A Proteomic Study of Pectin Degrading Enzymes Secreted by *Botrytis cinerea* Grown in Liquid Culture

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Abstract

Botrytis cinerea is a pathogenic filamentous fungus which infects more than 200 plant species. The enzymes secreted by *B. cinerea* play an important role in the successful colonization of a host plant. Some of the secreted enzymes are involved in the degradation of pectin, a major component of the plant cell wall. A total of 126 proteins secreted by *B. cinerea* were identified by growing the fungus on highly or partially esterified pectin, or on sucrose in liquid culture. Sixty-seven common proteins were identified in each of the growth conditions, of which 50 proteins exhibited a Signal P motif. Thirteen *B. cinerea* proteins with functions related to pectin degradation were identified in both pectin growth conditions, while only four were identified in sucrose. Our results indicate it is unlikely that the activation of *B. cinerea* from the dormant state to active infection is solely dependent on changes in the degree of esterification of the pectin component of the plant cell wall. Further, these results suggest that future studies of the *B. cinerea* secretome in infections of ripe and unripe fruits will provide important information that will describe the mechanisms that the fungus employs to access nutrients and decompose tissues.

Keywords

Host-pathogen interactions; *Botrytis cinerea*; Matrix polysaccharides; Proteomics; Mass spectrometry

1. INTRODUCTION

B. cinerea is a pathogenic filamentous fungus which infects more than 200 plant species in a variety of organs including fruit, flowers, and leaves. The host range for *B. cinerea* infection includes economically important crops such as tomato, berries, chickpeas, french beans, and grapes as well as cut flowers [1]. On certain fruit hosts *B. cinerea* initially infects while the fruits are green and remains dormant [2–4]. This quiescent infection is superceded by a resumption of fungal growth activity once the fruit ripens [2–4]. This resumption of growth and infection leads to postharvest losses and reduction in the shelf life of perishable products. Attempts to prevent postharvest disease lead to the use of fungicides in addition to those already in use for treatment of preharvest infections. The development of new strategies against postharvest infection would be of benefit from both an economic and an environmental standpoint because of the high cost and intrusive impact of the fungicides and the loss of consumable products.

Changes during the ripening process appear to play an important role in the activation of the dormant infection. One of the major processes of ripening involves the enzymatic de-

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esterification and depolymerization of the cell wall components, resulting in softening of the fruit [5,6]. All the major components of the fruit cell wall, the pectin, cellulose and hemicellulose, undergo changes during ripening. Pectin is a major component of the plant cell wall, providing mechanical stability and influencing pH and ionic properties of the wall. The pectin backbone consists mainly of α-(1–4)-linked D-galacturonic acid [7]. The galacturonic acid is highly esterified in the pectin of unripened fruit [8]. A decrease in the degree of esterification of the galacturonic acid backbone, combined with changes in the average molecular weight and neutral sugar content of cell walls, is consistent with softening and ripening [5,8–10].

B. cinerea secretes a battery of enzymes utilized for the degradation and consumption of the host plant. Pectin degradation by *B. cinerea* is enabled by enzymes, including pectin methyl esterases (PMEs), exopolygalacturonases (exo PGs), endopolygalacturonases (endo PGs), pectate and pectin lyases (PLs), and rhamnogalacturonan lyase and hydrolase [11–14]. The genome of *B. cinerea* has multiple isoforms for most of the above enzymes, for example six isoforms of endo PG have been previously reported [15]. During plant pathogen interactions, pectin fragments called oligogalacturonides are produced which act as defense elicitors. In *Fragaria vesca,* a partial degree of demethylation of oligogalacturonides may be required for eliciting defense responses to *B. cinera* infection [16]. Furthermore, in Arabidopsis, the overexpression of the pectin methylesterase inhibitor (PMEI) resulted in an increased resistance to *B. cinerea,* implying the importance of the degree of esterification of pectin to plant resistance [17].

To better understand at the molecular level the complex interaction between pathogen and host, we propose to use an idealized model system that is accessible and easily manipulated, and whose results can subsequently be incorporated into a biological model. Here we demonstrate the impact of the degree of esterification of pectin on fungal secretion and report on the secretome of *B. cinerea* when grown in liquid culture on three different carbon nutrient sources. Two of these conditions simulate fungal interactions with expected host nutrient sources.

Specifically, *B. cinerea* was grown in liquid cultures with highly esterified pectin, partially esterified pectin, and sucrose as sole carbon sources. Sucrose was used to define those enzymes which can be considered constitutively expressed. Shotgun proteomics was used to study the *B. cinerea* secretome. We observed changes in the profile of secreted proteins that were nutrient dependant, indicating an adaptability of *B. cinerea* to the growth conditions.

2. MATERIALS AND METHODS

Microorganisms and culture conditions

B cinerea strain (BO5.10) was a kind gift of the laboratory of John Labavitch, University of California-Davis. Fungal stock cultures were maintained on potato dextrose agar, PDA (BD Biosciences, MD, USA) at 37°C for 14 days. Conidia were harvested with a sterile 0.01% Tween 20 (w/v) solution and spores were gently suspended with a magnetic stirrer. Erlenmeyer flasks containing 150 ml of liquid media (described below) were inoculated with the spore suspension at 10^6 spores ml^{-1}, and incubated at 24°C in an orbital shaker at 200 rpm. The growth medium contained KH_2PO_4 (0.29 g), K_2HPO_4 (0.94 g), $(NH_4)_2SO_4$ (1.20 g), NaCl (0.15 g), $CaCl_2 H_2O$(0.40 g), $MgSO_4$ (0.150 g), $FeSO_4 7H_2O$ (0.015 mg), $ZnSO_4 7H_2O$ (0.015 g), and $MnSO_4 H_2O$ (0.150 mg). The medium was supplemented with one of the three following carbon sources: Citrus pectin (0.5% w/v) (Sigma-Aldrich, MO, USA) labeled as HE pectin, 30% esterified pectin (0.5% w/v) (Hercules DL USA) labeled as PE pectin, and sucrose (0.5% w/v) (Fisher Scientific, NJ, USA). The pH was adjusted to 4 with H_2SO_4. The medium was sterilized at 121°C for 15 min. After seven days the

supernatant cultures were collected by filtration through a No. 41 Whatman filter paper and lyophilized. After lyophilization, the cultures were resuspended in 2 mL of deionized water prior to further desalting using a HiPrep 26/10 desalting column (GE, Pistcataway, NJ). The desalted fractions containing the secreted proteins were lyophilized and stored at −20 °C until further analysis.

Secreted protein isolation and separation by 1D-SDS -PAGE

Lyophilized secreted proteins were resuspended in 100 μl of deionized water. 30 μl of the sample was mixed with 10 μL of 2X Laemmli buffer (Sigma-Aldrich, MO, USA) and boiled at 95°C for 5 min before loading onto the gel. Proteins were separated on a 4–12% Bis-Tris preacasted gel (Invitrogen, Carlsbad, CA) using the 1x MOPS SDS buffer (Invitrogen, Carlsbad, CA) as running buffer. SeeBlue® Plus2 Prestained Standard molecular weight standards (Invitrogen, Carlsbad, CA) were used. After electrophoresis, proteins were visualized by staining with Coomassie blue. Each gel lane was excised into three sections of equal length and destained.

In gel digestion

Excised bands were first cut into smaller pieces (1 × 1 mm), dried by vacuum centrifugation, and reduced by submerging the gel pieces in 100 mM ammonium bicarbonate solution containing 10 mM dithiothreitol for 1 h at 55°C. Excess dithiothreitol/ammonium bicarbonate was removed and the same volume of 100 mM ammonium bicarbonate containing 55 mM iodoacetamide was added and incubated for 45 min in the dark. After alkylation, the gel pieces were treated with 100 mM ammonium bicarbonate and acetonitrile sequentially and then dried by vacuum centrifuge. To the dried gel pieces, 2 μg of trypsin was added in sufficient 100 mM ammonium bicarbonate solution to submerge the gel pieces in the solution. Digestion was carried out at 37°C overnight. The gel was washed once with ammonium bicarbonate followed by acetonitrile, and twice with 5% formic acid followed by acetonitrile. Peptides were collected from the washings, dried by vacuum centrifugation, and resuspended in 0.1% formic acid solution for mass spectrometric analysis.

LC-MS/MS analysis

The peptides from each sample were analyzed in duplicate. An Agilent 1100 capillary LC (Palo Alto, CA) was attached to the mass spectrometer via a T splitter to allow infusion at ηL flow rates. Five μm diameter C_{18} beads (Rainin, Woburn, MA) were packed into a pulled fused silica capillary (10.5 cm × 100 μm ID) under 1000 psi pressure using nitrogen gas. Peptide samples were loaded onto the column for 45 min under the same pressure. Peptides were then eluted with a gradient using 0.1% formic acid (buffer A) and 99.9% acetonitrile/ 0.1% formic acid (buffer B). Following the initial wash with 95% buffer A for 10 min, peptides were eluted from the column during a 90 min linear gradient of 5–60% of buffer B at a flow rate of ~200 ηL/min directly into a LTQ linear ion trap mass spectrometer (Thermo Fisher, San Jose, CA) using a voltage of 2500V.

The instrument was set to acquire MS/MS spectra on the nine most abundant precursor ions from each MS scan with a repeat count set of 3 and repeat duration of 5 sec. Dynamic exclusion was enabled for 160 sec. Raw tandem mass spectra were converted into a peak list using ReAdW followed by mzMXL2Other algorithms [18]. The peak lists were then searched using Mascot 1.9 (Matrix Science, Boston, MA).

Database searching and protein identification

A target database was created from *B. cinerea BO5.10* genes (Broad Institute, MA). A decoy database was then constructed by reversing the sequences in the normal database. Searches

were performed against the target and decoy databases using the following parameters: 1) fully tryptic enzymatic cleavage with two possible missed cleavages, 2) peptide tolerance of 800 parts-per-million, 3) fragment ion tolerance of 0.8 Da, and 4) variable modifications due to carboxyamidomethylation of cysteine residues (+ 57 Da) and deamidation of asparagine residues (+1 Da). Following the database searches, statistically significant proteins were determined for each of the four samples at a 1% protein FDR using the ProValT algorithm [19].

Protein functional annotation

For proteins with no assigned functions, homology searches were performed using the BlastP program against all non-redundant protein sequences present in the NCBI database (http://www.ncbi.nlm.nih.gov/blast). Protein alignments were considered significant if they were below an e-value of 10^{-50}. The mechanism of secretion was predicted using SignalP and SecretomeP to identify classical motifs [20].

3. RESULTS

Visualization of secreted proteins

The production of *B. cinerea* secreted proteins was carried out in liquid culture conditions having either partially esterified (PE) pectin, highly esterified (HE) pectin, or sucrose as the carbon source. Analysis of the 1D SDS-PAGE used for the separation of the secreted proteins (Figure 1) revealed the presence of numerous proteins. The protein band patterns for *B. cinerea* cultured in the PE pectin and HE pectin media were very similar, while there were certain significant differences for *B. cinerea* grown in sucrose. To identify the *B. cinerea* secreted proteins in each growth condition, each gel lane was cut into three equal parts and analyzed by shotgun proteomics.

Shotgun proteomics

Three segments from each lane of the 1D SDS-PAGE were digested in-gel and resulting peptides were subjected to LC-MS/MS analysis. Each fraction was analyzed in duplicate. All together, a total of 126 protein groups were identified (Supplementary Information Table 1), indicating that a minimum of 126 *B. cinerea* proteins were present in the liquid culture after *B. cinerea* had grown for seven days. Each protein group had at least one discriminating peptide in that group compared to all the other identified proteins (Supplementary Information Table 2). At a 1% FDR, *B. cinerea* grown in PE pectin produced 105 *B. cinerea* proteins identified by 822 MS/MS spectra, while *B. cinerea* grown in HE pectin or on sucrose produced 95 and 89 proteins identified by 791 and 638 MS/MS spectra, respectively. The BlastP algorithm was used to determine the putative function of the *B. cinerea* proteins based on homology with other proteins, which in turn was used to classify the proteins into different categories: carbohydrate metabolism and transport (catabolism), pectin degrading enzymes (pectinases), peptidases, pathogenicity factor, hypothetical and others. More than half of the identified proteins belonged to the category of carbohydrate metabolism and transport (Figure 2). Thirty-one identified proteins did not match any annotated protein with sufficient homology to assign a putative function and were classified as hypothetical proteins.

This study was designed to provide a qualitative global secretome analysis. In the present work we provide researchers with access to descriptive proteomic analysis. A limit of the shotgun approach is that it detects only protein fragments and not intact proteins and therefore cannot discern isomeric forms of proteins and posttranslational modifications.

In the current study dynamic exclusion, complexity of the sample and disparity of the protein secretions in various growth conditions impact the spectral count of the protein. Observation with small spectral counts cannot be correlated to changes in the level of protein secretion. For reasonable semi-quantification there is a requirement of the observation of many spectra for a given protein [21]. At least a three-fold difference is required for less than 4 spectral counts to determine any significant change in the protein [22]. Thus any differences in protein level that are below 4 spectral counts need to be viewed in a qualitative manner.

Proteins secreted by *B. cinerea* in different growth conditions

The proteins were assumed to be extracellular because they were obtained from culture media after simple filtration. The extracellular localization of the identified proteins was confirmed using the Signal P algorithm. Based on amino acid sequences, Signal P predicted that 87 proteins were secreted out of the 126 (Supplementary Information Table 1) total identified proteins. Of the 67 common proteins identified under all three growth conditions (Figure 3, Table 1), 50 were predicted to be secreted by signal P. Eighty-eight proteins were observed to be in common in both of the pectin growth conditions.

Pectin degrading enzymes

Thirteen pectin degrading enzymes were identified in the culture filtrates following *B. cinerea* growth on the three carbon sources (Table 2 and Figure 4). The pectinases included three PMEs, three PLs, two endo PGs, two exo PGs, and two PGs of unknown mode of action. These 12 proteins were found in culture filtrates following growth on either source of pectin. Only one pectate lyase was uniquely observed in the highly esterified pectin growth medium. The two *B. cinerea* endo PGs, PG2 and PG6, were only observed when *B. cinerea* was grown in either pectin source but not when grown in sucrose. After growth in sucrose, we were able to identify only four enzymes, BC1G_00617.1, BC1G_013137.1, BC1G_01617.1 and BC1G_06840.1, out of 13 pectin degrading enzymes identified from pectin growth conditions, and these had spectral counts at least three-fold less than those observed when *B. cinerea* was grown with either source of pectin. Spectral counts are the number of MS/MS spectra identified as belonging to specific peptides and provide a semi-quantitative estimate of the relative protein abundance in the analyzed sample [22]. Only one *B. cinerea* PME was identified as secreted when *B. cinerea* was grown in sucrose and it had a similar number of spectral counts compared to the cultures grown in pectins. Three proteins, BC1G_06840.1, BC1G_07946.1 and BC1G_00799.1, were identified with pectin degrading functions yet lacked a classical signal peptide for secretion.

4. DISCUSSION

Proteomics

Proteomics was used to study the impact of different degrees of pectin esterification on the proteins secreted by *B. cinerea* when pectin was the sole carbon source for growth in culture. Previously, proteomic studies in other filamentous fungi have shown substrate dependent secretion. *Aspergillus flavus*, a filamentous fungus, has shown unique rutin degrading enzymes when grown in the presence of rutin in liquid culture, and absence of those enzymes when grown in potato dextrose in liquid culture [23]. Although fruit, which serves as a natural host for *B. cinerea,* is a much more complex environment than a single carbon source liquid culture medium, changes in the extent of pectin esterification occur as fruits ripen. Therefore, differences observed in protein secretion following infection with *B. cinerea* could be a consequence of the differences in the degree of esterification. Modeling the changes in fruit pectins required a simple system.

Studying the changes in a mixture of proteins between two or more similar systems generally requires the use of equal concentrations of sample. In the present study, changes occur both in terms of number and in overall concentration of secreted proteins depending on the growth conditions. Fungi are known to exhibit substrate dependant secretion; therefore, a comparison based on equal protein concentration might result in an inaccurate representation of the relative amounts of the secreted proteins. To accommodate this concern we chose to use equal volume as the basis for our comparison. The proteins are collected in a standard volume of solution, and analyses were conducted on a volume-to-volume basis. If the proteins secreted by the fungus in different systems results in too large a change in concentration, then quantitative comparison of individual proteins using this method may not be possible. However a comparison between profiles of proteins is possible and the results from an equal volume analysis represent the most accurate reflection of the secretion profile.

The gel analysis suggested that the total concentrations of proteins secreted in the different media were similar, validating our comparison between profiles. Overall we were able to identify 126 *B. cinerea* proteins. Sixty-seven proteins were observed in all three systems, indicating that these proteins may be constitutively secreted at a minimal detectable level in all growth conditions and thus their presence is not dependent on the carbon source. Among these proteins, 50 had an N-terminal signal peptide motif, confirming their secretion. On the basis of putative function, 35 of these were classified as carbohydrate metabolism and transport proteins, 11 as hypothetical proteins, one as a peptidase, one as a pathogenicity factor and two others. Thirty-five proteins were identified with only one peptide identification. Generally proteins secreted by the fungi are small and have some post translation modification. Hence it is difficult to get high percentage coverage of the amino acid sequence of the protein using mass spectrometry.

Pectin degrading enzymes

Pectinases play an important role in cell wall degradation and successful invasion. Endo PGs are one of the most widely studied classes of pectin degrading enzymes. Endo PGs hydrolyze the internal (1–4) linkage between D-galacturonic acid units of pectin [24]. Previously, six endo PGs have been identified from *B.* cinerea [25]. However, it has been suggested that *B. cinerea* can secrete up to 13 endo PG isoenzymes when post translational modifications are taken into account [26]. The secretion of different isoforms can be explained by differences in substrate, pH, and environmental conditions [11]. The deletion mutants of both BcPG1 and BcPG2 showed reduced virulence on multiple hosts [25]. In this study we identified endoPG 2 (BC1G_13667.1) and endoPG 6 (BC1G_08033.1) when the fungus was grown in either HE or PE pectin as a carbon source in liquid culture. The absence of all endo PG isoforms following growth in sucrose indicates the secretion of endo PGs is carbon source dependant. This is consistent with previous work demonstrating that the expression of the endo PG gene family has a differential pattern that depends on host tissue, stage of infection and temperature [27]. There was no significant change in the secretion of endo PGs as a result of the differences in the degree of pectin esterification. Previously, it had been suggested that a basic level of gene expression exists for two PGs (BcPG1 and BcPG2) in liquid culture growth conditions [11]. In the current study, we were unable to detect any BcPG1 in the culture media. There is a possibility that the BcPG1 protein was present, but not in sufficient quantity for detection via shotgun proteomics, or it is possible that it was not detected as the result of extensive post translation modifications. Post translational modifications have been reported on other endo PG isoforms, notably BcPG6 [28], which were, in fact, detected in our analysis. The controlled secretion of endo PGs and the role of the PG inhibiting proteins in the plant wall as a defense mechanism against *B. cinerea* infections emphasize the importance of BcPG [29,30].

PMEs de-esterify pectin, releasing methanol and polygalacturonic acid. This de-esterification facilitates the subsequent action of PGs and PLs [24]. On certain hosts, BCPME1 has been shown to be an essential determinant of *B. cinerea* virulence [24,25]. We were able to identify three different PMEs, BC1G_00617.1, BC1G_6840.1 and BC1G_00799.1, which were secreted by *B. cinerea*. Two PMEs, BC1G_00617.1 and BC1G_6840.1, were identified as secreted in all three growth conditions, implying that these PMEs may be a constituent of the secretion profile which is independent of the carbon substrate.

PL cleaves polygalacturonic acid into oligogalacturonides via beta-elimination [24], but the role of pectate lyases in *B. cinerea* infections has not been investigated previously. Three pectate lyases, BC1G_07052.1 BC1G_09000.1 and BC1G_12517.1, were identified as secreted only under the pectin growth conditions. Of interest is a pectate lyase, BC1G_07052.1, which was only observed in HE pectin.

Other pectinases identified were two exo PGs, BC1G_01617.1 and BC1G_00240.1, identified in both pectin growth conditions. BC1G_01617.1 was identified in the sucrose growth medium, but with a spectral count of 2 as compared to 29 and 33 spectral counts for material from the cultures grown in both PE and HE pectin. Two other PGs, BC1G_13137.1 and BC1G_07496.1 were detected in both pectin growth mediums. BC1G_13137.1 was also identified in the sucrose growth medium, but with a spectral count of 1 compared to spectral counts of 12 and 16 in PE pectin and HE pectin medium. BC1G_03464.1 is a putative rhamnogalacturonase that hydrolyzes the internal (1–2) linkage between units of pectin in rhamnogalacturonan I, and was identified in both of the pectin growth conditions and not in sucrose [14].

When grown in the sucrose media, *B. cinerea* produced 12 proteins which were not observed in the other media. Of these 12 proteins, five (BC1G_00882.1, BC1G_14570.1, BC1G_05168.1, BC1G_10120.1 and BC1G_15832.1) were classified as hypothetical proteins. Of the remaining proteins, two were alpha amylases (BC1G_02623.1 and BC1G_02333.1), one was an alpha glucosidase (BC1G_11115.1), one was a member of the glycosyl hydrolase family 95 (BC1G_08975.1), one was a putative 3-isopropylmalate dehydrogenase Leu2A (BC1G_14880.1), one was acatalase (BC1G_12856.1), and one was a Cu/Zn superoxide dismutase (BC1G_00558.1). Thus, while the secretome of *B. cinerea* in sucrose growth medium has similar characteristics compared to secretomes in pectin media, the total number of pectinases and the total spectral count of pectinases secreted were lower. Hence, the secretion of pectinases, except for PMEs, depends on the carbon source of growth.

Secreted proteins

Out of the 126 proteins identified in culture filtrates following fungal growth, only 87 proteins had a signal peptide motif according to the signalP algorithm [20]. This N-terminal motif indicating that the "protein is likely to be secreted" is frequently used to confirm the extracellular nature of proteins identified. The absence of such a predicted N-terminal motif in 39 proteins indicates that these might be intracellular proteins resulting from cell lysis, as the identification of internal proteins in secretome studies due to cell lysis is an inherent feature of secretome proteomics. However, the handling of the material here did not involve any treatments likely to result in cell lysis. In addition, there were three pectinases predicted not to contain the signal peptide and hence predicted by the algorithm to not be secreted. The pectinases are known to be secreted and thus we postulate that a nonconventional N-terminal motif exists in certain *B. cinerea* proteins for secretion. A non-classical method of secretion is known to exist in yeast and the Cu/Zn superoxide dismutase of *B. cinerea,* which plays an important role in french bean virulence, had previously been confirmed to be

secreted by a non-classical pathway in such organisms as *Aspergillus fumigates* and *Claviceps purpurea* [31,32].

Identification of function

Most of the proteins identified from the *B. cinerea* B05.10 database are hypothetical proteins with unknown function; therefore the BlastP algorithm was used to assign putative functions to proteins by comparing the identified hypothetical proteins to the proteins in the NCBI nr database. Almost three-quarters of the identified proteins were assigned putative functions using an e value threshold of e^{-50}; however, one-quarter of the identified proteins remained hypothetical. Most of these hypothetical proteins were similar to other fungal hypothetical proteins below the required threshold score of e^{-50}; however, there were a few proteins with no significant alignments with any other proteins in the NCBI nr database. These hypothetical proteins, if unique, might be good targets for future biological studies.

Conclusion

Shotgun proteomics was successfully used to identify the secretome of *B. cinerea* grown in three culture conditions which differed by the carbon nutrients provided. We were able to identify a total of 126 *B. cinerea* proteins, 67 of which were observed in all three growth conditions. Thirteen pectinases were identified as secreted by *B. cinerea* when grown in all culture conditions. The secretion of most of the pectinases depended on the carbon substrate used by the fungus for growth. However, secretion of two pectin methyl esterases is independent of the carbon substrate. There were no major differences in protein secretion when *B. cinerea* was grown in liquid culture with 30% *vs* 80% esterified pectin. Because both the growth of *B. cinerea* and the secretion of proteins were similar in cultures containing differently esterified pectins, it is likely that the activation of *B. cinerea* from the dormant state to active infection is not solely dependant on changes in the degree of esterification of the pectin component of the plant cell wall. However, these results suggest that future studies of the *B. cinerea* secretome in infections of ripe and unripe fruits will provide important information that will describe the mechanisms that the fungus employs to access nutrients and decompose tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

List of abbreviations

B	*Botrytis*
PMEs	pectin methyl esterases
exo PGs	exopolygalacturonases
endo PGs	endopolygalacturonases
PLs	pectate and pectin lyases
PMEI	pectin methylesterase inhibitor

| PE	partially esterified pectin
HE	highly esterified pectin

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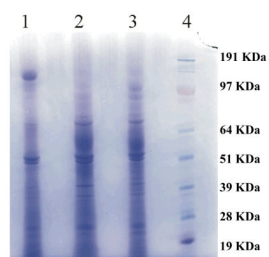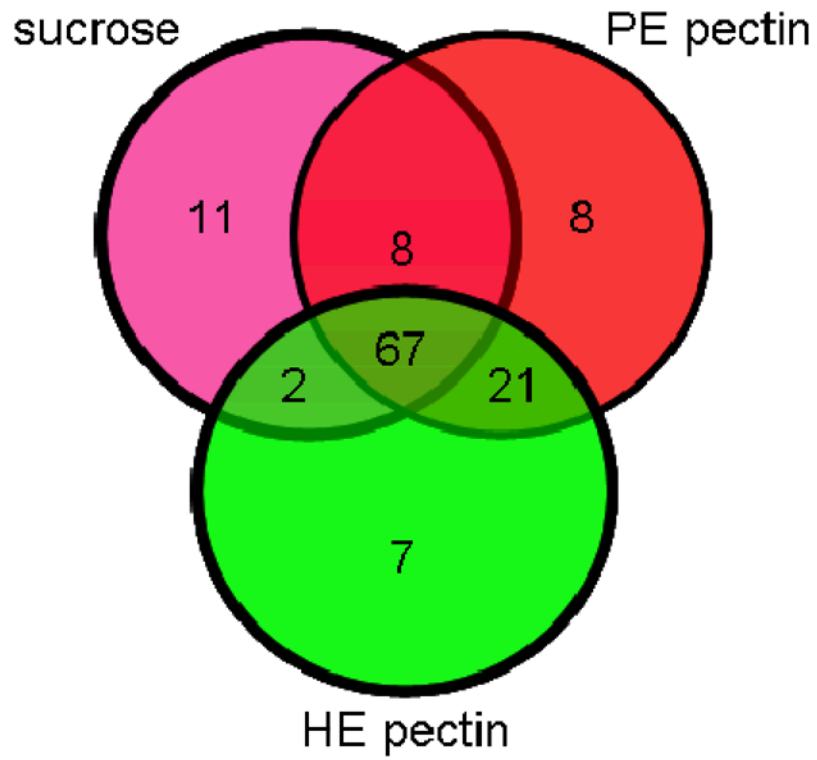
Figure 1.
Secreted proteins from *B. cinerea*, grown in liquid cultures with sucrose (Lane 1), HE pectin (Lane 2) or PE pectin (Lane 3) as the sole carbon source, were separated on one-dimensional SDS-PAGE and stained with Coomassie blue as described in the Materials and Methods section. Molecular weight standards are shown in Lane 4.
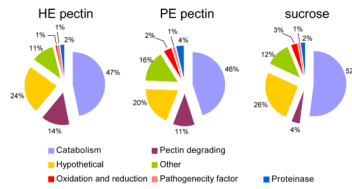
Figure 2.
Classifications of *B. cinerea* proteins into different functional categories based on their putative function, when fungus was grown in liquid culture with HE pectin, PE pectin or sucrose as the sole carbon source. Identified proteins classification categories include proteins involved in carbohydrate metabolism and transport (catabolism), pectin degrading enzymes, hypothetical proteins (proteins with unknown function), oxidation and reduction, pathogenecity factor and others.

Figure 3.
Venn diagram of the identified *B. cinerea* proteins using LC-MS/MS, when fungus was grown in liquid cultures using HE pectin, PE pectin or sucrose as the sole carbon source.
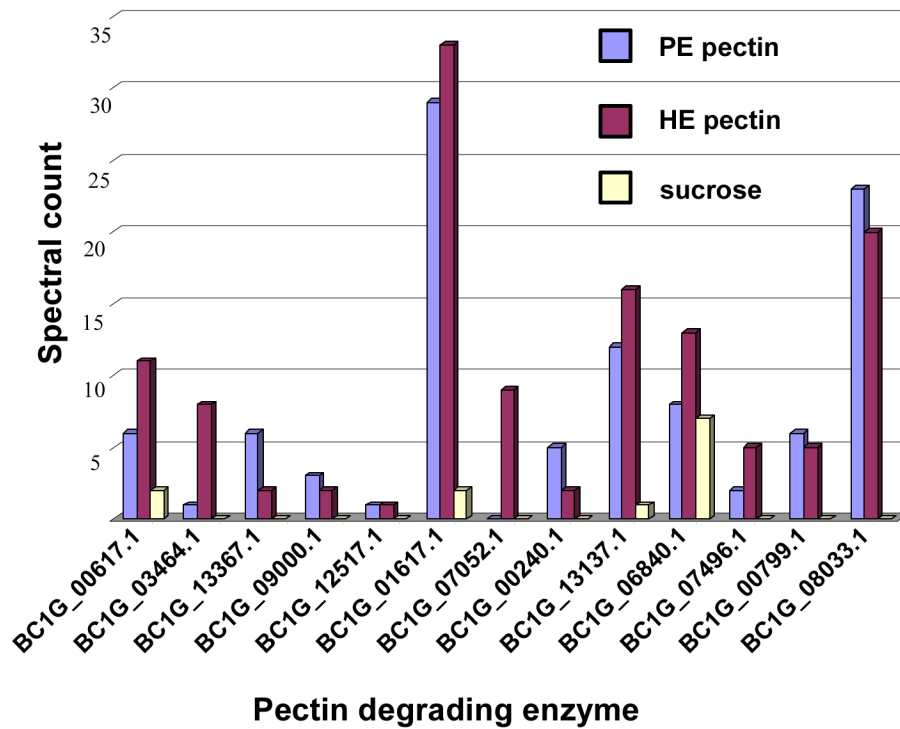
Figure 4.
Spectral count of thirteen PDEs secreted by *B. cinerea* grown in three different liquid cultures (sucrose, HE pectin or PE pectin as the sole carbon source). The spectral count for each PDE is the sum of the spectra identified to that PDE obtained from two replicate LC-MS/MS analyses at 1% FDR.

Table 1

Results of *B. cinerea* proteins with signal P identified in all three growth conditions.

Gene Id[a]	Putative Function[b]	Signal P[c]	PE pectin		HE pectin		sucrose	
			Score[d]	Spectra[e]	Score[d]	Spectra[e]	Score[d]	Spectra[e]
BCIG_13137.1	Polygalacturonase	0.986Y	492.13	12	791.28	16	73.04	1
BCIG_00617.1	Pectin methylesterase	1.000Y	247.49	6	296.65	11	122.37	2
BCIG_06546.1	alpha-galactosidase precursor (Melibiase)	0.999Y	247.44	7	47.29	1	129.34	2
BCIG_07622.1	beta-glucosidase	0.995Y	523.25	13	396.44	5	461.52	11
BCIG_07319.1	1,3-beta glucanase	0.999Y	792.22	16	808.75	17	532.25	9
BCIG_10455.1	1,3-beta-glucanosyltransferase Gel1	0.998Y	254.54	4	172.44	3	263.19	10
BCIG_12859.1	alpha-glucosidase precursor	1.000Y	443.98	10	559.85	10	552.39	19
BCIG_04994.1	alpha-L-arabinofuranosidase	0.999Y	816.83	15	460.12	10	50.56	2
BCIG_08372.1	alpha-L-arabinofuranosidase A	0.995Y	100.63	2	336.39	4	102.4	1
BCIG_06328.1	alpha-L-rhamnosidase	0.992Y	218.77	6	265.45	5	231.9	6
BCIG_14030.1	beta-1-3-glucanosyltransferase	1.000Y	409.21	12	525.03	10	420.58	9
BCIG_10247.1	beta-fructofuranosidase	1.000Y	518.21	11	331.52	6	57.64	2
BCIG_03567.1	beta-galactosidase	0.998Y	792.51	17	309.73	6	266.3	6
BCIG_02364.1	beta-glucosidase	0.991Y	608.27	16	602.61	9	273.37	5
BCIG_10221.1	beta-glucosidase 1 precursor	0.816Y	1203.24	29	651.09	13	1544.26	48
BCIG_13690.1	Cell wall glycosyl hydrolase YteR, putative	0.998Y	243.78	4	495.08	13	110.69	3
BCIG_06509.1	Chitin binding protein	1.000Y	307.19	5	308.5	4	572.64	11
BCIG_00594.1	Endoglucanase	0.999Y	586.93	28	516.98	26	290.77	9
BCIG_13938.1	Exo-arabinanase	1.000Y	396.29	10	604.94	15	92.18	2
BCIG_01033.1	Exo-beta-1,3-glucanase	0.929Y	69.41	1	137.36	3	67.03	1
BCIG_01617.1	Exo-polygalacturonase	0.996Y	1098.98	29	833.38	33	116.36	2
BCIG_07215.1	Family 20 chitobiase	0.999Y	170.5	3	99.4	2	141.94	3
BCIG_11018.1	Family of alpha-1,6-mannanases	0.999Y	480.62	7	411.05	9	631.97	11
BCIG_00409.1	Glcosyl transferase/cell wall glucanase	1.000Y	347.29	6	271.42	7	246.29	4
BCIG_08755.1	Glucoamylase P precursor	0.997Y	359.15	7	126.38	2	366.87	6
BCIG_11898.1	Glucosidase	0.999Y	507.88	12	767.12	17	748.96	12
BCIG_12132.1	Glycosyl hydrolase family 65 protein	0.997Y	152.95	4	186.69	4	286.58	5

Gene Id[a]	Putative Function[b]	Signal P[c]	PE pectin		HE pectin		sucrose	
			Score[d]	Spectra[e]	Score[d]	Spectra[e]	Score[d]	Spectra[e]
BC1G_09079.1	GPI-anchored cell wall beta-1,3-endoglucanase	0.999Y	664.29	12	621.5	13	588.23	9
BC1G_01204.1	Glyoxal oxidase	0.983Y	347.32	6	156.57	2	366.68	5
BC1G_00455.1	Mannosyl-oligosaccharide alpha-1,2-mannosidase precursor	0.998Y	1016.64	29	950.03	29	593.02	12
BC1G_02314.1	Extracellular phytase	0.995Y	460.62	12	230.08	4	427.18	14
BC1G_10486.1	Glutaminase GtaA	0.992Y	475.9	11	465.49	14	380.16	11
BC1G_11266.1	Laccase	0.999Y	550.72	17	418.69	14	565.69	16
BC1G_10329.1	Laccase	0.989Y	266.26	4	497.02	8	186.98	4
BC1G_11835.1	Phytase	0.978Y	84.38	1	61.01	3	191.12	4
BC1G_02965.1	Acid phosphatase/phosphoesterase	0.996Y	354.4	5	310.9	6	100.75	2
BC1G_02163.1	Ceratoplatanin	0.996Y	238.17	8	238.86	8	237.05	9
BC1G_07521.1	Aspartate protease	0.999Y	413.97	13	318.55	15	417.04	8
BC1G_03710.1	Carboxypeptidase S1	0.989Y	52.47	1	58.78	1	79.96	1
BC1G_01393.1	Hypothetical protein	1.000Y	461.2	14	486.53	14	510.93	9
BC1G_15201.1	Hypothetical protein	1.000Y	240.06	10	211.51	14	329.78	13
BC1G_12279.1	Hypothetical protein	1.000Y	41.65	1	47.4	1	66.02	3
BC1G_12374.1	Hypothetical protein	0.999Y	207.74	5	189.88	4	358.51	8
BC1G_08393.1	Hypothetical protein	0.999Y	150.34	3	258.01	3	155.89	3
BC1G_14136.1	Hypothetical protein	0.999Y	36.38	1	128.38	2	60.58	3
BC1G_11139.1	Hypothetical protein	0.999Y	61.09	2	72.08	3	72.26	1
BC1G_00198.1	Hypothetical protein	0.999Y	1004.38	21	736.07	15	1005.62	29
BC1G_08801.1	Hypothetical protein	0.982Y	199.37	5	86.34	1	51.09	1
BC1G_10095.1	Hypothetical protein	0.951Y	156.41	2	48.69	1	76.79	1
BC1G_13215.1	Hypothetical protein	0.820Y	144.78	2	113.98	1	122.66	2

[a] Protein identification number provided by the broad institute *B. cinerea* BO5.10 database

[b] Name/function was assigned based on sequence similarity when blasted using NCBI non redundant database

[c] Signal P prediction value using algorithm SignalP3.0 server (http://www.cbs.dtu.dk/services/SignalP/)

[d] Protein score calculated by adding individual non redundant peptide Mascot score over a threshold score at 1% False Discovery Rate as calculated by ProValT

[e] Total number of spectra matched to proteins in all replicates in a growth condition of *B. cinerea*

Table 2

Results of the pectinases identified in the current study.

Gene Id[a]	Putative Function[b]	Signal P[c]	PE pectin Score[d]	PE pectin Spectra[e]	HE pectin Score[d]	HE pectin Spectra[e]	sucrose Score[d]	sucrose Spectra[e]
BCIG_00617.1	Pectin methylesterase	1.000Y	247.49	6	296.65	11	122.37	2
BCIG_03464.1	Rhamnogalacturonase	1.000Y	59.08	1	142.64	8	-	-
BCIG_13367.1	Endopolygalacturonase 2	1.000Y	179.84	6	53.75	2	-	-
BCIG_09000.1	Pectate lyase	1.000Y	141.08	3	160.45	2	-	-
BCIG_12517.1	Pectate lyase, putative	1.000Y	44.42	1	55.61	1	-	-
BCIG_01617.1	Exo-polygalacturonase	0.996Y	1098.98	29	833.38	33	116.36	2
BCIG_07052.1	Pectate lyase	0.997Y	-	-	384.47	9	-	-
BCIG_00240.1	Similar to exopolygalacturonase	0.990Y	389.71	5	143.36	2	-	-
BCIG_13137.1	Polygalacturonase	0.986Y	492.13	12	791.28	16	73.04	1
BCIG_06840.1	Pectin methylesterase	0.232N	332.97	8	436.87	13	363.34	7
BCIG_07496.1	Polygalacturonase	0.000N	106.26	2	230.48	5	-	-
BCIG_00799.1	Pectin methylesterase	0.002N	204.22	6	225.48	5	-	-
BCIG_08033.1	Polygalacturonase 6	0.999Y	712.92	23	564.83	20	-	-

[a] Protein identification number provided by the broad institute *B. cinerea* BO5.10 database

[b] Name/function was assigned based on sequence similarity when blasted using NCBI non redundant database

[c] Signal P prediction value using algorithm SignalP3.0 server (http://www.cbs.dtu.dk/services/SignalP/)

[d] Protein score calculated by adding individual non redundant peptide Mascot score over a threshold score at 1% False Discovery Rate as calculated by ProValT

[e] Total number of spectra matched to proteins in all replicates in a growth condition of *B. cinerea*