Carbon, nitrogen and pH regulate the production and activity of a polygalacturonase isozyme produced by *Penicillium expansum*


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The influence of carbon, nitrogen and pH on polygalacturonase (PG) activity produced by *Penicillium expansum* were investigated. *P. expansum* mycelial growth was greatest on lyophilized fruit tissue and the highest PG activity occurred in apple pectin medium. Nitrogen source influenced PG activity and was highest with ammonia while the greatest mycelial mass was supported by glutamate or glutamine. PG activity and mycelial mass peaked 5 days after inoculation as polyuronide content decreased and the pH and ammonium levels increased in apple pectin medium. A single active PG isozyme with an isoelectric point of ~7.6 was produced in apple pectin medium and a partial cDNA clone was obtained that was most homologous to the pggII gene from *Penicillium griseoroseum*. The results from this study indicate that *P. expansum* can modulate the activity of PG in response to nutrient sources and ambient pH through signalling pathways that modulate nutrient acquisition, uptake and metabolism.

Keywords: blue mold; postharvest decay; virulence factor; nutrient sensing; polygalacturonase; *P. expansum*

**Introduction**

*Penicillium expansum* (Link) Thom., is the causal agent of blue mould and is one of the most important postharvest pathogens of apple fruit (Wilson and Nuovo 1973; Ogawa and English 1991). Blue mould decay leads to significant economic losses during storage and also impacts fruit destined for processing by the production of the carcinogenic mycotoxin patulin (Ciegler et al. 1971; Wilson and Nuovo 1973). *P. expansum* infects fruit primarily through wounds caused by stem punctures or bruises occurring at harvest or during postharvest handling but also enters through natural openings, i.e. lenticels, stem ends and the calyx end (Janisiewicz and Peterson 2004). Primary control of this pathogen is achieved through the application of postharvest fungicides, such as Scholar®, Penbotec® and Mertec® (Errampalli 2004); however, increased use of these compounds eventually leads to development
of resistance and reduces their efficacy (Li and Xiao 2008). Manipulation of environmental and nutritional factors that regulate virulence factors in *P. expansum* may lead to desirable control measures as alternatives to fungicides.

The plant cell wall serves as the primary barrier to the establishment of phytopathogens, and is composed of four complex polysaccharides (cellulose, hemicellulose, lignin and pectin) (Walton 1994). Pectin is the main component of the middle lamella, which causes adhesion of adjacent primary cell walls in dicots and non-graminaceous monocots. Biochemical evidence has shown that plant pathogens produce a variety of cell wall degrading enzymes which are involved in postharvest decay (Yao et al. 1996; Zhang et al. 1999; Jurick et al. 2009, 2010). Polygalacturonases (EC 3.2.1.15) (PGs) are produced by bacteria and fungi to degrade pectin and facilitate the invasion and colonisation of host tissue (Zhang et al. 1999).

Fungi have evolved elegant mechanisms of regulating catabolic enzyme production through the use of signalling pathways that are responsive to external cues. Carbon, nitrogen and ambient pH have been shown to act as regulators of fungal gene expression in model fungi like *Saccharomyces cerevisiae* (Marzluf 1997; Peñalva and Arst 2002). Studies in filamentous fungal plant pathogens have focused on the regulation of cell wall degrading enzymes which are also under the control of ambient pH, carbon and nitrogen (Cotton et al. 1996; Mey and Fevre 2003). However, the impact of nutritional cues on PG activity has not been explored in *P. expansum*. Therefore, the objectives of this study were to: (1) determine the effect of carbon and nitrogen sources and ambient pH on *P. expansum* PG enzyme activity and growth in vitro, (2) determine the PG isozymes produced over time and under different pH conditions and (3) obtain a cDNA clone from *P. expansum* grown in pectin–ammonia medium and compare its sequence with PG genes from other *Penicillium* species.

**Materials and methods**

**Fungal culture and chemicals**

The *P. expansum* isolate used in this study was isolated from naturally infected apple fruit and maintained on potato dextrose agar (PDA). All chemicals used in this work were purchased from Sigma Chemical Co. (St. Louis, MO) unless indicated otherwise.

**Polygalacturonase production by *P. expansum* in vitro**

The fungus was grown in modified Richard’s solution containing, 5 g of KH₂PO₄, 2.5 g MgSO₄ 7H₂O, 0.02 g and FeCl₃ H₂O per 1 l of distilled water and different carbon and nitrogen sources. Carbon sources were 1% (wt/vol) apple pectin (without sugars, 72% galacturonic acid, 7% methoxy content), citrus pectin (without sugars, 78% galacturonic acid, 8% methoxy content), esterified citrus pectin (79% galacturonic acid, 31% esterification), polygalacturonic acid (25-50 000 Mw), D-galacturonic acid, lyophilised “Golden Delicious” apple or “Anjou” pear tissue. For media with various carbon sources, 10 g/l of KNO₃ was used as a nitrogen source. Nitrogen sources were 1% (wt/vol) urea, glutamine, glutamate, asparagine and ammonium chloride. For media with various nitrogen sources, 1% apple pectin was used as the carbon source. Conidia of *P. expansum* were harvested from a five day-old culture grown on PDA using 1 ml of 0.01% Tween-treated water.
The conidial suspension was adjusted to $10^4$ conidia/ml using a compound light microscope and a hemacytometer. One millilitre of the conidial suspension was added to each 250 ml flask containing 50 ml of an autoclaved medium. The flasks were placed on an orbital shaker (150 rpm) for seven days at 25°C. Culture filtrates were vacuum harvested using a sterile Buchner funnel attached to a sidearm flask. The mycelium was collected and mycelial masses were recorded. Filtrates were placed in 50-ml screw cap conical tubes and centrifuged at 11,500 $\times$ g for 20 min. The supernatants were then sterilised using a disposable 0.2 μm filter sterilisation unit attached to a vacuum source. Filtrates were stored in 50 ml conical tubes at 4°C.

**PG enzyme activity assay**

PG enzyme activity assays were carried out on filter sterilised *P. expansum* culture supernatants from varying carbon, nitrogen and pH conditions. PG activity was determined by measuring reducing sugars released from polygalacturonic acid, and using D-galacturonic acid to construct a standard curve as described by Yao et al. (1996). An aliquot of the enzyme preparation was mixed with 0.1 ml assay buffer (100 mM sodium acetate, pH 5.0, containing 0.4% polygalacturonic acid) and adjusted to a final volume of 0.2 ml with water in 13 mm $\times$ 100 mm borosilicate tubes. This mixture was incubated at 37°C for 20 min and 1 ml of 100 mM borate borax buffer (pH 9.0) was added to stop the reaction. A 0.2 ml solution of 0.1% 2-cyanoacetamide was added and samples were placed into a boiling water bath for 10 min. Samples were then equilibrated to 20°C and absorbance at 276 nm was measured. Control reactions were carried out by adding borate borax buffer with 2-cyanoacetamide prior to the addition of substrate buffer, and were assayed for each sample to determine background levels of reducing sugars. One unit of PG activity was defined as the amount of enzyme required to release 1 nmol of reducing sugar per min per millilitre under the assay conditions employed.

**Determination of culture pH, total soluble polyuronides and ammonium content**

Apple–pectin ammonia medium was used for the time course evaluation and assessment of culture pH on PG activity. The apple pectin–ammonia medium was adjusted to an initial pH of 3.0, 4.0 and 5.0 with 10 N NaOH and then inoculated with a *P. expansum* conidial suspension as stated above. The pH of the medium following seven days incubation was then measured using a pH meter and recorded. PG activity and mycelial mass were also evaluated as described in the materials and methods section. Time course experiments were performed in the same manner as above, except 1 ml of culture medium was removed after inoculation with the fungus one, three, five and seven days after inoculation. One millilitre of culture medium was then used to analyse pH, ammonia levels and total soluble polyuronides. Total soluble polyuronides were assayed according to Blumenkrantz and Asboe-Hansen (1973) as 1 μl of culture filtrate was added to 99 μl of water in a 13 mm $\times$ 100 mm borosilicate tube and briefly vortexed. The samples were placed in an ice bath and 600 μl of sulphuric acid tetraborate solution (12.5 mM tetraborate in concentrated sulphuric acid) was added. All samples were vortexed briefly and placed in a boiling water bath at 100°C for
5 min. Samples were then cooled immediately on ice to room temperature and 10 μl of m-phenylphenol reagent (0.15% m-phenylphenol in 0.5% sodium hydroxide) was added and vortexed. Absorbance (520 nm) of the samples was determined using a spectrophotometer (SmartSpec, Bio-Rad Laboratories). Concentration of polyuronides in the culture filtrates was determined according to a standard curve developed for galacturonic acid. Ammonium concentration was determined in culture filtrates using a method previously described by Prusky et al. (2004). Briefly, ammonium was measured with an ammonia electrode Model 95-12 (Orion) in 1-ml aliquots after addition of 10 μl of 10 N NaOH. Different concentrations of NH₄Cl in the range of 7–700 μM were used to construct a standard curve. There were four biological replicates along with an uninoculated media control and all experiments were repeated.

**Detection of PG isozymes**

Isoelectric focusing-polyacrylamide gel electrophoresis (IEF-PAGE) of PG was performed using a pre-cast Ampholine IEF gel (pH 3.5-9.5, GE Life sciences) in a Pharmacia Biotech Multiphor II system following the manufacturer’s instructions (Amersham–Pharmacia). The electrode strips were soaked with 1 M phosphoric acid (anode) and 1 M sodium hydroxide (cathode) and placed on opposing sides of the gel. Protein samples were applied to application strips that were then placed directly on the gel. IEF protein standards (pI range 3.5–10.7, SERVA, USA.) were diluted 1:5 with water prior to loading. Isoelectric focusing was then conducted at 1500 v, 25 ma, 25 w for 1.5 h. PG activity overlays were used to detect PG isozymes and were conducted using the following method described by Zhang et al. (1997). A thin agarose gel (0.75 mm) containing a 0.1% solution of polygalacturonic acid pH 5.0 was made and poured onto a Gel-Bond PAG film (FMC Bioproduct, Rockland, Maryland). The agarose and the IEF gel were placed between two glass plates held together with four binder clips and allowed to incubate at 37°C for 1 h. The agarose overlay was then stained with 0.05% ruthenium red solution for 1 h and destained with distilled water for 1 h. Clear zones on a red background, indicating PG activity, were visualised using a light box.

**Molecular cloning of a polygalacturonase cDNA**

Total RNA was isolated from lyophilised seven day-old mycelial mats obtained from cultures grown on apple pectin–ammonia medium at pH 4.0 using the Qiagen Total RNA isolation kit according to the manufacturer’s instructions (Qiagen, USA). Total RNA was also subjected to DNAse digestion in solution followed by RNA clean up protocols included with the kit according to the manufacturer’s instructions (Qiagen, USA). Quantity and quality of the RNA were assessed by absorbance at 260 and 280 nm using a nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE), and quality was determined based on the appearance of 28 S and 18 S rRNA bands on an ethidium bromide stained 1% agarose gel. Five micrograms of total RNA were used to make cDNA using the 3’ RACE kit according to the manufacturer’s instructions (Invitrogen, USA). The cDNA was used as a template for conventional PCR using degenerate primers (DPG F3 5’ ACAACTCBGA YGSTTWCG 3’ and DPG R3 5’ TTRATRCGGACACCGTTG 3’) designed to
the highly conserved glycosyl hydrolase domain from 15 PG gene sequences obtained from nine fungal species that were deposited in Genbank (*Sclerotinia sclerotiorum* (AY49677.1, AJ620513.1, L29040, AJ620514.1, XM001594302.1), *Botrytis fuckeliana* (AY665552.1), *Venturia nashicola* (AB430864.1, AB430831.1, AB430830.1, AB430863.1), *Penicillium griseoroseum* (AF047713.1), *Penicillium expansum* (AF047713.1), *Aspergillus oryzae* (XM001820901.2), *Aspergillus parasiticus* (L23523.1) and *Galactomyces geotrichum* (AB994909.1). Amplicons generated from cultures grown on apple pectin–ammonia medium were sequenced and analysed using BLAST (Altschul et al. 1990). The partial cDNA was cloned using the TOPO-TA cloning kit (Invitrogen, US). Multiple sequence alignments were carried out using ClustalW (Thompson et al. 1994).

**Analysis of data**

Each experiment was repeated once, and all experiments had four replicates per treatment. Analysis of variance of data was performed using a general linear model (Proc GLM) using SAS (SAS institute, Cary, NC). Treatment means were separated using Fishers LSD ($p = 0.05$).

**Results**

The source of carbon affected PG activity and fungal growth of *P. expansum* in liquid shake cultures. The highest PG activity was obtained when apple pectin was used as the sole carbon source, followed by glucose, and then lyophilised apple and pear tissue (Figure 1(a)). However, fungal growth was the highest on lyophilised apple and pear tissue followed by apple pectin, glucose and galactose (Figure 1(b)).

Varying nitrogen sources in the apple pectin medium influenced PG activity and mycelial growth of *P. expansum in vitro*. The greatest PG activity was achieved in culture with ammonium chloride while medium containing urea, glutamine, glutamate, and asparagine all had significantly lower levels of PG enzyme activity (Figure 2(a)). Fungal growth did not coincide with PG activity as glutamine and glutamate were the best nitrogen sources for mycelial growth followed by aspargine, ammonium chloride and urea (Figure 2(b)).

The culture pH of the apple pectin–ammonia medium affected total PG activity and mycelial growth of *P. expansum in vitro*. Optimal PG activity was observed at pH 4.0 followed by 5.0 and 3.0 (Figure 3(a)). Mycelial mass followed a similar trend as the highest growth was also achieved when the culture pH was initially adjusted to pH 4.0 (Figure 3(b)). The pH of the culture medium following seven days incubation also varied. Culture pH after seven days for the pH 3.0 treatment remained unchanged, but for pH 4.0 increased to 5.6 and for pH 5.0 rose to 5.98.

Both fungal growth and PG activity differed with various forms and sources of pectin, polygalacturonic and D-galacturonic acid. PG activity was greatest in apple pectin followed by citrus pectin, and methyl esterified citrus pectin while activity in polygalacturonic acid and D-galacturonic acid were undetectable (Figure 4(a)). Fungal growth was the highest in citrus pectin and polygalacturonic acid followed by methyl esterified citrus pectin, apple pectin with D-galactuonic acid producing the least amount of growth (Figure 4(b)).
PG activity and fungal growth in liquid medium containing apple pectin with ammonium chloride were evaluated over time. Both PG activity and mycelial growth followed similar trends as the greatest activity and growth occurred five days post inoculation (Figure 5(a) and (b)). Culture pH also increased over time from 4.0 to 5.98 after seven days incubation (Figure 6(a)). Total soluble polyuronides declined steadily and reached the lowest level after seven days while ammonium levels initially decreased, but then increased over time (Figure 6(b) and (c)).

PG activity overlays were used in conjunction with isoelectric focusing to detect active polygalacturonase isozymes from *P. expansum* culture filtrates containing pectin and ammonia as sole carbon and nitrogen sources. A single active PG isozyme with an apparent isoelectric point (pI) of 7.9 was detectable in pectin ammonia medium over time and at pH 3.0, 4.0 and 5.0 (Figure 7(a) and (b)). A partial PG clone was obtained from mycelium grown for seven days in liquid medium containing pectin and ammonia (Figure 8(a) and (b)). This clone was 347 nucleotides in length encoding 115 amino acids and was 95% identical to the pggII gene from *P. griseoroseum* at the nucleotide and amino acid levels (Genbank Accession

Figure 1. The effect of carbon source on (a) polygalacturonase activity and (b) mycelial weight of *Penicillium expansum* grown for seven days in liquid shake culture pH 4.0. Bars represent the means from eight biological replicates from two experiments. Means with the same letter are not significantly different (*p* = 0.05) according to Fishers LSD.
AF195113). When the sequence was analysed by BLAST, the conserved glycosylhydrolase 19 domain was detected.

**Discussion**

The main focus of this study was to investigate the effects of carbon and nitrogen source and ambient pH on the activity of a polygalacturonase enzyme produced by *P. expansum in vitro*. PG activity was greatest when the fungus was grown on apple pectin as the sole carbon source, while other simple (glucose and galactose) and complex carbon sources (dried apple and pear tissue) yielded significantly lower levels of PG activity. The ability to grow on a variety of carbon sources coupled with the production of PG is hypothesised to afford the fungus the flexibility to degrade various substrates during necrotrophic and saprophytic phases of the life cycle. Our findings are consistent with those reported for *Phomopsis cucurbitae*, a postharvest pathogen of cantaloupe fruit, in which both optimal PG activity and fungal growth
did not coincide (Zhang et al. 1999). They are also congruent with other studies showing that fungal PGs are induced by their substrate, and exhibit activity on carbon sources other than pectin (Wagner et al. 2000).

Nitrogen source also influenced PG activity with the most statistically significant increase occurring in culture when the fungus was grown with pectin and ammonium chloride. Ammonia is the preferred nitrogen source in fungi as it is readily metabolised and used for the production of amino acids and nucleotides (Meti et al. 2011). A study by Prusky et al. (2004) showed that ammonia levels were higher in healthy apple fruit tissue than in *P. expansum*-decayed apple fruit tissue. Therefore, ammonia sensing, uptake and/or metabolism may be a part of the mechanism by which the fungus controls virulence through increasing the secretion of PG to facilitate tissue maceration and decay of healthy fruit tissue.

Fungal growth and PG activity were variable when the fungus was cultured in different forms of pectin or galacturonic acid. Fungal growth occurred on polygalacturonic and galacturonic acid, showing that the fungus is capable of using both of them as a carbon source. However, incubation on polygalacturonic acid did not result in detectable levels of PG activity seven days after inoculation which may have been the result of complete digestion of the substrate. A lack of PG activity in media containing galacturonic acid is not unexpected because PG is not necessary for its utilisation as a carbon source. It is therefore reasonable to hypothesise that a similar set of metabolic machinery exists in *P. expansum* based on data from this

Figure 3. The effect of initial culture pH on (a) polygalacturonase activity and (b) mycelial weight of *Penicillium expansum* in liquid shake cultures with apple pectin and ammonium chloride after seven days. Bars represent the means from eight biological replicates from two experiments. Means with the same letter are not significantly different (*p* = 0.05) according to Fishers LSD.
study which allows the fungus to metabolise pectin when supplied as a sole carbon source.

Culture pH affected fungal PG activity and mycelial growth as optimal levels for both were achieved at pH 4.0. This is in agreement with a previous study conducted by Prusky et al. (2004) where it was shown that the *P. expansum* polygalacturonase PEPG1 transcript level was most abundantly expressed at pH 4.0 and less at pH 3.0 and 5.0. Optimal PG activity at pH 4.0 may provide the fungus with a fitness advantage as the pH of most healthy apple tissue ranges from pH 3.0 to 5.0 with an average of ~4.0 (Prusky et al. 2004). Therefore, the fungus would have the capacity to secrete optimal amounts of the cell wall-degrading enzyme, such as PG into an environment that is most conducive for colonisation (Yao et al. 1996).

Figure 4. The effect of poly and d-galacturonic acid on (a) polygalacturonase activity and (b) mycelial weight of *Penicillium expansum* grown for seven days in liquid shake culture. Bars represent the means from eight biological replicates from two experiments. Means with the same letter are not significantly different (*p* = 0.05) according to Fishers LSD.
Optimal PG activity occurred when the fungus was grown on pectin and ammonia as the sole carbon and nitrogen sources. The observed pH increase in the culture medium was most likely due to the decrease in polyuronide content (composed of apple pectin) resulting from pectin degradation and concomitant secretion of ammonia by the fungus. Ammonia depletion by *P. expansum* has been previously demonstrated by Prusky et al. (2004) in decayed apple fruit and they hypothesised that this led to the decrease in the pH of *P. expansum*-decayed fruit tissue by removing the ammonia and thus reducing the buffering power of the substrate. Our results also indicate a similar trend *in vitro* during the initial phases of fungal growth (one to three days post inoculation) which may have caused ammonia levels to decrease compared to the uninoculated control. However, as fungal biomass increased with time (five to seven days post inoculation), ammonia levels and pH both increased most likely due to efflux of ammonia from deaminated proteins into the culture medium.

*P. expansum* produced a single detectable isozyme in culture over time and at different pHs with an apparent pI of 7.9. Production of a single isozyme in culture differs from what was demonstrated for *P. cucurbitae* as it produced eight isozymes with pIs ranging from 3.7 to 8.6 (Zhang et al. 1999). The isozyme that was secreted by *P. expansum in vitro* had a different pI than the one reported by Yao et al. (1996) from *P. expansum*-decayed apple fruit with a pI of 8.1. Inclusion of a positive control in the PG activity overlays used in this study (using a commercial PG preparation...
from *A. niger*) indicates that our system is capable of detecting multiple PG isozymes of varying pIs ranging from 3.0 to 9.0. Since the pI of the PG produced in culture differed from the one reported in decayed apple fruit, we hypothesise that the fungus has a mechanism to sense and respond to its local environment which may be under control of nutrient assimilation pathways.

Using degenerate primers, we have obtained a partial cDNA sequence corresponding to a PG gene which is distinct from the PEPG1 gene previously cloned and characterised from *Penicillium expansum*-decayed apple fruit tissue (Yao et al. 1996). We are naming this new partial PG cDNA clone, PEPG2 for *P. expansum* polygalacturonase gene 2. Yao et al. (1996) demonstrated that PEPG1 was undetectable in healthy apple fruit which supports the claim that the PG was

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Figure 6. Time course analysis of (a) pH, (b) polyuronides and (c) ammonium levels of culture filtrates of *Penicillium expansum* grown for one, three, five, and seven days in liquid shake culture with apple pectin and ammonium chloride at initial pH 4.0. Bars represent the means from eight biological replicates from two experiments. Means with the same letter are not significantly different ($p = 0.05$) according to Fishers LSD.
produced by the fungus and was not an apple PG. Definitive evidence that the PEPG2 is not of host origin comes from the fact that it was cloned from fungal mycelium grown in sterile liquid culture in complete absence of apple or pear fruit tissue. Expression of PEPG2 in culture further supports the hypothesis that *P. expansum* is capable of secreting a specific PG based on either the host or the environment that the fungus is colonising. Production of host specific PGs has previously been demonstrated by Jurick et al. (2010) where they showed that a PG produced in decayed pear fruit tissue was capable of macerating pear fruit and producing higher levels of soluble polyuronides then when PG was incubated with apple fruit tissue *in vitro*.

Results from this study have demonstrated for the first time that the production of a PG, secreted by *P. expansum in vitro*, is affected by both the culture pH and the form of available carbon and nitrogen. We have also shown that a distinct PG isozyme is produced in culture and have obtained a partial PG cDNA clone that is different from the one produced in decayed apple fruit. Together, both biochemical and molecular data suggest that *P. expansum* is capable of sensing its environment...
via pH and nutrient assimilation pathways to modulate the deployment of specific PG gene products. Various sensing mechanism(s) may then be implemented by the fungus to secrete specific PG isozymes during decay for optimal substrate degradation and host colonisation. Future studies involving the functional genetic analysis of PG genes in *P. expansum* will elucidate the precise role that PGs play in postharvest decay of apple and pear fruit and may lead to specific decay control strategies.

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References


