonases produced *by Colletotrichum acutatum* grown in Richard's Solution amended with cell wall extracts from 0 or 4% calcium chloride infiltrated apples. Lane containing protein standards is labeled STD. The arrows point to faint bands unique to one or the other growth conditions. The two panels on the right are larger digitally enhanced sections of the left panel presented to show the faint bands more clearly

in the CTCW medium compared to the NTCW medium, implying that CAPG1 might play an important role during the initial stages of infection. This effect on PG expression occurred not only during germination and appressorium formation when biotrophic invasion takes place, but also during mycelium elongation when the fungus changes to the necrotrophic phase. It is known that many *Colletotrichum* species initially establish infection through a brief biotrophic phase, during which the host cells remain alive, followed by a more destructive necrotrophic phase characterized by cell death in the host (O'Connell *et al*., 2000). Thus, PG may play essential virulence roles during various stages of infection. Present results show for the first time a negative correlation between the Ca-mediated resistance and PG expression. This would suggest a possible role for cell wall bound Ca in the reduction of PG activity and PG expression.

As an additional approach, zymograms were used to examine the effects of increasing host cell wall Ca content on PG expression. Isozyme profiles were obtained from fungal growth in CTCW or NTCW medium. Four similar major isozymes were produced in both media. However, in the NTCW medium, two additional isozymes were produced by the fungus with pIs of 6.5 and 6.9. In CTCW medium, one additional isozyme was produced with a pI of 7.6. It is not clear which of these isozymes, correspond to products of two cloned CAPG genes (E. Park, *unpublished data)*. Only two PG genes had been previously reported in *Colletotrichum* species (Centis *et al*., 1996, 1997; Li and Goodwin, 2002) and their products were predicted to have basic pIs, although Prusky *et al*. (1989) and English *et al*. (1972) reported an acidic isozyme (pI-4.95, MW-62 kDa) which is significantly larger than the predicted molecular weight of the two PG genes (Centis *et al*., 1996, 1997). It should be noted that in a previous study (Mason and Jefferies, 1993) zymograms showed that the carbon source influenced the differential production of isozymes. Therefore, it is also possible that other isozymes may be produced when the fungus is grown under different growth conditions.

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