PROTEOMICS APPROACH TO STUDY BOTRYTIS CINEREA SECRETED PROTEINS UNDER DIFFERENT GROWTH CONDITIONS

by

PUNIT SHAH

(Under the Direction of Ron Orlando)

ABSTRACT

Botrytis cinerea is a filamentous fungus infecting more than 200 plant species, causing significant economic losses worldwide. Filamentous fungi have been known to secrete enzymes involved in polymer, oligosaccharides and protein decomposition. The ability of *B. cinerea* to infect a broad range of plants suggests that the fungus secretes a variable profile of enzymes to attack in different cellular environments. Recent advances in mass spectrometry, high pressure liquid chromatography, bioinformatics, and the availability of a multitude of genome sequences have made it possible to study the secretome of fungi in a high throughput manner.

In this study, proteomic analysis of the *B. cinerea* secreted proteins was performed under various growth conditions. *B. cinerea* was grown on solid substrate and submerged liquid cultures with access to one of various carbon growth sources (sucrose, partially esterified pectin, highly esterified pectin, strawberry fruit extract, tomato fruit extract, *Arabidopsis* leaves extract). Solid substrates of nylon or cellophane membrane were utilized for *B. cinerea* secretome studies. More than 200 *B. cinerea* proteins were identified in various secretome studies. Differences in *B. cinerea* secretome profiles were observed between solid and liquid culture growth conditions. Differences were also observed depending on the carbon source and solid substrate used for

B. cinerea growth. However there were proteins identified which were constituent in all the secretome of *B. cinerea.* We discuss the dependence of *B. cinerea* secretion on growth conditions.

INDEX WORDS: Botrytis; Filamentous fungi; Secretome; Mass spectrometry; Proteomics; Electrospray ionization; Liquid chromatography

PROTEOMICS APPROACH TO STUDY BOTRYTIS CINEREA SECRETED PROTEINS UNDER DIFFERENT GROWTH CONDITIONS

by

PUNIT SHAH

B.S.C, Ramnarian Ruia College, University of Mumbai, India, 2001

M.S.C, Ramnarian Ruia College, University of Mumbai, India, 2003

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2008

© 2008

Punit Shah

All Rights Reserved

PROTEOMICS APPROACH TO STUDY BOTRYTIS CINEREA SECRETED PROTEINS UNDER DIFFERENT GROWTH CONDITIONS

by

PUNIT SHAH

Major Professor: Ron Orlando

Committee: Carl Bergmann
Jonathan Amster

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
August 2008

DEDICATION

To my late Grandfather, I respect and admire him; He has been my role model. His hard work and dedication has been my inspiration. His last advice of not to be lazy is my driving force in life

To my Grandmother, Her beliefs and unshaking dedication in religion has been.

To my parents; For their encouragement to pursue career in science.

I can never hope to express how grateful I am for your support in this venture.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank to my major advisor, Dr. Ron Orlando, for giving me an opportunity to work under his guidance. I would like to thank for his support and encouragement when they were most needed. I would like to thank Dr. Carl Bergmann, for his guidance, support and encouragement. I have learned scope of my research from his advice and interest in my research. I would like to thank James Atwood whose guidance helped me deal with the problems I faced during my graduate school experience.

I would like to thank all the individuals with whom I had privilege of working Gerardo Gutierrez-Sanchez, Peggie Angel, Arthur Nuccio, Tasneem Bahrainwala, Min Xie, Lei Cheng, Caroline Watson, Fernanda Ludolf, Nicholas Seyfried, Kumar Kolli, Todd Mize, Lin Lin, Shreyal Patel. I would like to acknowledge all the contributions you all have made in my graduate school experience. Finally, I would like to thank my family and friends for their support.

TABLE OF CONTENTS

Page

CHAPTER 1

INTRODUCTION

Botrytis cinerea is a necrotrophic plant pathogen, infecting different plant organs on more than 200 plant species[1,2]. It is the causal agent of pre-and post-harvest diseases on many economically important crops, and is responsible for rot on fruits and vegetables and blight on leaves and flowers[3, 4]. *B. cinerea* secretes an array of enzymes that could be used for destruction, degradation, transportation and consumption of host cell carbohydrates and proteins[3,4]. The fungus needs to digest the nutrient that required for its growth. Therefore, fungal secreted enzymes play an important role in generation and digestion of plant fragments before consumption by the fungus. With over 25 fungal genomes completed within them Botrytis genome, now the major challenge in modern fungal biology is to understand the expression, function and regulation of the entire set of proteins encoded by fungal genomes. The ability to perform large-scale protein identification could help in the elucidation of gene function at protein level and to understand the host-pathogen interaction mechanism and the large host range of *B. cinerea.*

In recent years advancements in mass spectrometry and various hyphenated mass spectrometric techniques have propelled characterization of bio-molecules[5-9]. It has been possible to study large bio-molecules like proteins, due to the advent of soft ionization methods such as Matrix Assisted Laser Desorption Ionization (MALDI) and Electrospray Ionization (ESI)[10]. High throughput proteomics employs ever-advancing tools like Liquid chromatography, mass spectrometry and bioinformatics in an attempt to characterize every proteins expressed in the analyte sample. The recent developments in mass spectrometry have led to efficient, effective, and accurate method for the proteome of biomolecules[11].

The research presented in this dissertation focuses on the application of mass spectrometry to the study secreted proteins by *B. cinerea*. Chapter 3 represents the first

secretome of *B. cinerea* grown on solid substrate using a mass spectrometry. Chapter 3 also focuses on the biological interpretation of large amounts of data generated by high throughput proteomics using bioinformatics tools like Mascot Search algorithm, ProValT algorithm, Blast P algorithm, signal P algorithm, and Secretome P algorithm[12-15]. Chapter 4 is a study of the secretome response of *B. cinerea* when grown on different solid substrate in mock interaction with different growth media. Chapter 5 focuses on the secretome of the fungi grown in liquid culture. The carbon sources for the growth of fungi in liquid culture are highly esterified pectin, low esterified pectin and sucrose. The primary goal of the research described here was to identify as many *B. cinerea* secreted proteins with a combination of mass spectrometry and bioinformatic softwares.

REFERENCES

1. Williamson, B.; Tudzynski, B.; Tudzynski, P.; Van Kan, J. A. L., *Botrytis cinerea*: the cause of grey mould disease. *Molecular plant pathology* 2007, 8, (5), 561-580.

2. Elad, Y., *Botrytis: Biology, Pathology and Control*. Kluwer Academic Pub: 2004.

3. Elad, Y.; Shtienberg, D., Botrytis cinerea in greenhouse vegetables: chemical, cultural, physiological and biological controls and their integration. *Integrated Pest Management Reviews* 1995, 1, (1), 15-29.

4. van Kan, J. A. L., Licensed to kill: the lifestyle of a necrotrophic plant pathogen. *Trends in Plant Science* 2006, 11, (5), 247-253.

5. Gygi, S. P.; Corthals, G. L.; Zhang, Y.; Rochon, Y.; Aebersold, R., Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. *Proceedings of the National Academy of Sciences* 2000, 97, (17), 9390.

6. Matthiesen, R., *Mass spectrometry data analysis in proteomics*. Humana Press: 2007.

7. Quadroni, M.; James, P., Proteomics and automation. *Electrophoresis* 1999, 20, (4-5), 664-677.

8. Simpson, R. J., *Proteins and Proteomics: A Laboratory Manual*. CSHL Press: 2003.

9. Veenstra, T. D.; Yates Iii, J. R., Proteomics for biological discovery. *Proteomics* 2006, 6, 6384-6387.

10. Vestal, M. L., Methods of ion generation. *Chem Rev* 2001, 101, (2), 361-75.

11. Aebersold, R.; Mann, M., Mass spectrometry-based proteomics. *Nature* 2003, 422, 198-207.

12. Perkins, D. N.; Pappin, D. J.; Creasy, D. M.; Cottrell, J. S., Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 1999, 20, (18), 3551-67.

13. Bendtsen, J. D.; Jensen, L. J.; Blom, N.; von Heijne, G.; Brunak, S., Feature-based prediction of non-classical and leaderless protein secretion. In Oxford Univ Press: 2004; Vol. 17, pp 349-356.

14. Dyrløv Bendtsen, J.; Nielsen, H.; von Heijne, G.; Brunak, S., Improved prediction of signal peptides: SignalP 3.0. *Journal of Molecular Biology* 2004, 340, (4), 783-795.

15. Weatherly, D. B.; Atwood, J. A.; Minning, T. A.; Cavola, C.; Tarleton, R. L.; Orlando, R., A heuristic mehod for asigning a flse-discovery rate for protein identifications from Mascot database search results. *Molecular & Cellular Proteomics* 2005, 4, (6), 762-772.

CHAPTER 2

LITERATURE REVIEW

Botrytis cinerea

Botrytis cinerea is a filamentous fungus which infects plant species. This fungus has been known to infect more than 200 different kinds of plants including some commercially viable plants like berry fruits, tomatoes, lettuces, cabbages, grapes, and flowers[1,2]. Botrytis has been reported to infect different organs of the plant including the roots, stem, leaves, fruits and flowers. Botrytis causes large economic losses all around the world from Australia to India, Turkey, Hungary, USA, and Argentina[3] as well as Europe and Africa. *B. cinerea* infections have been reported in climates varying from Alaskan cold to hot, arid climates. In India alone, *B. cinerea* has been known to destroy 20000 hectares of chickpeas in one season[3]. Botrytis is also known to cause disease in economically important crops while they are being cultured in greenhouses or kept in storage[4]. Botrytis can thrive under temperatures ranging from 2–30 °C, hence causing post-harvest losses and reduction of shelf life of perishable goods[3,5]. Botrytis is generally believed to demonstrate optimal growth at 15-20 °C; however considerable infection has also been observed at 4 °C [3,6]. It also has a beneficial role in the production of rare wines, resulting in fine and concentrated sweet wine; thus another name for *B. cinerea* is "Noble Rot"[7]. *B. cinerea* is also used as a model for the study of fungal infections processes and is studied widely by many research groups. This fungus causes infections which are characterized by the rapid destruction of the tissues of the host plant as it proceeds to colonize it in a process called necrotrophy[8]. The fight against fungal diseases provoked by *B. cinerea* requires the use of pre- and post-harvest fungicides. The magnitude of the fungicidal treatments has resulted in the appearance of resistant strains. For example the use of benzimidazole and dicarboximide fungicides has led to the development of resistant strains to these fungicides[9], thereby

necessitating the development of new antifungal molecules, novel methods of cultivation techniques and the development of resistant cultivars [9, 10, 11, 12].

Infection Process

In the case of *B. cinerea*, infection is primarily spread by multi-nucleate, asexual conidia. The condia on the host surface germinate and develop into the germ tube. The germ tube swells at the tip, supporting a collection of enzymes and metabolites, which are finally secreted on to the host cell. Generally in filamentous fungi, the condia produces the germ tube which develops into an appresorium that facilitates mechanical penetration of the host cell[8]; this is followed by the secretion of an array of enzymes. However, in the case of *B. cinerea*, no physical damage is observed at the site of penetration. This observation, along with smooth edges at the penetration site suggests the principle mechanism of attack is enzymatic degradation of the primary barrier of the host cell[13]. After successful invasion of the initial host cell by the pathogen, it spreads into the adjacent healthy tissues. At a certain stage of infection, the fungus produces conidiophores which bear conidia. The fungus eventually releases the conidia, resulting in inoculums for the next infection[3].

It has been proposed that Botrytis is an opportunistic pathogen which attacks senescent tissues or fully mature tissues which have some structural damage[14]. However Botrytis is fully capable of infecting plants with no structural damage. This fungus utilizes a large range of pathogenic factors including enzymes, activated oxygen species, toxins, and plant hormones to attack its host plants[15]. *B. cinerea* then rots or necrotizes the healthy tissues initially by penetrating host cell walls, which are biphasic composites of cellulose microfibrils, the pectin network and the hemicellulose network.

Plant cell walls

Plant cell walls are a primary defense system of the plant. They function as mechanical support for the plants,, but they also provide a physical and chemical barrier to protect the cell from the external environment[16] as well as preventing intracellular fluid from leaking out of the cell. The cell wall is generally composed of three carbohydrate polymeric networks of pectin, cellulose and hemicellulose[17,18]. Cellulose is composed of long chains of beta-(1,4)-linked glucose[19]. Cellulose is the most abundant organic polymer in nature. Cellulose fibrils consist of bunches of closely packed cellulose chains with close lateral associations. Hemicelluloses are rather similar to cellulose. The difference between cellulose and hemicelluloses is the existence of small side chains to the backbone preventing close packing into microfibrils. The most abundant hemicelluloses are xyloglucans. The pectin backbone primarily consists of alpha-(1-4)-linked D-galacturonic acid[20]. Pectin is generally composed of three major domains of homogalacturonan (HGA), rhamogalacturonan-I (RG-I) and rhamogalacturonan-II (RG-II)[19, 20]. Other than the polysaccharides, plant cell walls also contain proteins, glycoproteins, phenolic and inorganic compounds.

Botrytis cinerea secreted proteins

An array of enzymes and metabolites are secreted by *B. cinerea* to invade the plant tissue, kill the plant cell and convert the plant cell into fungal biomass. Many of these enzymes act extracellularly at the plant-fungal interface. Filamentous fungi have been known to secrete enzymes involved in polymer decomposition, oligosaccharide decomposition and protein decomposition. Cell wall degradation is often observed during the initial steps of pathogenesis and has been suggested to be instrumental in penetration. Some of the *B. cinerea* secreted enzymes previousy reported are cutinases, lipases, pectin methyl esterases (PME), endopolygacturonases, exopolygalacturonases, pectin lyases, pectate lyases, rhamnogalacturonan

hydrolases, glucosidases, superoxide dismutase, glyoxal oxidase, arabinases, xylanases, aspartic proteases and laccases [3,21-34]. Endopolygalacturonases (EPG) are one of the early classes of enzymes secreted during infection for degradation of the pectin component of plant cell walls. EPGs are one of the most widely studied classes of pectin degrading enzymes , and hydrolyze the internal (1-4) linkage between D-galacturonic acid units of pectin[35]. Previously 6 EPGs have been identified from *B. cinerea*[8]; however it has been suggested that *B. cinerea* can secrete up to 13 polygalacturonase isoforms[36].

Wubben et al. have shown that a basic level of gene expression exists for two endoPGs (*Bcpg*1 and *Bcpg*2) in liquid cultures when fungus is grown on different media. The expression of certain *Bcpg* genes depended on the pH of the liquid culture. They studied the impact of galacturonic acid in liquid culture on gene expression of *Bcpgs,* and it was determined that the expression of the *Bcpg*s depended on the carbon source, including the presence or absence of galacturonic acid[37]. The gene expression of endoPG was studied on tomato, broad bean, apple and courgette infected by *B. cinerea*. All endo*Bcpg* genes are differentially expressed depending on the host tissue, the stage of infection, and the temperature. The expression of endo*Bcpg* genes in plants was partly explained by the result obtained in liquid culture[36].

Ten et al have shown that deletion of the PG-encoding gene named *Bcpg1* in a Botrytis mutant resulted in a weaker infection compared to wild-type strains[38]. The results indicated that *Bcpg1* is important for virulence[38]. The endoPG gene family exists in all *Botrytis cinerea* strains and Botrytis species, although some of these Botrytis species can infect only one host plant species. Therefore how these genes are implicated in the infection by Botrytis of more than 200 species is still unclear. Out of the six endoPGs, two deletion mutants reduced virulence on

multiple host, whereas the other 4 endo*BcPG* deletion mutants demonstrated no effect on virulence of multiple host compared to wild type[1].

The *Bcpg1* deletion mutant exhibited reduced virulence on d'Anjou pear fruit compared to wild type, however deletion of the PME-encoding gene named *Bcpme1* did not.[39] Thus, *Bcpg1* increases the rate of gray mold progression in pear fruits, but *Bcpme1* appears to be dispensable[39]. PMEs de-esterify the pectin component of cell walls, releasing methanol and polygalacturonic acid. This de-esterification facilitates the subsequent action of polygalacturonases and pectate lyases[35]. Endo-beta-(1,4) xylanase and *Bcsod1* deletion mutants showed reduction in virulence compared to wildtype, however deletion of *Bcpme1*, *Bcpme2, lipase, Bclcc1, Bclcc2, cutinase, cellulase* and *gloxal oxidase* genes from Botrytis had no effect on virulence[3,8,32,33,35]. The results obtained in these experiments are influenced by the isoenzymes of the deleted gene. Most of these studies are based on a single gene, one gene family or a single protein. It is speculated that Botrytis is capable of producing isoenzymes for all of the above cell wall degrading enzymes and hence it is suggested that these isoenzymes might compensate for deletion mutants of single genes. To study the true significance of the protein function, gene deletion of the whole family of genes should be conducted. Nonetheless, the targeted mutagenesis of *B. cinerea* proteins has facilitated an initial level of understanding of the individual gene involvement in pathogenesis. The *B. cinerea* genome must encode several different enzymes to act in concert to invade, destroy and feed from the host.

Necrosis and ethylene inducing proteins (NEP) are small proteins (<30 KDa) known to be related to pathogenicity. NEP or NEP-like proteins are known to elicit a cell defense response and necrosis in large numbers of dicotyledonous plants. Ethylene production is an active response of plants to a perceived pathogen attack and is associated with the induction of the

defense response[40]. Recently it has been shown that *B. cinerea* NEP proteins are present in the nuclear compartment of the host cell post-infection[41]. As is evident from the above discussion, a comprehensive study to identify all secreted *B. cinerea* proteins involved in pathogenecity is required.

Proteomics

The successful mapping of genes in genomics has in large part been responsible for the current rise of proteomics. One of the major goals of proteomics is to identify and quantify proteins in a biological system which is often supplemented with studying the structure of proteins, protein-protein interactions, and protein-carbohydrate interactions. For characterizing complex protein samples, 2D gel electrophoresis and high throughput proteomics are the most widely used methods[42, 43].

Two dimensional gel electrophoresis

Two dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis (2D SDS-PAGE) is a protein separation technique. Separation in the first dimension is based on isoelectric points [43-45] and is called isoelectric focusing. An immobilized pH gradient strip is generally used for this procedure which creates a stable pH gradient in the separation medium. When proteins are placed in the pH gradient and an electric field, they may acquire charge and migrate to their isoelectric point, defined as the point at where the net protein charge is zero. . Any protein which migrates away from its isoelectric point acquires charge and migrates back to its isoelectric point[43].

In the second dimension the proteins are separated by sodium dodecylsulfate polyacrylamide gels on the basis of molecular weight[45]. The gel from the first dimension separation is soaked in a denaturing solution containing the detergent sodium dodecylsulphate

(SDS). SDS has a net negative charge and binds to the proteins. The number of SDS molecules binding to the protein depends on the protein size, effectively making the mass to charge value of every protein the same. A voltage is then applied across the gel. All the proteins move towards the cathode but smaller proteins move faster through the gel whereas larger molecules move slower due to friction, thus separating proteins according to size[43, 46].

Protein spots resolved on the second gel can be visualized using dyes like Coomassie Blue or by silver staining. Staining enables a semi-quantitative comparison between proteome samples as well as selection of a particular feature for further analysis. Coomassie Blue is easy to use and shows linearity of detection over a 10-50 fold range[47]. The low sensitivity of Coomassie Blue limits the visualization to only highly abundant proteins. Silver staining is a very sensitive technique for protein visualization. However it is a complex multistep procedure and so gel to gel reproducibility may be difficult[46, 48, 49]. Not all proteins are stained equally by this technique and linearity of detection is around 10 fold and hence difficult to use for quantitative analysis[46, 48-50]. 2-D gel electrophoresis is a powerful method for the separation of proteins and when coupled with the use of enzymes such as glycosidases and phosphatases allows the investigator to detect the size of proteins that may undergo a variety of posttranslational modifications, such as glycosylation or phosphorylation. While the ability to quantify proteins is an advantage of 2D gels, the in gel resolution of small proteins can be difficult.

Liquid Chromatography and Tandem Mass Spectrometry (LC-MS/MS)

LC-MS/MS proteomic analysis is one of the most powerful high throughput approaches for characterizing complex proteome samples. The analysis includes four principal steps: sample preparation (digestion), separation of the peptides, generation of spectra of peptides by mass spectrometry and data mining (protein identification, functional classification or comparative analysis)[51].

In proteomics, proteins are converted into peptides through enzymatic digestion which cleaves a complicated mixture of proteins to an even more complicated mixture of peptides. Trypsin is the most common enzyme used in proteomics[43, 51]. Trypsin cleaves after the basic residues arginine and lysine if they are not followed by proline. Other enzymes used for protein digestion are Lys C (cleaves after Lys), and Asp-N (cleaves before aspartate). The resulting peptides are generally separated using high pressure reverse phase or strong cation exchange liquid chromatography. In reverse phase chromatography the peptide separation is based on hydrophobicity and in strong cation exchange chromatogrphy the peptide separation is based on number of basic residues present on the peptides[52-55]. Generally the separation is performed online with the HPLC connected directly to the mass spectrometer. The separated peptides elute directly into the mass spectrometer for analysis. The mass spectrometer consists of three main components: an ion source, a mass analyzer and the detector. The two most common ionization sources used in proteomics are matrix assisted laser desorption ionization (MALDI) and electrospray ionization[56]. Tryptically digested peptides gain charge in the ionizaton source and corresponding ions are transferred to the mass analyzer.

Ion trap, time of flight (TOF), Quadrupole and Fourier transform ion cyclotron resonance mass spectrometer (FTICR) are four types of mass analyzers currently used[57-60]. Some of these analyzers are used in tandem to obtain proteomics data. In the mass analyzer the mass to charge value for the various peptide ions is obtained. These peptide ions may be subjected to fragmentation to obtain fragment ion spectra. The precursor and fragment ion spectra are often enough to obtain an amino acid sequence of the peptide. Due to the generation of thousands of spectra in one proteomic analysis search algorithms and protein databases are used to obtain the

amino acid sequence of the peptide. For the purpose of the current study, only electro spray ionization and Ion trap Mass Spectrometers will be discussed.

Electro Spray Ionization (ESI)

Soft ionization techniques such as ESI and MALDI, have opened up the high throughput characterization of peptides using mass spectrometry, and along with the genomics revolution and algorithm for database searching, gave birth to the field of proteomics. In ESI high voltage is applied to the capillary through which the analyte solution is flowing. The result of the voltage is the addition of charge to the analyte ions. The charged droplets sprayed from the capillary travel to the mass spectrometer due to potential and pressure differential[61, 62]. The mass spectrometer is at a lower pressure compared to droplets that are under atmospheric pressure. During the migration, the droplets size is reduced by evaporation of the solvent or by splitting of droplets into smaller drops due to high charge density, resulting into a beam of ions which is analyzed by the mass spectrometer[61] (Figure 2.1). ESI is accomplished at atmospheric pressure, thus allowing online coupling of high pressure liquid chromatography and mass spectrometers like the Ion trap[62, 63]. The low flow rates and ionization efficiency make ESI an ideal source for high throughput proteomics study[63].

Ion Trap Mass Spectrometer

Analyte Ions generated from ESI are guided into the ion trap mass analyzer. The ion trap consists of three electrodes uniquely positioned, two end cap electrodes and between them a ring electrode with a hyperbolic profile (Figure 2.2).

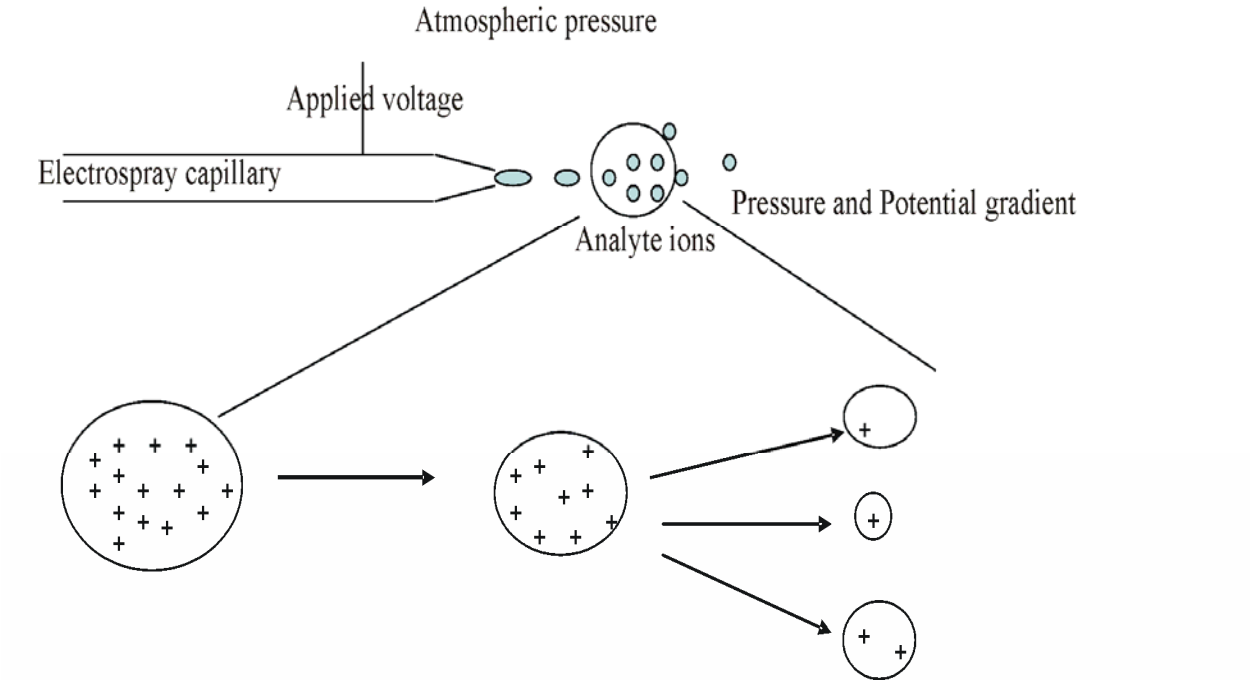

Figure 2.1: is a general scheme of electro spray ionization process.

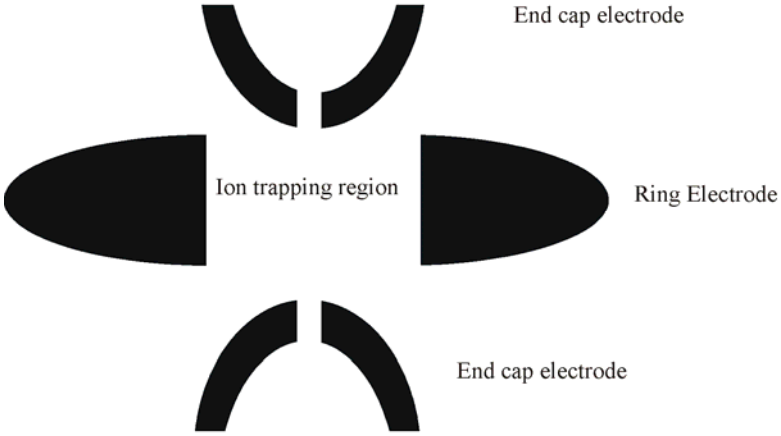

Figure 2.2 A cross sectional schematic of ion trap mass spectrometer

The theory of ion trap[60, 64-67] is the ions are initially trapped inside the mass analyzer in a three dimensional electric field. The electric field is generated by applying RF and DC voltage

between the ring and end cap electrodes. The incoming analyte ion's kinetic energy is reduced with helium molecules already present in the trap. W. Paul and H. Steinwedel predicted the stability of the ions trajectories inside the three dimensional electric field by a stability diagram[68].

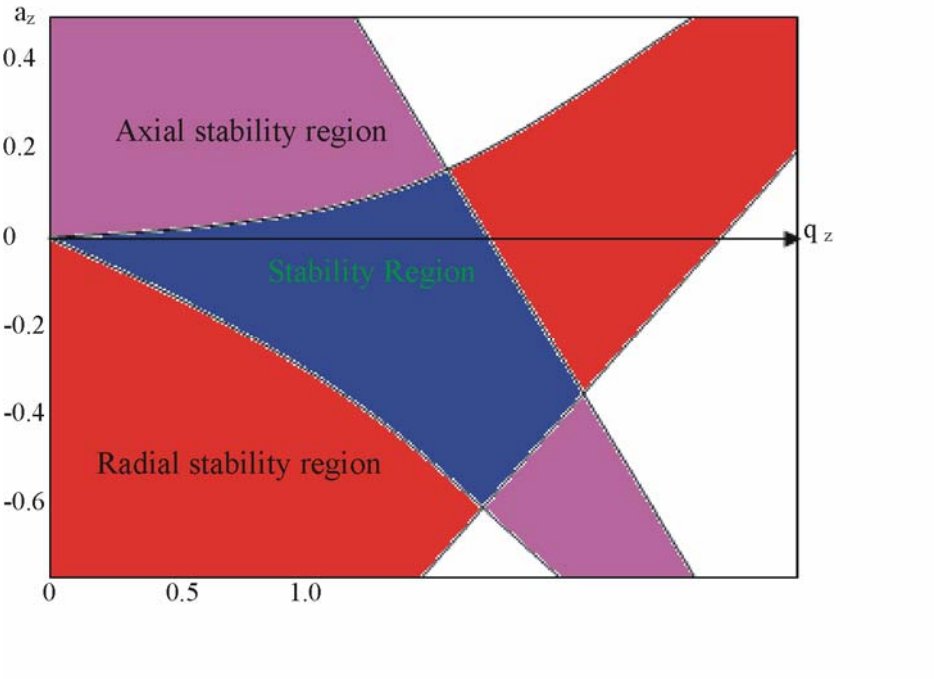

Figure 2.3 Stability diagrams of ions inside the ion trap[68]

Position of an ion in a stability diagram can be determined by the "a" and "q" coordinates obtainable from following equation[60, 64, 65, 67, 68].

$$q_z = \frac{4eV}{mr_o^2\omega^2}$$

$$a_z = \frac{8eU}{mr_o^2\omega^2}$$

V = amplitude of radio frequency (RF) voltage applied to ring electrode, U= amplitude of direct current (DC) voltage applied to ring electrode, r_o = hyberbolic radius of the ring electrode, M = mass of the ion, E = electric charge, ω = angular frequency. q_z =dimensionless parameter that determines stability of ion trajectories depending upon their mass-to-charge ratio, the size of the ion trap, and the amplitude and frequency of the fundamental RF.

The central region in the stability diagram represents where the ion will be stably trapped in both dimensions. By adjusting the RF and DC voltage at the electrodes ions can be made to have stability in only one field and hence ejecting the ions from a specific direction (mass selective ejection). The MS spectrum can be obtained by detecting the ejected ions. Ion traps have the ability to isolate and store a particular precursor ion. By using a dipolar Field, an isolated precursor ion can be accelerated[60, 64, 66, 67]. The accelerated ion having higher energy brings about harder and frequent collision with helium ions causing fragmentation of the precursor ion. The MS/MS spectrum can be obtained by ejecting and detecting fragment ions. Ion traps are robust, sensitive and relatively inexpensive. In the trap, a limited number of ions are accumulated in the center before space charge effects reduce the accuracy of mass measurement. In linear or two-dimensional ion traps, ions are stored in a cylindrical volume that is considerably larger than that of a 3D trap[65, 69]. Technical advancements in mass spectrometers and LC systems have increased sensitivity and throughput of proteome analysis. The scan time for one spectrum is small, resulting in the generation of thousands of spectra in a typical high throughput proteomics study using LC-MS/MS.

Data Analysis

A typical proteomics study generates tens of thousands of spectra. Manual assignment of these spectra to peptide sequences is a time consuming process and not feasible. In high

throughput proteomics, mass spectrometric data analysis is conducted using software that searches the database. Databases contain all the probable amino acid sequences of proteins that can be expressed in the sample from the genome or expressed sequenced tags (EST). Mascot and Sequest are the two most commonly used algorithms. In the software the experimental spectra is compared with the theoretically predicted spectra of peptides in the database [70, 71]. Mascot and Sequest assign peptides to spectra with a score indicating the amount of certainty regarding identification, like Xcorr value in Sequest and Mowse score in Mascot[70, 71]. While these scores are useful for peptide identification, an interpretation of the validity of the results is desired. For an accurate interpretation of results, it is necessary to distinguish between correct assignments and false identification [53, 72-75]. Different methods have been suggested to filter correct assignments from incorrect peptide assignments based on Sequest and Mascot scores by using various concepts like p-value, Q score, false positive rate, family wise error rate [FWER], false negative rate, and false discovery rate [53, 71, 76].

Secretome of filamentous fungi

The secretome is the complement of proteins secreted from the cell. In case of filamentous fungi there had been very few studies on their secretome at the onset of this century. However the availability of complete genome sequences from different filamentous fungi has made secretome study plausible. As more genome sequences become available, identification and functional classification of proteins from filamentous fungi will become easier to perform, and this approach will become an important research tool in functional fungal biology. Recently there has been increase in the number of studies conducted on the secretomes of different filamentous fungi. Filamentous fungi have a number of properties that make them model organisms to study secretomes, These include their use as producers of industrial enzymes, their

ability to infect different kinds of host, and their impact as agents of biodegradation, spoilage and decay; all these provide a compelling rationale for secretome research[77]. Wilson Francisco and colleagues provided pioneering contributions to this field, establishing a sample preparation protocol for the fungal secretome[77]. Secretomes of the filamentous fungi which have been reported are *Fussarium graminearum, Aspergillus flavus, Aspergillus oryzae, Pleurotus spadius and Phanerochate chrysosporium*[77-81]. In *Fussarium graminearum* secretome studies, 89 proteins were identified when fungi were grown on liquid culture of glucose and hop plant cell wall[82]. In *Aspergillus flavus* secretome 51 unique proteins identified from 3 different growth conditions[78]. It was observed that depending upon the growth conditions, the secretome of *Aspergillus flavus* changed[78]. However one third of the proteins identified were present in all three secretomes.

Twenty nine proteins were identified from *Aspergillus oryzae* secretome using 2D gel electrophoresis and MALDI TOF analysis, and it was suggested that there is difference in protein profile of secretion when fungus is grown in liquid and solid medium[79]. Twenty four secreted proteins have been identified from *Phanerochate chrysosporium* secretome[80]. The edible fungus *Pleurotus sapidus* was grown in submerged cultures either on peanut shells or on glass wool as a carrier material[81]. Proteins from the cultures with a carrier medium of peanut shells were TCA precipitated. Their analysis by 2-DE was severely complicated by a pronounced smear in the acidic pH range which was not observed with supernatants from the glass wool cultures. Most probably this interference was caused by phenolic constituents extracted from the peanut shells. A difference in protein profile of secretion was also observed when fungus was grown in above mediums; accordingly the secretion of this fungus also depends on the type of growth substrate. A proteomic analysis was used to differentiate and identify the extracellular rutin-induced and non-induced proteins secreted by *A. flavus*[78]. Secreted proteins were purified by TCA

precipitation and washed with 70% ethanol. The measured protein concentration was not analogous to the amount of protein appearing on the gels. The authors suggested the possibility of the presence of interfering agents in the samples.

A comprehensive proteomics study of *B. cinerea* secreted proteins has not been achieved to date, even though *B. cinerea* secreted enzymes play an important role in host pathogen interaction. Advancement in liquid chromatography, mass spectrometry and bioinformatics techniques and availability of genomes has made the study of filamentous fungi secretomes plausible. In this work following three manuscripts deal with investigation of *B. cinerea* secretome.

REFERENCES

1. Williamson, B.; Tudzynski, B.; Tudzynski, P.; Van Kan, J. A. L., *Botrytis cinerea*: the cause of grey mould disease. *Molecular plant pathology* 2007, 8, (5), 561-580.
2. Jarvis, W. R., Botryotinia and Botrytis species: taxonomy, physiology, and pathogenicity. *Can. Dep. Agric. Monogr* 1977, 15.
3. Elad, Y., *Botrytis: Biology, Pathology and Control*. Kluwer Academic Pub: 2004.
4. Elad, Y.; Shtienberg, D., *Botrytis cinerea* in greenhouse vegetables: chemical, cultural, physiological and biological controls and their integration. *Integrated Pest Management Reviews* 1995, 1, (1), 15-29.
5. Lahlali, R.; Serrhini, M. N.; Friel, D.; Jijakli, M. H., Predictive modelling of temperature and water activity (solutes) on the in vitro radial growth of *Botrytis cinerea* Pers. *International Journal of Food Microbiology* 2007, 114, (1), 1-9.
6. Bondoux, P., *Maladies de conservation des fruits à pépins. Pommes et poires*. Editions Quae: 1992.
7. Nelson, K. E.; Amerine, M. A., Use of *Botrytis cinerea* for the production of sweet table wines. *American Journal of Enology and Viticulture* 1956, 7, (4), 131-136.
8. van Kan, J. A. L., Licensed to kill: the lifestyle of a necrotrophic plant pathogen. *Trends in Plant Science* 2006, 11, (5), 247-253.
9. Myresiotis, C. K.; Karaoglanidis, G. S.; Tzavella-Klonari, K., Resistance of *Botrytis cinerea* isolates from vegetable crops to anilinopyrimidine, phenylpyrrole, hydroxyanilide, benzimidazole, and dicarboximide fungicides. *Plant Disease* 2007, 91, (4), 407-413.

10. Raposo, R.; Delcan, J.; Gomez, V.; Melgarejo, P., Distribution and fitness of isolates of *Botrytis cinerea* with multiple fungicide resistance in Spanish greenhouses. *Plant Pathology* 1996, 45, (3), 497.

11. LaMondia, J. A.; Douglas, S. M., Sensitivity of *Botrytis cinerea* from Connecticut greenhouses to benzimidazole and dicarboximide fungicides. *Plant Disease* 1997, 81, (7), 729-732.

12. Rosslenbroich, H. J.; Stuebler, D., *Botrytis cinerea*—history of chemical control and novel fungicides for its management. *Crop Protection* 2000, 19, (8-10), 557-561.

13. Salinas, J.; Verhoeff, K., Microscopical studies of the infection of gerbara flowers by *Botrytis cinerea*. *European Journal of Plant Pathology* 1995, 101, (4), 377-386.

14. Prusky, D., Pathogen quiescence in postharvest diseases. *Annual Review of Phytopathology* 1996, 34, (1), 413-434.

15. Staples, R. C.; Mayer, A. M., Putative virulence factors of *Botrytis cinerea* acting as a wound pathogen. *FEMS Microbiology Letters* 1995, 134, (1), 1-7.

16. Vorwerk, S.; Somerville, S.; Somerville, C., The role of plant cell wall polysaccharide composition in disease resistance. *Trends in Plant Science* 2004, 9, (4), 203-209.

17. Carpita, N. C.; Gibeaut, D. M., Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J* 1993, 3, (1), 1-30.

18. Carpita, N.; McCann, M., The cell wall. *Biochemistry and Molecular Biology of Plants, BB Buchanan, W. Gruissem, and RL Jones, eds (Rockville, MD: American Society of Plant Physiologists)* 2000, 52–108.

19. Carpita, N. C., Structure and biogenesis of the cell walls of grasses. *Annual Reviews in Plant Physiology and Plant Molecular Biology* 1996, 47, (1), 445-476.

20. Willats, W. G. T.; Orfila, C.; Limberg, G.; Buchholt, H. C.; van Alebeek, G.; Voragen, A. G. J.; Marcus, S. E.; Christensen, T.; Mikkelsen, J. D.; Murray, B. S., Modulation of the degree and pattern of methyl-esterification of pectic homogalacturonan in plant cell walls. Implications for pectin methyl esterase action, matrix properties, and cell adhesion.. *Journal of Biological Chemistry* 2001, 276, (22), 19404-19413.

21. Kars, I.; Krooshof, G. H.; Wagemakers, L.; Joosten, R.; Benen, J. A. E.; van Kan, J. A. L., Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. *Plant Journal* 2005, 43, (2), 213-225.

22. Chen, H. J.; Smith, D. L.; Starrett, D. A.; Zhou, D.; Tucker, M. L.; Solomos, T.; Gross, K. C., Cloning and characterization of a rhamnogalacturonan hydrolase gene from *Botrytis cinerea*. *IUBMB Life* 1997, 43, (4), 823-838.

23. Kars, I.; McCalman, M.; Wagemakers, L.; van Kan, J. A. L., Functional analysis of *Botrytis cinerea* pectin methylesterase genes by PCR-based targeted mutagenesis: Bcpme1 and Bcpme2 are dispensable for virulence of strain B05. 10. *Molecular Plant Pathology* 2005, 6, (6), 641-652.

24. Schouten, A.; Tenberge, K. B.; Vermeer, J.; Stewart, J.; Wagemakers, L.; Williamson, B.; van Kan, J. A. L., Functional analysis of an extracellular catalase of *Botrytis cinerea*. *Molecular Plant Pathology* 2002, 3, (4), 227-238.

25. Wubben, J. P.; Mulder, W.; ten Have, A.; van Kan, J. A. L.; Visser, J., Cloning and partial characterization of endopolygalacturonase genes from *Botrytis cinerea*. *Applied and Environmental Microbiology* 1999, 65, (4), 1596.

26. van Kan, J. A. L.; van't Klooster, J. W.; Wagemakers, C. A. M.; Dees, D. C. T.; van der Vlugt-Bergmans, C. J. B., Cutinase a of *Botrytis cinerea* is expressed, but not essential, during penetration of gerbera and tomato. *Molecular Plant-Microbe Interactions* 1997, 10, (1), 30-38.

27. Comménil, P.; Belingheri, L.; Sancholle, M.; Dehorter, B., Purification and properties of an extracellular lipase from the fungus *Botrytis cinerea*. *Lipids* 1995, 30, (4), 351-356.

28. Cabanne, C.; Doncche, B., Purification and characterization of two isozymes of polygalacturonase from *Botrytis cinerea*. Effect of calcium ions on polygalacturonase activity. *Microbiological Research* 2002, 157, (3), 183-189.

29. Sasaki, I.; Nagayama, H., ß-glucosidase from *Botrytis cinerea*: Its relation to the pathogenicity of this fungus. *Biosci. Biotechnol. Biochem* 1994, 58, 616-620.

30. ten Have, A.; Dekkers, E.; Kay, J.; Phylip, L. H.; van Kan, J. A. L., An aspartic proteinase gene family in the filamentous fungus *Botrytis cinerea* contains members with novel features. In Soc General Microbiol: 2004; Vol. 150, pp 2475-2489.

31. Slomczynski, D.; Nakas, J. P.; Tanenbaum, S. W., Production and characterization of laccase from *Botrytis cinerea* 61-34. *Applied and Environmental Microbiology* 1995, 61, (3), 907.

32. Brito, N.; Espino, J. J.; Gonzalez, C., The endo-ß-1, 4-xylanase Xyn11A is required for virulence in *Botrytis cinerea*. *Molecular Plant-Microbe Interactions* 2006, 19, (1), 25-32.

33. Rolke, Y.; Liu, S.; Quidde, T.; Williamson, B.; Schouten, A.; Weltring, K. M.; Siewers, V.; Tenberge, K. B.; Tudzynski, B.; Tudzynski, P., Functional analysis of H2O2-generating systems in *Botrytis cinerea*: the major Cu-Zn-superoxide dismutase(BCSOD

1) contributes to virulence on French bean, whereas a glucose oxidase(BCGOD 1) is dispensable. *Molecular Plant Pathology* 2004, 5, (1), 17-27.

34. Kapat, A.; Zimand, G.; Elad, Y., Biosynthesis of pathogenicity hydrolytic enzymes by *Botrytis cinerea* during infection of bean leaves and in vitro. *Mycological Research* 1998, 102, (08), 1017-1024.

35. Valette-Collet, O.; Cimerman, A.; Reignault, P.; Levis, C.; Boccara, M., Disruption of *Botrytis cinerea* pectin methylesterase gene Bcpme 1 reduces virulence on several host plants. *Molecular Plant-Microbe Interactions* 2003, 16, (4), 360-367.

36. ten Have, A.; Breuil, W. O.; Wubben, J. P.; Visser, J.; van Kan, J. A. L., *Botrytis cinerea* endopolygalacturonase gnes are differentially expressed in various plant tissues. *Fungal Genetics and Biology* 2001, 33, (2), 97-105.

37. Wubben, J. P.; ten Have, A.; van Kan, J. A. L.; Visser, J., Regulation of endopolygalacturonase gene expression in *Botrytis cinerea* by galacturonic acid, ambient pH and carbon catabolite repression. *Current Genetics* 2000, 37, (2), 152-157.

38. ten Have, A.; Mulder, W.; Visser, J.; van Kan, J. A. L., The endopolygalacturonase gene Bcpg1 is required for full virulence of *Botrytis cinerea*. *Mol. Plant-Microbe Interact* 1998, 11, 1009-1016.

39. Akagi, A.; Stotz, H. U., Effects of pathogen polygalacturonase, ethylene, and firmness on interactions between pear fruits and *Botrytis cinerea*. *Plant Disease* 2007, 91, (10), 1337-1344.

40. van Loon, L. C.; Geraats, B. P. J.; Linthorst, H. J. M., Ethylene as a modulator of disease resistance in plants. *Trends in Plant Science* 2006, 11, (4), 184-191.

41. Staats, M.; van Baarlen, P.; Schouten, A.; van Kan, J. A. L.; Bakker, F. T., Positive selection in phytotoxic protein-encoding genes of Botrytis species. *Fungal Genetics and Biology* 2007, 44, (1), 52-63.

42. Gygi, S. P.; Corthals, G. L.; Zhang, Y.; Rochon, Y.; Aebersold, R., Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. *Proceedings of the National Academy of Sciences* 2000, 97, (17), 9390.

43. Matthiesen, R., *Mass Spectrometry Data Analysis in Proteomics*. Humana Press: 2007.

44. Gorg, A.; Obermaier, C.; Boguth, G.; Harder, A.; Scheibe, B.; Wildgruber, R.; Weiss, W., The current state of two-dimensional electrophoresis with immobilised pH gradients. *Electrophoresis* 2000, 21, (6), 1037-1053.

45. O'Farrell, P. H., High resolution two-dimensional electrophoresis of proteins. *Journal of Biological Chemistry* 1975, 250, (10), 4007-4021.

46. Veenstra, T. D.; Yates Iii, J. R., Proteomics for Biological Discovery. *Proteomics* 2006, 6, 6384-6387.

47. Simpson, R. J., *Proteins and Proteomics: A Laboratory Manual*. CSHL Press: 2003.

48. James, P.; Quadroni, M.; Carafoli, E.; Gonnet, G., Protein identification by mass profile fingerprinting. *Biochem Biophys Res Commun* 1993, 195, (1), 58-64.

49. Quadroni, M.; James, P., Proteomics and automation. *Electrophoresis* 1999, 20, (4-5), 664-677.

50. Goldberg, H. A.; Warner, K. J., The Staining of Acidic Proteins on Polyacrylamide Gels: Enhanced Sensitivity and Stability of “Stains-All” Staining in Combination with Silver Nitrate. *Analytical Biochemistry* 1997, 251, (2), 227-233.

51. Patterson, S. D.; Aebersold, R. H., Proteomics: the first decade and beyond. *Nature Genetics* 2003, 33, (3 s), 311-323.

52. Florens, L.; Washburn, M. P.; Raine, J. D.; Anthony, R. M.; Grainger, M.; Haynes, J. D.; Moch, J. K.; Muster, N.; Sacci, J. B.; Tabb, D. L., A proteomic view of the *Plasmodium falciparum* life cycle. *Nature* 2002, 419, 520-526.

53. Peng, J.; Elias, J. E.; Thoreen, C. C.; Licklider, L. J.; Gygi, S. P., Evaluation of multidimensional chromatography coupled with tandem mass spectrometry (LC/LC-MS/MS) for large-scale protein analysis: the yeast proteome. *J. Proteome Res* 2003, 2, (1), 43–50.

54. Wang, H.; Hanash, S., Multi-dimensional liquid phase based separations in proteomics. *Journal of Chromatography B* 2003, 787, (1), 11-18.

55. Wolters, D. A.; Washburn, M. P.; Yates Iii, J. R., An automated multidimensional protein identification technology for shotgun proteomics. *Anal. Chem* 2001, 73, (23), 5683–5690.

56. Yates, J. R., Mass spectral analysis in proteomics. *Annual Review of Biophysics and Biomolecular Structure* 2004, 33, (1), 297-316.

57. Wiley, W. C.; McLaren, I. H., Time-of-Flight Mass Spectrometer with improved resolution. *Review of Scientific Instruments* 2004, 26, 1150.

58. Marshall, A. G., Fourier transform ion cyclotron resonance mass spectrometry. *Accounts of Chemical Research* 1985, 18, (10), 316-322.

59. Syka, J. E. P., Mass spectrometer. In EP Patent 0,237,259: 1987.

60. March, R. E., Quadrupole ion trap mass spectrometry: Theory, simulation, recent developments and applications. *Presented at the 16th Informal Meeting on Mass Spectrometry* 1998, 4, 6.

61. Vestal, M. L., Methods of ion generation. *Chem Rev* 2001, 101, (2), 361-75.

62. Gaskell, S. J., Electrospray: Principles and practice. *Journal of Mass Spectrometry* 1997, 32, (7), 677-688.

63. Griffiths, W. J.; Jonsson, A. P.; Liu, S.; Rai, D. K.; Wang, Y., Electrospray and tandem mass spectrometry in biochemistry. *Biochem J* 2001, 355, (Pt 3), 545-61.

64. March, R. E., An introduction to quadrupole ion trap mass spectrometry. *Journal of Mass Spectrometry* 1997, 32, 351È369.

65. Schwartz, J. C.; Senko, M. W.; Syka, J. E. P., A two-dimensional quadrupole ion trap mass spectrometer. *Journal of the American Society for Mass Spectrometry* 2002, 13, (6), 659-669.

66. Cooks, R. G.; Kaiser Jr, R. E., Quadrupole ion trap mass spectrometry. *Accounts of Chemical Research* 1990, 23, (7), 213-219.

67. March, R. E.; Todd, J. F. J.; Glish, G. L., Quadrupole ion trap mass spectrometry. *Journal of the American Society for Mass Spectrometry* 2006, 17, (5), 746-747.

68. Paul, W., Electromagnetic traps for charged and neutral particles. *Reviews of Modern Physics* 1990, 62, (3), 531-540.

69. Hager, J. W., A new linear ion trap mass spectrometer. *Rapid Communications in Mass Spectrometry* 2002, 16, (6), 512-526.

70. Perkins, D. N.; Pappin, D. J.; Creasy, D. M.; Cottrell, J. S., Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 1999, 20, (18), 3551-67.

71. Keller, A.; Nesvizhskii, A. I.; Kolker, E.; Aebersold, R., Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal Chem* 2002, 74, (20), 5383-5392.

72. Nesvizhskii, A. I.; Aebersold, R., Analysis, statistical validation and dissemination of large-scale proteomics datasets generated by tandem MS. *Drug Discovery Today* 2004, 9, (4), 173-181.

73. Resing, K. A.; Meyer-Arendt, K.; Mendoza, A. M.; Aveline-Wolf, L. D.; Jonscher, K. R.; Pierce, K. G.; Old, W. M.; Cheung, H. T.; Russell, S.; Wattawa, J. L., Improving reproducibility and sensitivity in identifying human proteins by shotgun proteomics. *Anal. Chem* 2004, 76, (13), 3556–3568.

74. MacCoss, M. J.; Wu, C. C.; Yates Iii, J. R., Probability-based validation of protein identifications using a modified SEQUEST algorithm. *Anal. Chem* 2002, 74, (21), 5593–5599.

75. Klose, J.; Blüggel, M., Evaluation of algorithms for protein identification from sequence databases using mass spectrometry data. *Proteomics* 2004, 4, 619-628.

76. Moore, R. E.; Young, M. K.; Lee, T. D., Qscore: an algorithm for evaluating SEQUEST database search results. *Journal of the American Society for Mass Spectrometry* 2002, 13, (4), 378-386.

77. Kim, Y.; Nandakumar, M. P.; Marten, M. R., Proteomics of filamentous fungi. *Trends in Biotechnology* 2007, 25, (9), 395-400.

78. Martha L. Medina, P. A. H. L. B. W. A. F., Analysis of secreted proteins from *Aspergillus flavus. Proteomics* 2005, 5, (12), 3153-3161.

79. Oda, K.; Kakizono, D.; Yamada, O.; Iefuji, H.; Akita, O.; Iwashita, K., Proteomic analysis of extracellular proteins from *Aspergillus oryzae* grown under submerged and solid-state culture conditions. *Appl. Environ. Microbiol.* 2006, 72, (5), 3448-3457.

80. Wymelenberg, A. V.; Sabat, G.; Martinez, D.; Rajangam, A. S.; Teeri, T. T.; Gaskell, J.; Kersten, P. J.; Cullen, D., The *Phanerochaete chrysosporium* secretome: Database predictions and initial mass spectrometry peptide identifications in cellulose-grown medium. *Journal of Biotechnology* 2005, 118, (1), 17-34.

81. Zorn, H.; Peters, T.; Nimtz, M.; Berger, R. G., The secretome of *Pleurotus sapidus*. *Proteomics* 2005, 5, (18), 4832-4838.

82. Phalip, V.; Delalande, F.; Carapito, C.; Goubet, F.; Hatsch, D.; Leize-Wagner, E.; Dupree, P.; Dorsselaer, A. V.; Jeltsch, J. M., Diversity of the exoproteome of *Fusarium graminearum* grown on plant cell wall. *Current Genetics* 2005, 48, (6), 366-379.

CHAPTER 3

SECRETEOME PROFILE OF *BOTRYTIS CINEREA*: A SHOTGUN APPROACH TO STUDY PATHOGEN INFECTION[1]

.

[1] Punit Shah, Hind El Mubarek, Ron Orlando, Gopi K. Podila and Maria R. Davis. To be submitted to *Open Spectroscopy*

ABSTRACT

To better understand general mechanisms of pathogen infection, we applied a shot-gun proteomics approach to study fungal infection of plants. The secretome of the phytopathogen, *Botrytis cinerea* was determined using a mock infection of full red strawberry extract. Plant host cultures were created by growing *B. cinerea* for 4-5 days on a nylon film overlaid on petri dishes containing growth media, with and without 4% red strawberry pulp. Afterwards, the membranes were transferred to Petri dishes containing polygalacturonic acid in acetate buffer for an additional 10 days to collect pectin induced protein secretions. Each sample was trypsin digested in triplicate then analyzed using Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) to obtain peptide sequences. Peptides were screened against the publicly available *B. cinerea* database at the BROAD Institute to identify each gene product. Putative peptide signal sequences were further analyzed using SignalP, TargetP and SecretomeP algorithms to assist in identifying predicted mechanisms of cellular secretion. The utility of this strategy is demonstrated by the comprehensive identification of over 59 putative classical and 38 nonclassical secreted proteins including 27 catabolic, 32 hypothetical, 10 involved in proteolysis/peptidolysis, and a variety of proteins with domain motifs that are conserved but have not been well characterized for fungal proteins. These putative secreted proteins are likely to be factors that *B. cinerea* uses to trigger pathogenic infection of strawberries.

List of abbreviations:

Carbohydrate Transport and Metabolism (CTM), Cellulose Binding Domain (CBD), Glycolipid Anchored Surface protein (GAS), Glycosyl hydrolase (Glyco_Hydro), Peroxiredoxin family (PRX), Dipeptidyl Aminopeptidase (DPPIV), Alpha Amylase (Aamy), Inorganic ion transport and metabolism (Kat), Protease Associated (PA), Aspartyl Protease (Asp), Dipeptidyl aminopeptidases/acylaminoacyl-peptidases (DAP2), Prolyl oligopeptidase family (Peptidase_S9), Acetyl xylan esterase (AXE1), Peptidase family M20/M25/M40 (Peptidase M20), Cyanate Hydratase (CynS), Chitin Binding Domain (ChtBD1), Peroxiredoxin family (PRX), Ran Binding Domain (RanBD), Serine Carboxypeptidase S28 (Peptidase S28), Ribosomal protein (RPP), Acetohydroxy acid isomeroreductase & Ketol-acid reductoisomerase (llvC), RNA Recognition Motif (RRM), PolyC Binding Protein (PCBP), AMP-activated protein kinase (AMPK), beta subunit glycogen binding domain (GBD), Plant protein of unknown function (DUF946), peptidyl-prolyl cis-trans isomerase (FKBP_C), Gamma subunit of Elongation Factor 1B (GST_C_EFB1gamma), Cyclophilin A, B and H-like cyclophilin-type peptidylprolyl cis- trans isomerase (PPIase) domain (cyclophilin_ABH_like), arabinofuranosidase B (AbfB)

INTRODUCTION

Botrytis cinerea is a filamentous phytopathogenic fungus that causes gray-mold rot of berries, tomatoes, peas and a number of leafy vegetables and fruits with low pH. Strawberries are one of the prominent low pH crops which incur considerable economical losses due to the activities of this pathogen. The ability for *B. cinerea* to cause disease is attributed, in part, to the fungi's arsenal of pathogenic tools. Secreted proteins are the core component of the degrading lifestyle of filamentous fungi and are particularly relevant when considering a study of fungal pathogens since these molecules trigger the initial interaction with the host plant. Another phytopathogenic fungus, *Sclerotinia sclerotiorum* is among the world's most successful plant pathogens with a host range of greater than 400 plant species. *S. sclerotiorum*, like Botrytis, also belongs to the class Leotiomycetes. *S. sclerotiorum* differs in many biological and molecular characteristics but is closely related taxonomically to *B. cinerea*. A comparison of genetic features between pathogenic fungal genomes can lead to a more comprehensive study of the degrading lifestyle of each. Both genome sequences from *B. cinerea* strain BO5.10 (provided by Syngenta AG.) and *S. sclerotiorum* strain '1980' genome sequences can be found at the Broad Institute site (www.broad.mit.edu). With an increasing availability of genome sequence data and mass spectrometric tools, it is becoming more feasible to use high throughput methods to identify a broader variety of fungal secreted proteins.

Typically, individual secreted proteins have been identified by 2-D gel electrophoresis followed by mass spectrometric analysis for a variety of fungi[1,2,3]. 2-D gel electrophoresis is a powerful method for the separation of proteins in a sample. While the ability to quantitate proteins is an advantage of 2D gels, the in gel resolution of small proteins (<30 kDa) can be

difficult. There are advantages in implementation of a proteomic approach based on multi-dimensional liquid chromatography–tandem mass spectrometry (LC-MS/MS) for the analysis of the secreteome[4,5,6].

Cellular protein secretion was once thought to traverse the endoplasmic reticulum (ER), Golgi apparatus, and plasma membrane; the well-accepted "classical" secretion pathway[7,8,9]. It is now recognized that other "non-classical" cellular secretion mechanisms exist that offer an alternate path that by-passes classical secretory routes – leading to proteins that do not have post-translational modifications such as N-glycosylation. These export processes are also resistant to brefeldin A, an inhibitor of the classical ER/Golgi dependent protein secretion mechanism[10,11]. Proteins that transverse this non-classical cellular secretion route have been found to be of particular biomedical relevance such as the galectin family, lectins of the extracellular matrix, involved in tumor-mediated immune suppression[12,13]. Other nonclassically secreted proteins that invoke cellular responses include proangiogenic growth factor fibroblast growth factor-2, FGF-2[14], inflammatory cytokines such as interleukin-1β, IL-1β[15] as well as hydrophilic acylated surface proteins (HASPs) from the *Leishmania* parasite implicated in host cell infection[16]. The presence or absence of a fully functional signal secretion sequence can be predicted with the use of several freely available computer software packages (http://www.cbs.dtu.dk/index.shtml) operated through the Center for Biological Sequence Analysis (CBS) located at the Technical University of Denmark DTU. The cellular relevance or existence of nonclassically secreted proteins is an important feature that has not been fully explored in the proteomic analysis of most fungal organisms.

Typically secreted proteins from filamentous fungi have included a variety of hydrolytic enzymes[2,17,18,19] and antifungal proteins[20]. Identified among *B. cinerea* secreted proteins include

aspartic proteases[21,22], β-glucosidases, laccases[23], pectinases[24] and possible bioactive proteins[25]. One of the best studied family of hydrolytic enzymes identified in *B. cinerea* are endopolygalacturonases. During all stages of infection, endopolygalacturonases (endoPGs) have been one family of secreted proteins that are acutely involved in the fungal pathogenic interaction with its host, to depolymerize pectin in the plant cell wall[26].

Filamentous fungi secrete these hydrolytic enzymes for penetration and feeding while colonizing solid substrates. The infection process of *B. cinerea* is initiated with an adhesion of conidia to the host surface that eventually forms a fungal sheath with more mature germling secretions[27]. This ensheathing film or fungal sheath enhances the organism's attachment to a host plant. In liquid culture with the production of the extracellular matrix (ECM), fungal biomass most often is centrifuged and/or filtered to recover the supernatant with secreted proteins[22,28]. Doss[29] has determined that 50-60% of the ECM dry weight is composed of carbohydrates, proteins and lipids. The carbohydrate fraction is composed of β-(1,3)(1,6)-D-glucans which is the sole polysaccharide found extracellular to the fungal mycelium[30,31]. We have used a simple method to allow sufficient quantities of secreted proteins, without any extraction from the ECM, to be reproducibly collected from *B. cinerea* (BO5.10) for biochemical and LC-MS/MS analysis [4, 5..

MATERIALS AND METHODS

Botrytis cinerea isolates and conidia collections

B. cinerea strain BO5.10 was obtained from Muriel Viaud (INRA, Versailles, FR). Stock spores were obtained after a single culturing of the primary culture on potato dextrose broth (Difco, Detroit, MI) for several weeks. Conidia were collected by gentle mixing with 0.05% Tween 80 in sterile water over the surface of the mature conidiophores. Frozen stocks of

isolated spores at10^6 spores/ml were prepared with sterile glycerol (15% final concentration) and stored at -80^{o}C in multiple aliquots.

Overall Methodology

A rapid throughput "shotgun proteomics" methodology for identifying fungal secreted proteins and signal sequences from the infection of plant hosts is outlined in Figure 3.1. Briefly this approach involves the following seven steps; 1) growing *B. cinerea* BO5.10 fungal cultures on nylon mesh overlaid on mock hosts, 2) floating fungal mesh on acetate buffer to collect secreted proteins for 10 days, 3) collecting buffer with fungal secreted proteins, 4) trypsin digestion of proteins, 5) identification of peptide sequences by mass spectroscopy, 6) blast similarity searches of the *B. cinerea* BO5.10 genome to obtain full-length protein sequences, and 7) analysis of the putative signal sequences to identify possible protein secretion mechanisms.

Inoculation of nylon membranes with B. cinerea spores

Ten micron pore size nylon membranes (Small Parts, Miami Lakes, FL) were used to immobilize the fungus. Nylon membranes cut to 100mm circular diameters were surface sterilized in 200 proof ethanol, rinsed thoroughly in sterile water and dried thoroughly in a laminar flow hood on sterilized Whatman 3MM paper prior to use. *B. cinerea* (strain BO5.10) was inoculated on sterile nylon membranes by streaking 1 x 10^4 spores gently over the surface of the membranes overlaid on NY agar (2 g/L malt extract, 2 g/L yeast extract, 15 g/L agar) containing the pulp of full red strawberries (4% strawberry pulp in NY, 96% NY media 15 g/L agar) and grown for 5 days at 22^{o}C. Controls were treated identically but contained only NY agar without any added plant material. The NY agar served as minimal media for healthy fungal growth.

Transfer of nylon grown B. cinerea to acetate buffer at pH 4.4

Membranes from both the control and NY with strawberry plates were carefully lifted from the agar plate and floated onto a plate containing 35 ml of 37.5 mM sodium acetate at pH 4.4 with 0.1% polygalacturonic acid sodium salt (Sigma Chemical, St. Louis, MO) and left to incubate in the dark for 10 days. Since media carry-over was not observed to an appreciable extent, rinsing the membranes prior to transfer was avoided to minimize fungal manipulation. One percent polygalacturonic acid was added to the buffer to induce polygalacturonases (PGs). If PGs were detected with biochemical assays, it was ensured that proteins smaller than the apparent molecular weight of 36-60 kDa for PGs[32] could be accumulated to reasonable levels in the buffer. The final volume of buffer containing *B. cinerea* protein secretions were collected in a 50 ml conical tube and analyzed further.

Proteolytic Digestion prior to MS analysis

In preparation for LC-MS/MS analysis, protein concentration was determined using Micro BCA Protein Assay Kit (Pierce, Rockford, IL). The sample was reduced by adding 1 M dithiothreitol, incubated at 55°C for two hours and carboxyamidomethylated in 10 mM iodoacetamide solution. 100 mM ammonium bicarbonate was used to adjust the pH of the solution to 8. Finally sequencing grade trypsin was added to each sample using a ratio of 1:50 enzyme to substrate and incubated overnight at 37°C with gentle shaking. The samples were then vacuum dried and reconstituted in 100 µl of 0.1% formic acid.

LC-MS/MS analysis

The peptide samples obtained from proteolytic digestion were analyzed in triplicate on an Agilent 1100 capillary LC (Palo Alto, CA) interfaced directly to a LTQ linear ion trap mass spectrometer (Thermo Electron, San Jose, CA). Mobile phase A and B were H_2O/0.1% formic

acid and ACN/0.1% formic acid, respectively. The peptide samples were loaded for 30 min onto a PicoFrit 8 cm X 50 μm column (New Objective, Woburn, MA) packed with 5 μm diameter C_{18} beads using positive N_2 pressure. The peptides were then desalted for 10 min with 0.1% formic acid using positive N_2 pressure. Peptides were eluted from the column into the mass spectrometer during a 90 min linear gradient from 5-60% B at a flow rate of 200 ημl/min. The instrument was set to acquire MS/MS spectra on the nine most abundant precursor ions from each MS scan with a repeat count of three and repeat duration of 15 sec. Dynamic exclusion was enabled for 20 sec. Raw tandem mass spectra were converted into mzXML format and then into peak list using ReAdW followed by mzMXL2Other. The peak lists were then searched using Mascot 1.9 (Matrix Science, Boston, MA). Peptide fragments from the culture were subsequently identified using blast database searches of the *B. cinerea* BO5.10 genome sequence (Broad Institute, MA).

Database searching and protein identification

Two sequence databases were constructed. The first database (normal) consisting of annotated proteins from Botrytis genes (Broad Institute, MA) combined with annotated proteins from Arabidopsis, tomato and strawberry (National Center for Biotechnology Information) to assist in identifying plant proteins that may be present in the samples. *Botrytis cinerea* (B05.10) genome sequence was provided by Broad Institute, Cambridge, Massachusetts and Syngenta AG, http://www.broad.mit.edu/annotation/genome/botrytis_cinerea/ Home.html. A decoy database (random) was then constructed by reversing the sequences in the normal database. Searches were performed against the normal and random databases using the following parameters: fully tryptic enzymatic cleavage with 3 possible missed cleavage, peptide tolerance of 1000 parts-per-million, fragment ion tolerance of 0.8 Da, and variable modifications due to carboxyamidomethylation of

cysteine residues (+ 57 Da) and deamidation of asparagine residues (+1 Da). Following the database searches, the identified peptides were grouped into proteins and statistically validated using PROVALT[33]. This algorithm identified Mascot values of 48 for a single peptide, 41 for two peptides and 37 for three peptides as the cut-off points for a protein false discovery rate <1%. Only proteins meeting this criterion were considered to be a statistically significant identification. When performing blast searches of the *B. cinerea* BO5.10 genome, each peptide group that identified a corresponding gene sequence was used to find the predicted full length protein sequence. Protein annotations were further verified by searching the Conserved Domain Database – CDD[34] at the National Center for Biotechnology Information (NCBI) website, http://www.ncbi.nlm.nih.gov/.

Computer algorithm – prediction of classical versus non-classical secretion mechanism

The mechanism of secretion was predicted using software packages, SignalP[35,36], TargetP[37] and SecretomeP[38,39.] Analyzing the predicted amino acid sequence along with the SignalP, TargetP and SecretomeP programs identified whether the entire predicted protein sequence encoded a classical or non-classical signal motif or whether neither category was represented. SignalP and SecretomeP numerical values for each predicted protein sequence analyzed is shown in Table 3.3.

Additional Assays

Enzyme activity plate assays

Further functional analysis of the activity of secreted proteins incorporated the use of the pectin plate assay and carboxymethylcellulose plate assays as a visual confirmation of PGs and cellulase enzymes in the protein extract. Forty µg of secreted protein sample was pipetted directly onto a pectin agar plate (0.1% pectin, 0.1% maltose, 0.1% KNO_3, 0.5% agarose in

0.05M $NaHPO_4$, 0.01M citric acid at pH 6.3). The pectin plates were then incubated for four hours at 30ºC and stained with 0.02% ruthenium red for two hours at 4ºC[40.] After destaining the plates with deionized water for 15 min, plates were observed for areas of clearing resultant of PG enzymatic activity (Figure 3.2). The positive control for the pectin plate assay was 0.09 U (or 0.039 mg) pectinase enzyme from *Aspergillus niger* (Sigma Chemical, St. Louis, MO) suspended in 37.5 mM of acetate buffer, pH 4.4. Carboxymethylcellulose (CMC) plates were prepared by incorporating 0.1% CMC in buffer A (0.05 M $NaHPO_4$, 0.01 M citric acid; pH 6.3) by heating with continuous stirring. Each plate was sectioned into 5 quadrants with the center quadrant consisting of the positive control, *Trichoderma reesei* Cellulase (Calbiochem, La Jolla, CA), spotted at a concentration of 40 μg in 37.5 mM acetate buffer, pH 4.4. The plates were incubated at 30°C for 4 hours to allow time for the enzymes to hydrolyze the substrate. After incubation the plates were stained with 1% Congo Red in water at room temperature. The stain was removed after 15 minutes then destained in 1 M NaCl in buffer A.

Identification of Ceratoplatanin-like Protein in B. cinerea Secretions

Peptide Sequence

An intense band for a fractioned protein was excised from the 16.5% SDS denaturing tricine gel[41] and "in-gel" trypsin digested according to the method of Shevchenko et al. (1996)[42.] The peptides were sequenced by LC/MS-MS and found to correspond with the predicted amino acid sequence of BC1G_02163.1 identified from the shot gun proteomic analysis as a ceratoplatanin-like protein.

Cloning SNOD1 full-length cDNA

Total RNA was isolated from *B. cinerea* BO5.10 mycelium after being exposed to 4% full red strawberry pulp in NY media for 5-8 days at 30°C using Trizol Reagent (Invitrogen, San Diego,

California). One microgram of RNA was reversed transcribed to cDNA according to the SMART cDNA Amplification kit (Clontech Laboratories, Mountain view, CA) instructions. The full-length cDNA encoding a *B. cinerea* ceratoplatanin-like protein, designated SNOD1, was directionally cloned into pBluescript SKII^{+} (Stratagene, LaJolla, CA) and sequence verified.

Antibody and Western Blot Analysis

A polyclonal antibody was made in New Zealand male white rabbits to the antigenic peptide (GRIDASYTQVDKSACGL) of a *B. cinerea* ceratoplatanin-like protein by OpenBiosystems (Huntsville, AL). A SuperSignal WestPico kit (Pierce) was used with the antibody at a dilution of 1/100 to bind to SNOD1 in both the purified and crude samples.

Secreted Protein Purification

An AKTA Explorer FPLC (GE Healthcare, Piscataway, NJ) in tandem with a column (4 cm diam., 120cm length) packed with Sephadex G-50 resin, MW range 1,500-30,000 (GE Heathcare) was used for the purification of *B. cinerea* SNOD1 secreted protein. SNOD1 was eluted from the column in 20 mM Tris-HCl, pH 8.0 buffer. Peak 1 eluted at 75 mins and peak 2 with the SNOD1 protein eluted at 125 mins. SNOD1 protein elution was monitored at both 280nm and 215nm with only a few aromatic amino acids, 6 tyrosines and 1 tryptophan, present in the protein. An Amicon stirred cell (Fisher Scientific) with a membrane of 2 kDa MWCO was used to concentrated the protein.

Southern Blot Analysis of B. cinerea SNOD1

Genomic DNA was isolated from *B. cinerea* BO5.10 grown for 5-8 days on NY media using the procedure of Moller et al. (1992)[43.] Thirty micrograms of genomic DNA was digested completely with either BamHI or HindIII then fractionated on a 1% agarose gel and blotted. Membranes were probed under high stringency with a full length SNOD1 cDNA random primed

with a Ready-To-Go labeling kit (GE Healthcare) using αP^{32}dCTP. Autoradiographic images were produced by a Gel Doc XR PC phosphoimager (BioRad, Hercules, CA)

RESULTS AND DISCUSSION

To study pathogenic interactions of the phytopathogenic fungus, *B. cinerea*, we identified the proteins secreted by *B. cinerea* BO5.10 grown in the presence of pulped full red strawberry receptacles using a high throughput LC-MS/MS methodology. Immobilizing the fungus on a nylon mesh while exposing it to an activation media induces strawberry specific proteins which can be easily collected when transferred to a more defined but non-metabolisable buffer. The secretion profiles include proteins that are needed by the fungus for catabolic activities to cleave host plant polymers for carbon assimilation, as well as enzymes needed for fungal cell wall reconstruction for new synthesis and growth. In addition, we have found secreted enzymes that the fungus may selectively induce to fight off a variety of plant defense responses.

Identification of secreted proteins by LC-MS/MS: Secreted protein - nylon control

Mass spectrometric analysis of the *B. cinerea* secreted proteins from uninduced nylon samples identified very few proteins. The *B. cinerea* gene numbers for each protein are listed in Table 3.2. The secreted proteins are predicted from pI values to be acidic and range in size from 35 – 67 kDa. 6-phosphogluconolactonase is predicted to be secreted by a nonclassical mechanism, while versicolorin B synthase, glucoamylase and glucosidase are enzymes predicted to be secreted by a classical mechanism of secretion.

Analysis of Secreted proteins from B. cinerea induced with red strawberry

B. cinerea grown on NY with extract from full red strawberries induced a greater number of secreted proteins that were classified into 7 distinct and 9 functional categories including carbohydrate metabolism, proteolysis/peptidolysis, stress response, electron/proton transport,

fatty acid and phospholipid metabolism, miscellaneous and hypothetical proteins, shown in Table 3.3. Secretion signals have been identified as classical, “C”, or nonclassical, “NC” using the prediction software; SignalP, TargetP and SecretomeP. Actual scores for SignalP and SecretomeP are also shown in Table 3.3. Domain motifs were identified with the Conserved Domain Database Search[34] at the National Center for Biotechnology Information (NCBI) and the Motif Search at the Bioinformatics Center Institute for Chemical Research Kyoto University and shown in Table 3.4. All domains motifs were consistent with the corresponding annotation for the protein where identified.

Fifty-one proteins associated with peptides lacked any sort of secretion signal and probably come from the lysis of cells during the experiments. These 51 proteins were not further analyzed in this work. The SignalP/TargetP and SecretomeP software programs were used to identify proteins strictly possessing secretory signals.

Carbohydrate metabolism

Twenty seven proteins were identified as belonging to the carbohydrate catabolism category. In this category most secreted proteins are in fact cell wall degrading and modifying enzymes. We were able to identify at least 18 enzymes that were secreted by the fungus to breakdown glucose polymers of the plant cell wall. These were classified as either glucanases or glycosyl hydrolases with some containing cellulose binding domains (CBD). Of the CBD domains, BC1G_14944.1 was found to have a domain consistent with the fungal type of cellulose binding proteins. Fungal cellulases are a major class of plant cell wall degrading enzymes that specifically cleave plant cellulose as an abundant cell wall polymer. Contrary to a wide variety of plants however, cellulose was determined to be a minor cell wall component of the cortical cell wall polysaccharides (CWP) of strawberry fruit[44].

There was one putative *B. cinerea* arabinofuranosidase B (AbfB) that was identified by BLAST analysis showing homology to a S. *sclerotiorum* enzyme with similar classification[45.] The presence of an AbfB as a secreted protein with a putative classical secretion peptide, suggests extracellular processing of arabinofuranoses. AbfB hydrolyses 1,5-α, 1,3-α and 1,2-α linkages in both oligosaccharides and polysaccharides, which contain terminal non-reducing l-arabinofuranoses in side chains[46.] A glycolipid anchored surface protein (GAS1) was identified for two transferases, BC1G_10455.1 and BC1G_14030.1, both matching distinct *B. cinerea* BO5.10 sequences. The 1,3-β-glucanosyltransferases are important in the biosynthesis of disaccharides, oligosaccharides and polysaccharides. They catalyze the transfer of sugar moieties from activated donor molecules to specific acceptor molecules to form glycosidic bonds. The transferases may act in both a catabolic and anabolic capacity. Cell wall components are being degraded in the plant, concurrent with increased manufacturing in the fungus as the pathogen obtains the necessary carbon building blocks for new cell wall synthesis. Cutin is also a major structural component in the cell wall of plants. The identification of both a fungal cutinase and chitobiase supports the concept that the fungus has the ability to penetrate the plant cell wall by clipping at the outer barriers.

Pectin polymers are highly abundant in fruit cell walls, consistent with the identification of 5 pectin degrading and processing enzymes in the protein secretions. In the strawberry receptacle ripening changes are accompanied by depolymerization of all pectin classes during ripening[47.] In these experiments, *B. cinerea* was also found to secrete pectin methyl esterases, endopolygalacturonases, exopolygalacturonases, and two different pectate lyases to assist in breaking down the pectin. Also of functional interest, was the presence of pectate lyase C, BC1G_00912.1; some of the family members are considered allergens. The identification of a

single polygalacturonase Bcpg1, in the protein secretions continues to reinforce the role of this enzyme in the specific cleavage of abundant pectin polymers in strawberry receptacles. Bcpg1 was implicated by ten Have et al. (1998)[48] as being an important enzyme in the degradation of complex pectin structures in the cell wall of tomato leaves and fruit as well as on apple fruit. However, from their infection studies with a *Bcpg1* deletion mutant of *B. cinerea* B05.10, Bcpg1 was less involved in the initial penetration but found to be critical for the secondary spread of infection beyond the inoculation point.

The infection of *B. cinerea* on strawberry induces carbohydrate cleaving enzymes that are preferential to this attack. Out of the 6 characterized polygalacturonases by Wubben et al.[26], only Bcpg1 was identified in the protein secretions. With 18 glucanases or glycosyl hydrolases it is proposed that a fungal attack on strawberries may use a variety of enzymes for efficient glucose catabolism.

Proteolytic/peptidolytic

For the proteolysis/peptidolysis proteins, a variety of proteases were identified. Dipeptidyl peptidases were detected along with a variety of other peptidases including serine and aspartyl peptidases. Members of the dipeptidyl peptidase family cleave dipeptides from the N-termini of peptides or proteins. A dipeptidyl-peptidase IV was previously purified from the culture media of *Aspergillus fumigatus*, a human-pathogenic fungus[49.] The protein from *Aspergillus fumigatus* was identified as an antigenic glycoprotein with a cleavable signal peptide. Aspartyl proteases have been previously identified by multiple investigators[21,22,50,51] as secreted proteins from *B. cinerea* and shown to be important for degrading host cell wall proteins or as a defense against antifungal host secreted proteins.

Ten Have et al.[50] identified 5 distinct AP enzymes as being secreted from *B. cinerea* in axenic cultures. There were two AP enzymes identified by LC-MS/MS analysis from this work that are identical to the translated *B. cinerea* gene sequences, BC1G_06849.1 and BC1G_08393.1 in the BO5.10 genome sequence released by the Broad Institute (http://www.broad.mit.edu). Both are predicted by SignalP /TargetP analysis to have classical secretion peptides. Out of the two putatively identified *B. cinerea* aspartyl proteases only BC1G_06849.1 was shown to be identical to the NCBI submitted gene sequence, AY361913. Although BC1G_08393.1 is similar to Bc_AP1, Bc_AP2, Bc_AP3 and Bc_AP4, it is apparently unique from each of the previously submitted annotations for these APs while for Bc_AP5 there was no significant similarity found. Bc_AP was determined by ten Have et al.[50] to be contained within clade 3 of a CLUSTALX phylogenetic alignment of AP sequences. Clade 3 is proposed to contain all vacuole or 'cellular' localized APs while Clade 1 contains APs from saprophytic fungi where most are shown experimentally to be secreted. The secretion of protein cleaving factors is an indication of further processing of plant cellular components which is necessary for the fungus to obtain additional food sources during its attack on the plant. The secreted proteases and peptidolysis proteins may function to cleave plant proteins but may also function to recycle fungal proteins that are no longer required in the infection mechanism.

Stress Response Proteins

Other secretion categories included stress response proteins, such as a Cu/Zn superoxide dismutase, tyrosinases, catalase, glyoxal oxidase and others implicated in defending the fungus against the myriad of plant attack mechanisms. Most involve the detoxification of plant compounds that in turn damage the fungal cell wall as the plant's attempt to thwart the fungal attack factors.

Hydrogen peroxide has been shown to play an important role in infection of hosts by *B. cinerea*[52.] Hydrogen peroxide can be converted into unstable hydroxyl radical ions. Hydroxy ions are known to destroy cell integrity by starting a chain reaction of lipid peroxidation. Fungal extracellular sugar oxidases and superoxide dismutases (SODs) are candidate enzymes potentially responsible for generating the hydrogen peroxides. A total of three classes of SODs have been reported, based on their active site metals: Cu/Zn SOD, Fe SOD, Mn SOD[53] and a putative Ni SOD occurring in prokaryotes[54.] Active oxygen species have been suggested to be involved in the killing of host tissues in the initial stages of infection[55.] Catalase is associated with the degradation of hydrogen peroxide. Glyoxal is converted into oxalic acid and hydrogen peroxide by glyoxal oxidase. In the white rot fungus, *Phanerochaete chrysporiem,* glyoxal oxidase produces hydrogen peroxide that is involved in lignin degradation[56.] The superoxide dismutase corresponding to BC1G_00558.1 is a Cu/Zn containing metalloenzyme. Extracellular secreted and glycosylated Cu/Zn metalloenzymes have also been identified from fungi such as *Humicola lutea*[57.]

Electron/proton transport – Fatty Acid/phospholipid metabolism

Oxidoreductases are enzymes that catalyze the oxidation of one compound with the reduction of another. BC1G_14679.1, is the only protein identified in the strawberry induced secretions that is able to transport charges. While two proteins have been categorized as being involved in lipid metabolism. Only BC1G_ 07637.1, is clearly identified by computer analysis as a classically secreted enzyme. The roles of oxidoreductases and esterase lipase, if any, in the pathogenic response is yet to be determined.

Miscellaneous proteins

A majority of proteins in the miscellaneous category are generally accepted as being intercellular proteins. This list includes proteins important for sugar catabolism, protein synthesis, amino acid synthesis and related functions. Each protein in the miscellaneous category has either a classical or nonclassical secretory signal. This may be indicative of the inability of the software to accurately predict extracellular protein localization for these proteins.

Peptides from proteins presumed to be involved in some aspect of protein folding or stabilization of protein tertiary structure were detected by LC-MS/MS analysis and also categorized as miscellaneous. These enzymes have been implicated in protein folding processes, which depend on catalytic/chaperone-like activities. Included in this list are a putative molecular chaperone, cyclophilin, FK506 binding protein and a disulphide bond forming protein. The disulphide bond forming protein is a putative oxidase that catalyzes the formation of disulfide bonds of newly synthesized polypeptides and may also exhibit reductase activity since it is a known endoplasmic reticulum isomerase, in addition to, being a chaperone. With these protein activities, any non-native disulfide bonds will be corrected; while, preventing protein aggregation and facilitating the folding of newly synthesized proteins. The cyclophilin is an enzyme that accelerates protein folding by catalyzing the cis-trans isomerization of the peptide bonds preceding proline residues.

Hypothetical

The largest category of 30 proteins is listed as unknown/hypothetical. This includes 23 proteins which have no similarity matches found to NCBI proteins (above the threshold of e^{-40}) and 7 remaining proteins which matched hypothetical proteins from other fungal organisms also with an unknown function. Small (<30kDa) hypothetical proteins make up a subset of proteins

which account for over half of the unknown/hypothetical list. Many small proteins are thought to be involved in novel host plant attack mechanisms[58.] There are many gene products that remain unidentified in the *B. cinerea* genome that are well-conserved, but remain unknown, across other sequenced fungal genomes. The assignment of protein function for the hypothetical gene products of one organism may offer a tremendous opportunity to begin to assign function in many other sequenced fungal and possible non-fungal genomes.

At the time of this article, the *B. cinerea* genome has been completed to a 5.4X sequence coverage and the *S. sclerotiorum* genome has an 8X sequence coverage in the assembly. Out of 94 total proteins identified, as being present in the *B. cinerea* genome with a secretion motif, only two hypothetical proteins with gene numbers, BC1G_05476.1 and BC1G_05038.1, have no corresponding *Sclerotina sclerotiorum* orthologs. As hypothetical proteins, elucidating the function of these proteins may give additional clues as to the unique biology of *B. cinerea* as a pathogenic plant fungus. An additional uncharacterized but well-conserved domain, COG1359, was also found to be encoded in the hypothetical protein sequence of BC1G_07825.1. Understanding the conserved functional role of this domain may divulge a new class of proteins important in fungal pathogenesis.

For functional confirmation of the shot gun proteomics analysis of *B. cinerea* secretions, a ceratoplatain-like protein (SNOD1) categorized as hypothetical was further characterized. The protein sequence was determined by LC-MS/MS analysis from the pool of secreted proteins and again from a denaturing fractionation followed with an in gel trypsin digestion and sequence of the protein. Peptide sequence searches against the *B. cinerea* BO5.10 genome sequence at the BROAD identified a gene for BC1G_02163. The cDNA for BC1G_02163 was sequence verified and was found to exist as a single copy in the *B. cinerea* BO5.10 genome upon fractionation of

both the BamHI and HindIII digests (Figure 3.3). The SNOD1 protein was purified from *B. cinerea* extracellular secretions and shown to be recognized by an antibody to the terminal antigenic peptide of this protein. Chague et al.[25] identified a ceratoplatanin-like sequence, *bcspl1*, that was shown to be induced with ethylene after 24 but not after 48 hour treatment in a Gα null (ethylene insensitive) mutant strain. Ceratoplatanin-like proteins have been identified in fungal secretions from *Hypocrea atroviridis*[59,] *Ceratocystis fimbriata* f. sp. platani[60] and *Coccidioides immitis*[61.]

Plate assays to detect enzyme activities

The plate assays provide a rapid method to confirm the secretion of active marker enzymes, such as pectin cleaving and cellulase activities. These enzymes were qualitatively measured with either the pectin or CMC plate assays, respectively (Figure 3.2). Enzyme activity was observed upon clearing of the media when stained with Rubidium Red or Congo Red for either the pectin plate or CMC plate assay, Figure 3.2 A and B, respectively. The pectin-containing plates can detect the presence of pectin lyase, polygalacturonase, and pectin methyl esterase in the sample[40.] Pectin lyase depletes the plate of stain-binding polymer that results in clearing. Pectin methyl esterase activity enhances binding of the stain by increasing the number of free acid groups on the polymer. After staining protein secretions for the pectin plate assay, the presence of pectin methyl esterase activity appeared as a darker red halo surrounding colonies[62.] The intensity of each cleared circle was visibly greater than or comparable in intensity to the control spotted in the center sector for both plate assays. There was an indication of carboxymethylcellulose and pectin cleaving proteins accumulating as active proteins in the *B. cinerea* collected secretions.

CONCLUSION

This is the first reported application of LC-MS/MS to perform a direct analysis of protein secretions from the organism, *B. cinerea* without prior gel fractionation. We have identified a total of 97 new *B. cinerea* predicted secreted proteins that have not been reported elsewhere at the time of this publication. This high throughput proteomic analysis of extracellular secretions has been shown to be of tremendous value for identifying fungal proteins at lower concentrations and less than 30kDa in molecular weight; not easily detected with conventional gel-based methods. The proteins were induced upon simulated infection of full red strawberry receptacles. Since we are inducing fungal secretions with broken plant cellular components, it is most likely that all observed proteins were being produced at the same time. With our current experimental design, we would be unable to simulate the gradation of protein induction from the fungus' sequential penetration of the various plant tissue layers. Our method most likely allows a global view of proteins induced with immediate interaction of the fungus on the exposed cell and cellular components.

Identifying the full arsenal of tools that are used by fungal pathogens, to mount their cellular attack on host cells, will be essential for a more thorough study of fungal attack mechanisms in order to better understand the role of extracellular proteins in fungal biology as well as for the elucidation of novel targets for fungal control methods.

REFERENCES

1. Zorn H, Peters T, Nimtz M, Berger RG. The secretome of *Pleurotus sapidus*. *Proteomics* 2005; 5: 4832-4838.

2. Medina ML, Haynes PA, Breci L, Francisco WA. Analysis of secreted proteins from *Aspergillus flavus*. *Proteomics* 2005; 5: 3153-3161.

3. Fernandez-Acero FJ, Jorge I, Calvo E, Vallejo I, Carbu M, Camafeita E, Lopez AL, Cantoral JM, Jorrin J. Two dimensional electrophoresis protein profile of the phytopathogenic fungus *Botrytis cinerea*. *Proteomics* 2006; 6: S88 – S96.

4. Wolters, DA, Washburn MP, Yates III JR. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nature Biotechnol.* 2001; 19:242–247.

5. John III RY. Mass spectrometry and the age of the proteome. *J. Mass Spectrometry* 1998; 33: 1-19.

6. Skipp P, Robinson J, O'Connor CD, Clarke IN. Shotgun proteomic analysis of *Chlamydia trachomatis*. *Proteomics* 2005; 5: 1558-1573.

7. Rothman JE, Wieland FT. Protein sorting by transport vesicles. *Science* 1996; 272: 227-234.

8. Palade G. Intracellular aspects of the process of protein secretion. *Science* 1975; 189: 347-358.

9. Schekman R, Orci L. Coat proteins and vesicle budding. *Science* 1996; 271: 1526-1533.

10. Misumi Y, Miki A, Takatsuki A, Tamura G, Ikehara Y. *J Biol Chem.* 1986; 261: 11398-11403.

11. Lippincott-Schwartz J, Yuan LC, Bonifacio JS, Klausner, RD. Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: Evidence for membrane cycling from Golgi to ER. *Cell* 1989; 56: 801-813.

12. Perillo NL, Pace KE, Seilhamer JJ, Baum LG. Apoptosis of T cells mediated by galectin-1. *Nature* 1995; 378: 736-739.

13. Hughes RC. Secretion of the galectin family of mammalian carbohydrate-binding proteins. *Biochim. Biophys. Acta* 1999; 1473: 172-185.

14. Nugent MA, Iozzo RV. Fibroblast growth factor-2. *Int. J. Biochem. Cell Biol.* 2000; 32: 115-120.

15. Braddock M, Quinn A. Targeting IL-1 in inflammatory disease: new opportunities for therapeutic intervention. *Nat. Rev. Drug Discov.* 2004; 3: 330-339.

16. McKean PG, Denny PW, Knuepfer E, Keen JK, Smith DF. Phenotypic changes associated with deletion and overexpression of a stage-regulated gene family in Leishmania. *Cell Microbiol.* 2001; 3: 411-523.

17. Fu J, Prade R, Mort A. Expression and action pattern of *Botryotinia fuckeliana* (*Botrytis cinerea*) rhamnogalacturonan hydrolase in *Pichia pastoris*. *Carbohy. Res.* 2001; 330: 73-81.

18. Brutus A, Reca IB, Herga S, Mattei B, Puigserver A, Chaix JC, Juge N, Bellincampi D, Giardina T. A family 11 xylanase from the pathogen *Botrytis cinerea* is inhibited by plant endoxylanase inhibitors XIP-I and TAXI-I. *Biochem. and Biophy. Res. Comm.* 2005; 337:160-166.

19. te Biesebeke R, Boussier A, van Biezen N, van den Hondel CAMJJ, Punt PJ. Identification of secreted proteins of *Aspergillus oryzae* associated with growth on solid cereal substrates. *J. Biotechnol.* 2006; 121: 482-485.

20. Marx F. Small, basic antifungal proteins secreted from filamentous ascomycetes: a comparative study regarding expression, structure, function and potential application. *Appl. Microbiol. Biotechnol.* 2004; 65: 133-142.

21. Movahedi S, Heale JB. The roles of aspartic proteinase and endo-pectin lyase enzymes in the primary stages of infection and pathogenesis of various host tissues by different isolates of *Botrytis cinerea* Pers ex. Pers. *Physiol. Molec. Plant Pathol.* 1990; 36: 303-324.

22. Movahedi S, Heale JB, Purification and characterization of an aspartic proteinase secreted by *Botrytis cinerea* pers ex. pers in culture and in infected carrots. *Physiol. Molec. Plant Pathol.* 1990; 36: 289-302.

23. Staples RC, Mayer AM, Putative virulence factors of *Botrytis cinerea* acting as a wound pathogen. *FEMS Microbiol. Letters.* 1995; 134: 1-7.

24. Kars I, van Kan JAL.Extracellular enzymes and metabolites involved in pathogenesis of *Botrytis*. In: Elad Y, Williamson B, Tudzynski P, Nelen N, Eds. *Botrytis: Biology, Pathology and Control*, Kluwer Academic Publishers, Dordrech, 2004; pp. 99-118.

25. Chague V, Danit LV, Siewers V, Gronover CS, Tudzynski P, Tudzynski B, Sharon A. Ethylene sensing and gene activation in *Botrytis cinerea*: A missing link in ethylene regulation of fungus-plant interactions? *Molec. Plant-Microbe Interact.* 2006; 19: 33-42.

26. Wubben JP, Mulder W, ten Have AT, van Kan JAL, Visser J, Cloning and partial characterization of endopolygalacturonase genes from *Botrytis cinerea*. *Appl. Environ. Microbiol.* 1999; 65: 1596-1602.

27. Doss RP, Potter SW, Soeldner AH, Christian JK, Fukunaga LEA. Adhesion of germlings of *Botrytis cinerea*. *Appl. Environ. Microbiol.* 1995; 61: 260-265.

28. Johnston DJ, Williamson B. Purification and characterization of four polygalacturonases from *Botrytis cinerea*. *Mycol. Res*. 1992; *96*: 343-349.

29. Doss RP. Composition and enzymatic activity of the extracellular matrix secreted by germlings of *Botrytis cinerea*. *Appl. Environ. Microbiol.* 1999; 65: 404-408.

30. Dubourdieu D, Ribereau-Gayon P. Structure of the extracellular β-D-glucan from *Botrytis cinerea*. *Carbohy. Res.* 1981; 93: 294-299.

31. Leal JA, Ruperez P, Gomez-Miranda B. Extracellular glucan production by *Botrytis cinerea*. *Trans, British Mycol. Soc.* 1976; 72: 172-176.

32. Kars I, Krooshof GH, Wagemakers L, Joosten R, Benen JAE, van Kan JAL. Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. *Plant J.* 2005; 43: 213-225.

33. Weatherly DB, Atwood JA, Minning TA, Cavola C, Tarleton RL, Orlando R. 2005. A heuristic method for assigning a false discovery rate for protein identifications from mascot database search results. *Molec. Cell. Proteomics* 2005; 4: 762-772.

34. Marchler-Bauer A, Anderson JB, Cherukuri PF, DeWeese-Scott C, Geer LY, Gwadz M, He S, Hurwitz DI, Jackson JD, Ke Z, Lanczycki C, Liebert CA, Liu C, Lu F, Marchler GH, Mullokandov M, Shoemaker BA, Simonyan V, Song JS, Thiessen PA, Yamashita

RA, Yin JJ, Zhang D, Bryant SH. CDD: a conserved domain database for interactive domain family analysis. *Nucleic Acids Res.* 2005; 33, Database Issue:D192-6.

35. Nielsen H, Engelbrecht J, Brunak S, von Heijne G, Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Engineering* 1997; 10: 1-6

36. Bendtsen JD, Jensen LJ, Blo N, von Heijne G, Brunak S, Feature based prediction of non-classical and leaderless protein secretion. *Protein Eng. Des. Sel.* 2004; 17: 349-356.

37. Emanuelsson O, Nielsen H, Brunak S, von Heijne G, Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J. Molec. Biol.* 2000; 300: 1005-1016.

38. Bendtsen JD, Kiemer L, Fausbøll A, Brunak S. Non-classical protein secretion in bacteria. *BMC Microbiol.* 2005; 5: 58.

39. Bendtsen JD, Nielsen H, von Heijne G, Brunak S. Improved prediction of signal peptides: SignalP 3.0. *J. of Molec. Biol.* 2004; 340: 783-795.

40. Hagerman AE, Blau DM, McClure AL. Plate assay for determining the time of production of protease, cellulase, and pectinases by germinating fungal spores. *Anal. Biochem.* 1985; 151: 334-342.

41. Schagger H, von Jafow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100kDa. *Anal Biochem* 1987; 166: 368-379.

42. Wilm M, Shevchenko A, Houthaeve T, Breit S, Schweigerer L, Fotsis T, Mann M. Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. 1996; 379: 466-469.

43. Möller EM, Bahnweg G, Sandermann H, Geiger HH. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. 1992; *Nucl. Acids Res.* 20: 6115-6116.

44. Koh T H, Melton L D, Ripening-related changes in cell wall polysaccharides of strawberry cortical and pith tissues. *Postharvest Biol.Technol.* 2002; 26: 23-33.

45. Yajama W, Kav NVK. The proteome of the phytopathogenic fungus *Sclerotinia sclerotiorum*. *Proteomics* 2006; 6: 5995-6007.

46. Gielkens M, Gonzalez-Candelas L, Sanchez-torres P, Van de vondervoort P. The abfB gene encoding the major alpha-L-arabinofuranosidase of *Aspergillus nidulans*: nucleotide sequence, regulation and construction of a disrupted strain. *Microbiology* 1999; 145: 735-741.

47. Huber DJ. Strawberry fruit softening: The potential roles for polyuronides and hemicelluloses. *J. Food Sci.* 1984; 49: 1310-1315.

48. ten Have A, Mulder W, Visser J, van Kan JAL. The endopolygalacturonase gene *Bcpg1* is required for full virulence of *Botrytis cinerea*. *Molec. Plant Microbe Interact.* 1998; 11: 1009-1016.

49. Beauvais A, Monod M, Wyniger J, Debeaupuis JP, Grouzmann E, Brakch N, Svab J, Hovanessian AG, Latge JP. Dipeptidyl-peptidase IV secreted by *Aspergillus fumigatus*, a fungus pathogenic to humans. Infection and Immunity 1997; 65: 3042-3047.

50. ten Have A, Dekkers E, Kay J, Phylip LH, van Kan, JAL. An aspartic proteinase gene family in the filamentous fungus *Botrytis cinerea* contains members with novel features. *Microbiology* 2004; 150: 2475-2489.

51. Manteau S, Abouna S, Lambert B, Legendre L. Differential regulation by ambient pH of putative virulence factor secretion by the phytopathogenic fungus *Botrytis cinerea*. *FEMS Microbiol. Ecol.* 2003; 43: 359-366.

52. Edlich W, Lorenz G, Lyr H, Nega E, Pommer EH, New aspects on the infection mechanism of *Botrytis cinerea* Pers. *Eur. J. Plant Pathol.* 1989; 95: 53-62.

53. Fridovich I. Superoxide radical and superoxide dismutases. *Ann. Re.v Biochem.* 1995; 64: 97-112.

54. Mates JM, Sanchex-Jimenez F. Antioxidant enzymes and their implications in pathophysiologic processes. *Frontiers in Bioscience* 1999; 4: D339-D345.

55. Tiedemann AV. Evidence for a primary role of active oxygen species in induction of host cell death during infection of bean leaves with *Botrytis cinerea*. *Physiol. Molec. Plant Pathol.* 1997; 50: 155-166.

56. Lyon GD, Goodman BA, Williamson B. *Botrytis cinerea* perturbs redox processes as an attack strategy in plants. In: Elad Y, Williamson B, Tudzynski P, Delen N. (Eds.), *Botrytis: Biology, pathology and control*, Kluwer academic publishers, Dordrecht 2004, pp. 119-136.

57. Angelova M, Dolashka-Angelova P, Ivanova E, Serkedjieva J, Slokoska L, Pashova S, Toshkova R, Vassilev S, Simeonov I, Hartmann H-J, Stoeva S, Weser U, Voelter W. A novel glycosylated Cu/Zn-containing superoxide dismutase: production and potential therapeutic effect. *Microbiology* 2001;147: 1641-1650.

58. Rep M. Small proteins of plant-pathogenic fungi secreted during host colonization. *FEMS Microbiol. Letters* 2005; 253: 19-27.

59. Seidl V, Marchetti M, Schandl R, Allmaier G, Kubicek C. Epl1, the major secreted protein of *Hypocrea atroviridis* on glucose, is a member of a strongly conserved protein family comprising plant defense response elicitors. 2006; 273: 4346-4359.

60. Pazzagli L, Cappugi G, Manao G, Camici G, Santini A, Scala A. Purification, characterization, and amino acid sequence of ceratoplatanin, a new phytotoxic protein from ceratocystis fimbriata f. sp. platani. *J Biol Chem* 1999; 274: 24959-24964.

61. Pan S, Cole GT. Molecular and biochemical characterization of a *Coccidioides immitis*-specific antigen. 1995; 63: 3994-4002.

62. Kersten JT, Guan Y, Allen C, *Ralstonia solanacearum* pectin methylesterase is required for growth on methylated pectin by not for bacterial wilt virulence. *Appl. Environ. Microbiol.* 1998; 64: 4918-4923.

63. Schwartz R, Ting CS, King J. Whole proteome pI values correlate with subcellular localizations of proteins or organisms within the three domains of life. *Genome Res.* 2001; 11: 703-709.

64. Knight CG, Kassen R, Hebestreit H, Rainey PB. Global analysis of predicted proteomes: Functional adaptation of physical properties. *Proc. Nat. Acad.Sci.* USA 2004; 101: 8390-8395.

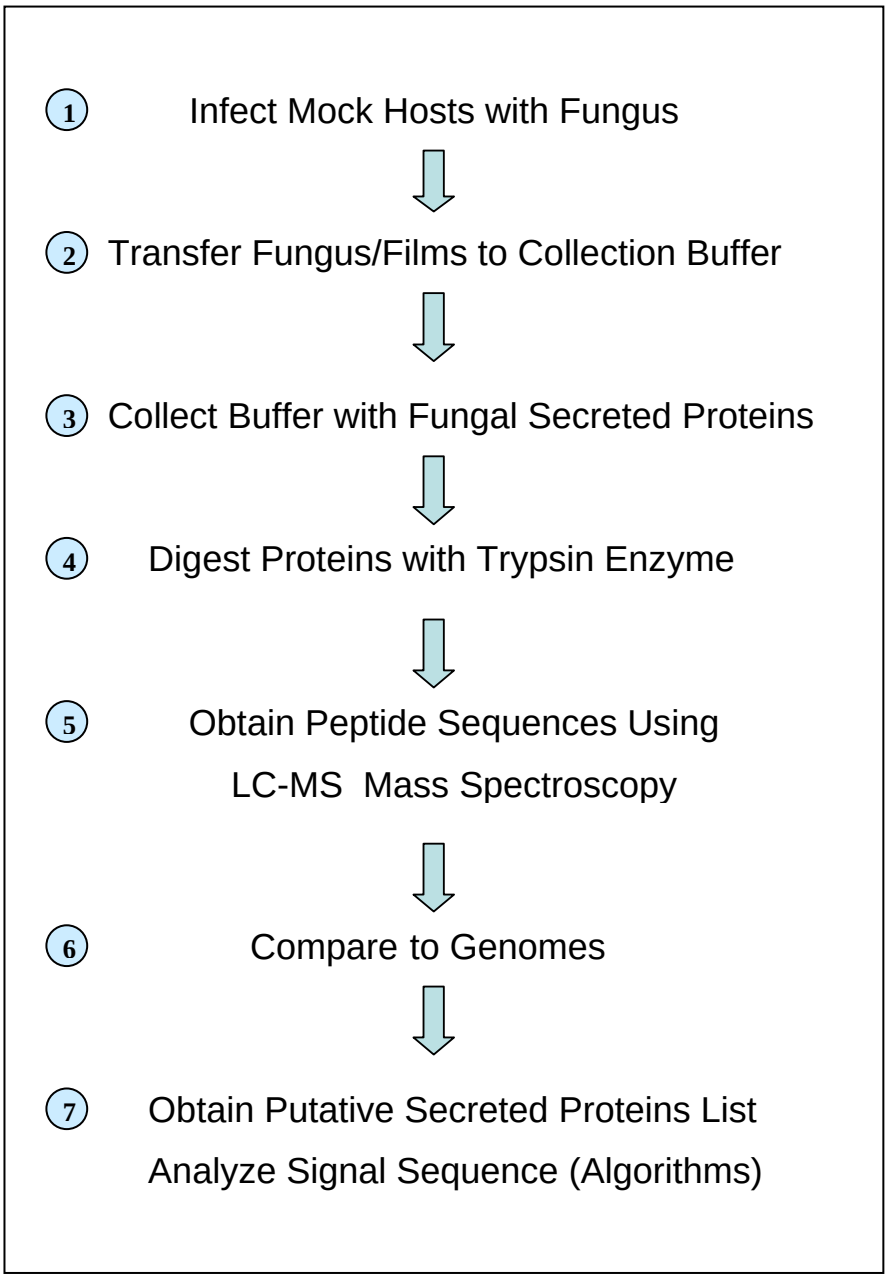

Figure 3.1: Procedural steps for the throughput proteomics analysis of fungal secreted proteins

Table 3.1: Nylon membrane, NY agar Control, Secreted proteins in 37.5 mM acetate buffer, pH 4.4 collected from *B. cinerea* grown on nylon membranes with prior exposure to NY agar with no added plant material. *B. cinerea* gene name with identifying peptide is listed along with associated *S. sclerotiorum* ortholog.

Protein	Gene Name	Peptide	Predicted Amino Acids	kDa*	pI*	*S. sclerotiorum* Ortholog
Versicolorin B Synthase	BC1G_02021.1	ALLGNSFGVPGR	628	66.8	4.61	SS1G_00730.1
Glucoamylase Precursor	BC1G_13215.1	LQTVDNPSGDLATGAGLAEPK	369	38.75	4.59	SS1G_08135.1
Glucosidase	BC1G_11898.1	GDTTASTLAQQIYDVR	294	34.92	4.5	SS1G_12930.1
6-Phosphogluconolactonase	BC1G_08719.1	IPEGAAAAECLLTPDGK +Carbamidomethyl	411	43.53	5.1	SS1G_08383.1

Table 3.2: Categories of Secreted Proteins Identified upon Exposure of *B. cinerea* to 4% pulped Full Red Strawberry in NY media. *Botrytis cinerea* BO5.10 and the corresponding *Sclerotina sclerotiorum* gene numbers can be found at http://www.broad.mit.edu/ along with the size of each protein in amino acids (A.A.). Number of peptides and percentage coverage were obtained from the LC-MS/MS data and used in the identification of each protein. kDa* and pI* values were determined from annotated genome sequence using ExPASY, http://us.expasy.org/tools/. Signal peptides were predicted by SignalP and SecretomeP software programs and the secretion mechanisms were determined to be Classical (C) or nonclassical (NC), scores are indicated.

	Botrytis						**Signal Peptide**		***Sclerotina***
	cinerea **Gene Number**	**No. of Peptides**	**% Coverage**	**Size A.A.**	**kDa***	**pI***	**Classical-C Nonclassical-NC**	**Score**	***sclerotiorum*** **Ortholog**
								C NC	
Carbohydrate Metabolism									
Cell Wall Degrading Endoglucanase	BC1G_09079.1	5	23	453	44.1	4.34	C	0.9990/0.9355	SS1G_04852.1
glucan 1,4-alpha-glucosidase	BC1G_08755.1	5	15	542	59.2	5.49	C	0.9970/0.9000	SS1G_13809.1
1,3-beta-glucanosyltransferase G...	BC1G_10455.1	4	13	457	47.6	4.76	C	0.9980/0.9468	SS1G_10353.1
pectate lyase A	BC1G_00912.1	3	17	329	34.4	7.8	C	1.0000/0.9580	SS1G_00238.1
alpha-glucosidase	BC1G_12859.1	3	6	997	109	4.54	C	1.0000/0.9359	SS1G_01083.1
endopolygalacturonase 1	BC1G_11143.1	3	14	382	37.9	8.05	C	1.0000/0.9083	SS1G_10167.1
glucosidase	BC1G_11898.1	3	10	294	31.9	4.5	C	0.9990/0.8968	SS1G_12930.1
family 20 chitobiase	BC1G_07215.1	3	7	599	65.9	5.46	C	0.9990/0.9292	SS1G_10038.1
beta-galactosidase	BC1G_03567.1	3	4	1012	110	5.42	C	0.9980/0.9458	SS1G_03647.1
glycosyl hydrolases Family	BC1G_14944.1	2	6	421	43.4	6.37	C	0.9950/0.9474	SS1G_08695.1
glucan 1,4-alpha-glucosidase	BC1G_04151.1	2	5	672	70.6	5.38	C	0.9120/0.9548	SS1G_10617.1
exo-polygalacturonase	BC1G_01617.1	2	7	472	51.3	5.22	C	0.9960/0.8863	SS1G_05832.1
beta (1-3) glucanosyltransferase (translation)	BC1G_14030.1	1	3	540	56.5	4.37	C	1.0000/0.9513	SS1G_12017.1
Glycosyl hydrolase 32 family	BC1G_02364.1	2	4	777	83.6	4.69	C	0.9910/0.7441	SS1G_09366.1
Glycosyl hydrolase	BC1G_13862.1	1	4	355	35.2	4.57	C	0.9990/0.7986	SS1G_13860.1
Cutinase	BC1G_02936.1	1	11	202	20.2	4.92	C	1.0000/0.9350	SS1G_07661.1
endo-1,4-beta-galactosidase	BC1G_03991.1	1	3	366	40.9	6.12	C	0.0000/0.8884	SS1G_01216.1
cell wall synthesis - Beta-glucosidase	BC1G_12627.1	1	3	471	47.9	4.91	C	0.9990/0.8821	SS1G_07480.1
	BC1G_00617.1	1	4	348	37.9	4.38	C	1.0000/0.8879	SS1G_00468.1

Table 3.2. Categories of Secreted Proteins Identified upon Exposure of *B. cinerea* to 4% pulped Full Red Strawberry in NY media.

	Botrytis cinerea **Gene Number**	**No. of Peptides**	**% Coverage**	**Size A.A.**	**kDa***	**pI***	**Predicted Signal Peptide Classical-C Nonclassical-NC**	**Score C NC**	***Sclerotina sclerotiorum*** **Ortholog**
Carbohydrate Metabolism (Cont'd)									
similar to pectin methylesterase									
Glycosyl hydrolases -15	BC1G_13215.1	1	6	369	38.7	4.59	C	0.8200/0.9564	SS1G_08135.1
Glycosylhydrolase family 32	BC1G_10247.1	1	4	511	55.6	4.25	C	1.0000/0.9321	SS1G_07184.1
arabinofuranosidase B	BC1G_04994.1	1	3	506	52.1	5.97	C	0.9990/0.9527	SS1G_02462.1
Glycosyl hydrolase 61	BC1G_07653.1	1	6	325	32.6	4.01	C	0.9960/0.9400	SS1G_14158.1
fungal beta-1,3-glucanases	BC1G_13534.1	1	4	559	58.5	4.38	C	0.9230/0.9090	SS1G_11977.1
similar to pectin methyl esterase	BC1G_06840.1	3	10	242	26.4	7.87	C	0.2320/0.8909	SS1G_03286.1
Glucanase	BC1G_12563.1	1	5	310	33.7	4.75	NC	0.0000/0.9434	SS1G_06401.1
alpha-amylase	BC1G_02623.1	1	3	427	47.2	6.24	C	0.9940/0.9091	SS1G_01776.1
Proteolysis & Peptidolysis									
dipeptidyl peptidase	BC1G_15663.1	5	15	694	76.1	4.74	C	0.9990/0.9347	SS1G_09909.1
peptidase - Serine Carboxypeptidase	BC1G_09564.1	2	3	544	60.2	5.57	C	1.0000/0.9482	SS1G_03361.1
peptidase - Aspartyl protease	BC1G_06849.1	2	7	398	42.9	4.92	C	0.9990/0.8263	SS1G_11818.1
proteases - Subtilase Family	BC1G_06836.1	1	4	521	55.2	5.49	C	0.9990/0.9466	SS1G_03282.1
Aspartyl protease	BC1G_08393.1	1	4	399	43.9	4.42	C	0.9990/0.9307	SS1G_03576.1
glutamate carboxypeptidase	BC1G_05299.1	6	19	488	54	4.97	NC	0.0000/0.8479	SS1G_04565.1
dipeptidyl peptidase	BC1G_10503.1	2	7	473	64.3	5.68	NC	0.0000/0.9172	SS1G_04140.1
Efp	BC1G_09782.1	1	8	165	17.8	5.04	NC	0.0000/0.6957	SS1G_01874.1
dipeptidyl aminopeptidase	BC1G_13641.1	1	2	921	103	5.13	NC	0.0000/0.8360	SS1G_07744.1
Peptidase	BC1G_07770.1	1	3	508	54.4	4.86	C	0.9980/0.7140	SS1G_07901.1
Stress Response									
choline dehydrogenase	BC1G_14012.1	3	8	678	73.6	4.54	C	0.9980/0.9256	SS1G_11988.1
Choline dehydrogenase	BC1G_02021.1	3	6	628	66.8	4.61	C	0.9990/0.8140	SS1G_00730.1
tyrosinase precursor	BC1G_04092.1	3	9	532	58.3	5.67	C	0.9990/0.9246	SS1G_01576.1
probable cyanate lyase	BC1G_08946.1	5	28	164	18	5.11	NC	0.0000/0.9284	SS1G_11485.1
Peroxiredoxin (PRX) family	BC1G_05133.1	3	29	156	16.3	5.37	NC	0.0010/0.8312	SS1G_07492.1

Table 3.2**:** Categories of Secreted Proteins Identified upon Exposure of *B. cinerea* to 4% pulped Full Red Strawberry in NY media.

	Botrytis cinerea **Gene Number**	**No. of Peptides**	**% Coverage**	**Size A.A.**	**kDa***	**pI***	**Predicted Signal Peptide Classical-C Nonclassical-NC Score**		***Sclerotina sclerotiorum*** **Ortholog**
							C	**NC**	
Stress Response (Cont'd)									
activator of heat shock protein 90	BC1G_11454.1	1	5	323	36.1	5.29	NC	0.0000/0.9431	SS1G_11648.1
Superoxide dismutase Cu/Zn	BC1G_00558.1	1	8	154	15.9	5.85	NC	0.0000/0.8703	SS1G_00699.1
Tyrosinase	BC1G_15125.1	1	2	686	75.4	5.99	NC	0.0000/0.9347	SS1G_14461.1
catalase	BC1G_01095.1	1	5	554	62	6.25	NC	0.0020/0.9301	SS1G_09141.1
glyoxal oxidase	BC1G_01204.1	3	6	816	84.6	5.11	C	0.9830/0.8892	SS1G_02582.1
Electron/Proton Transport									
oxidoreductase	BC1G_14679.1	3	12	515	54.7	8.49	NC	0.0570/0.9468	SS1G_03029.1
Fatty Acid & Phospholipid Metablism									
Esterase_lipase	BC1G_08184.1	1	3	446	50.6	4.43	NC	0.0000/0.9371	SS1G_08747.1
Esterase_lipase	BC1G_07637.1	1	2	556	58.8	5.25	C	1.0000/0.7128	SS1G_14146.1
Miscellaneous									
ribosomal protein P2	BC1G_08016.1	3	52	111	11.2	4.25	C	0.9190/0.2680	SS1G_01699.1
Phosphatidylglycerol / phosphatidylinositol transfer protein	BC1G_02986.1	1	13	168	18	4.64	C	0.9990/0.8291	SS1G_07613.1
Ketol acid reductoisomerase	BC1G_04443.1	1	4	398	44.1	8.78	C	0.8120/0.7077	SS1G_03855.1
Enolase 2 phosphoglycerate dehydratase	BC1G_00350.1	7	32	438	47.1	5.26	NC	0.0000/0.7547	SS1G_04072.1
Cell surface flocculin	BC1G_07647.1	4	6	1024	107	4.64	NC	0.2660/0.8884	SS1G_14153.1
inorganic pyrophosphatase	BC1G_11823.1	1	6	235	26.5	6.53	NC	0.0000/0.9077	SS1G_04783.1
glucose-repressible gene protein	BC1G_11685.1	1	26	71	73.7	6.26	NC	0.0000/0.9406	SS1G_00036.1
Ran GTPase-activation protein	BC1G_08895.1	1	5	261	28.7	4.94	NC	0.0000/0.9268	SS1G_11444.1
Glyceraldehyde-3-phosphate dehydrogenase	BC1G_11968.1	1	3	338	36.5	5.92	NC	0.0000/0.8767	SS1G_07798.1
elongation factor 1 beta subunit	BC1G_03337.1	1	11	158	17.7	4.73	NC	0.0000/0.9002	SS1G_06711.1
fructose-bisphosphate aldolase	BC1G_04836.1	1	4	360	39.2	5.41	NC	0.0000/0.7920	SS1G_06561.1
dnaK-type molecular chaperone BiP	BC1G_04390.1	2	6	670	73.1	4.9	C	0.9820/0.7611	SS1G_02266.1

Table 3.2: Categories of Secreted Proteins Identified upon Exposure of *B. cinerea* to 4% pulped Full Red Strawberry in NY media.

	Botrytis cinerea **Gene Number**	**No. of Peptides**	**% Coverage**	**Size A.A.**	**kDa***	**pI***	**Predicted Signal Peptide Classical-C Nonclassical-NC**	**Score C NC**	***Sclerotina sclerotiorum*** **Ortholog**
Miscellaneous (Cont'd)									
disulfide bond formation	BC1G_02223.1	1	2	531	57.4	4.64	C	0.9990/0.7454	SS1G_02967.1
Peptidyl-prolyl cis-trans isomerase (cyclophilin)	BC1G_01740.1	2	11	181	19.5	6.3	NC	0.0000/0.9369	SS1G_06284.1
peptidyl-prolyl cis-trans isomerase (FK506 binding protein)	BC1G_03430.1	1	9	149	16.4	9.4	NC	0.0020/0.8174	SS1G_08260.1
Hypothetical									
hypothetical Protein	BC1G_00448.1	9	27	722	78.1	4.77	C	0.9830/0.9322	SS1G_04473.1
No significant similarity found	BC1G_15542.1	2	26	193	20	4.47	C	0.9990/0.9034	SS1G_13668.1
No hits found (ceratoplatanin-like)	BC1G_02163.1	4	66	137	13.9	4.65	C	0.9960/0.9466	SS1G_10096.1
No hits found	BC1G_12374.1	2	15	187	19	4.49	C	0.9990/0.9304	SS1G_12361.1
No hits found	BC1G_00896.1	4	38	155	16.5	4.59	C	1.0000/0.9284	SS1G_00263.1
No hits found	BC1G_05033.1	3	27	234	25.9	5.92	C	0.9980/0.8917	SS1G_07262.1
No hits found	BC1G_08635.1	2	17	208	21.9	6.14	C	0.9990/0.9273	SS1G_09844.1
No significant similarity found	BC1G_02326.1	1	3	460	44.3	4.62	C	1.0000/0.7918	SS1G_09299.1
unknown no match	BC1G_04945.1	2	3	1053	108	4.08	C	0.9930/0.8264	SS1G_02504.1
No hits found	BC1G_02492.1	2	6	409	42	6.24	C	0.9960/0.9396	SS1G_01262.1
unknown	BC1G_02060.1	1	7	162	17.2	3.95	C	0.9990/0.9403	SS1G_00768.1
unknown	BC1G_07448.1	1	10	221	23.4	5.84	C	0.9990/0.9015	SS1G_08566.1
unknown	BC1G_14129.1	1	9	160	16.6	4.3	C	0.9970/0.9320	SS1G_12361.1
unknown	BC1G_13938.1	1	8	384	41.4	5.36	C	1.0000/0.9338	SS1G_11842.1
unknown	BC1G_13335.1	1	3	377	41	5.56	C	1.0000/0.6402	SS1G_01032.1
unknown	BC1G_01393.1	1	10	188	20.8	4.42	C	1.0000/0.6907	SS1G_06119.1
unknown	BC1G_08642.1	5	51	153	16.6	6.21	NC	0.0000/0.9481	SS1G_09838.1
unknown	BC1G_05503.1	2	29	125	14.5	5.05	NC	0.0000/0.8513	SS1G_01463.1
unknown	BC1G_07825.1	2	22	109	11.9	5.4	NC	0.0000/0.8695	SS1G_03388.1
unknown	BC1G_05476.1	2	15	185	20.4	5.03	NC	0.0000/0.9238	**No Hit**
unknown	BC1G_05980.1	1	7	208	22.8	4.29	NC	0.0040/0.7699	SS1G_02387.1
unknown	BC1G_10301.1	1	9	127	14.1	9.59	NC	0.0120/0.9075	SS1G_11910.1
unknown	BC1G_00769.1	1	2	790	85.9	5.51	NC	0.0000/0.9512	SS1G_00363.1
related to Plant protein of unknown function (DUF946)	BC1G_00044.1	1	10	115	12.6	5.63	NC	0.0000/0.7266	SS1G_04690.1
unknown	BC1G_02930.1	1	2	399	43.4	6.66	NC	0.0000/0.9277	SS1G_12879
unknown	BC1G_10587.1	1	2	774	79.4	4.68	NC	0.0000/0.8138	SS1G_10535.1

Table 3.2: Categories of Secreted Proteins Identified upon Exposure of *B. cinerea* to 4% pulped Full Red Strawberry in NY media.

	Botrytis cinerea **Gene Number**	**No. of Peptides**	**% Coverage**	**Size A.A.**	**kDa***	**pI***	**Predicted Signal Peptide Classical-C Nonclassical-NC**	**Score** **C** **NC**	***Sclerotina sclerotiorum*** **Ortholog**
unknown	BC1G_13428.1	1	3	371	39.8	5.82	NC	0.0000/0.9104	SS1G_06958.1
unknown	BC1G_05132.1	1	3	315	35.4	9.25	NC	0.0000/0.8556	SS1G_07495.1
unknown	BC1G_05038.1	1	3	356	39.3	9.91	NC	0.0000/0.8849	**No Hit**
unknown	BC1G_09672.1	1	3	349	37.8	5.53	NC	0.0000/0.9057	SS1G_14393.1
unknown	BC1G_10466.1	1	3	448	45.2	4.09	C	0.9990/0.8771	SS1G_08907.1
unknown	BC1G_07482.1	1	3	483	51.8	4.86	C	0.9940/0.2305	SS1G_08645.1

Table 3.3: Domains of Secreted Proteins Identified upon Exposure of *B. cinerea* to 4% pulped Full Red Strawberry in NY media. The most common domain motifs for each protein were identified with the Conserved Domain Database (CDD) at NCBI.

	***Botrytis cinerea* Gene Number**	**Domain Motif Code**	**Common Domain Name**
Cell Wall Degrading Endoglucanase	BC1G_09079.1	COG5309	CTM
glucan 1,4-alpha-glucosidase	BC1G_08755.1	CBM20	CBD
1,3-beta-glucanosyltransferase G...	BC1G_10455.1	pfam03198	GAS1
pectate lyase A	BC1G_00912.1	smart00656	Allergens
alpha-glucosidase	BC1G_12859.1	pfam01055	Glyco_hydro_31
endopolygalacturonase 1	BC1G_11143.1	pfam00295	Glyco_hydro_28
glucosidase	BC1G_11898.1	pfam00295, COG5309	Glyco_hydro_28, Ctm
family 20 chitobiase	BC1G_07215.1	pfam00728, pfam02838	Glyco_hydro_20
beta-galactosidase	BC1G_03567.1	pfam01301	Glyco_hydro _35
glycosyl hydrolases Family	BC1G_14944.1	smart00321, pfam00704	fungal type CBD, Glyco_hydro_18
glucan 1,4-alpha-glucosidase	BC1G_04151.1	pfam00723, pfam00686	Glyco_hydro_15, CBD_20
exo-polygalacturonase	BC1G_01617.1	pfam00295	Glyco_hydro_28
beta (1-3) glucanosyltransferase (translation)	BC1G_14030.1	pfam03198	GAS1
Glycosyl hydrolase 32 family	BC1G_02364.1	pfam00933, pfam01915	Glyco_hydro_3, Glyco_hydro_3
Glycosyl hydrolase 45	BC1G_13862.1	pfam02015	Glyco_hydro_45
cutinase	BC1G_02936.1	pfam01083	Cutinase
endo-1,4-beta-galactosidase	BC1G_03991.1	COG3867	CTM
cell wall synthesis - Beta-glucosidase	BC1G_12627.1	pfam03867	SUN family
similar to pectin methylesterase	BC1G_00617.1	pfam01095	Pectinesterase
Glycosylhydrolases - 15	BC1G_13215.1	pfam00723	Glyco_hydro_15
Glycosylhydrolase family 32	BC1G_10247.1	smart00640	Glyco_32
arabinofuranosidase B	BC1G_04994.1	pfam05270	AbfB
Glycosyl hydrolase 61	BC1G_07653.1	pfam03443	Glyco_hydro_61
fungal beta-1,3-glucanases	BC1G_13534.1	cd02181	GH16_MGL1_glucanase
similar to pectin methyl esterase	BC1G_06840.1	pfam01095	Pectin esterase
glucanase	BC1G_12563.1	cd02181	GH16_MGL1_glucanase
alpha-amylase	BC1G_02623.1	pfam00128, pfam09260, smart00642	Aamy catalytic domain, DUF1966
dipeptidyl peptidase	BC1G_15663.1	COG1506, pfam05448	DAP2, AXE1

Table 3.3: Domains of Secreted Proteins Identified upon Exposure of *B. cinerea* to 4% pulped Full Red Strawberry in NY media.

	***Botrytis cinerea* Gene Number**	**Domain Motif Code**	**Common Domain Name**
peptidase - Serine Carboxypeptidase	BC1G_09564.1	pfam05577	Peptidase_28
peptidase - Aspartyl protease	BC1G_06849.1	pfam00026	Asp
proteases - Subtilase Family	BC1G_06836.1	pfam00082, pfam05922	Peptidase_S8, Subtilisin_N
Aspartyl protease	BC1G_08393.1	pfam00026	Asp
glutamate carboxypeptidase	BC1G_05299.1	pfam01546, COG0624	Peptidase_M20, ArgE
dipeptidyl peptidase	BC1G_10503.1	pfam05448, COG1506	AXE1, DAP2
Efp	BC1G_09782.1	COG0231	Efp
dipeptidyl aminopeptidase	BC1G_13641.1	pfam00326, pfam00930	Peptidase_S9, DPPIV_N
Peptidase	BC1G_07770.1	pfam02225, pfam04389	PA Domain, Peptidase_M28
choline dehydrogenase	BC1G_14012.1	pfam00732, pfam05199, COG2303	GMC_oxred_N, GMC_oxred_C, BetA
Choline dehydrogenase	BC1G_02021.1	pfam00732, pfam05199, COG2303	GMC_oxred_N, GMC_oxred_C, BetA
tyrosinase precursor	BC1G_04092.1	pfam00264	Tyrosinase
probable cyanate lyase	BC1G_08946.1	pfam02560, COG1513	CynS
Peroxiredoxin (PRX) family	BC1G_05133.1	cd03013	PRX5_like
activator of heat shock protein 90	BC1G_11454.1	COG5580	
Superoxide dismutase Cu/Zn	BC1G_00558.1	cd00305	Cu-Zn_Superoxide_Dismutase
Tyrosinase	BC1G_15125.1	pfam00264	Tyrosinase
catalase	BC1G_01095.1	cd00328, COG0753	KatE
glyoxal oxidase	BC1G_01204.1	cd00035, pfam07250	ChtBD1, Glyoxal_oxid_N
oxidoreductase	BC1G_14679.1	pfam01565	FAD_binding_4
Esterase_lipase	BC1G_08184.1	cd00312	
Esterase_lipase	BC1G_07637.1	cd00312	
ribosomal protein P2	BC1G_08016.1	COG2058	RPP1A
Phosphatidylglycerol / phosphatidylinositol transfer protein	BC1G_02986.1	cd00917	PG-PI_TP
Ketol acid reductoisomerase	BC1G_04443.1	pfam01450, COG0059	llvC, llvC
Enolase 2 phosphoglycerate dehydratase	BC1G_00350.1	cd03313, COG0624	enolase
inorganic pyrophosphatase	BC1G_11823.1	cd00412	
Ran GTPase-activation protein	BC1G_08895.1	cd00835	RanBD
Glyceraldehyde-3-phosphate dehydrogenase	BC1G_11968.1	pfam02800, pfam00044, COG0057	Gp_dh_C, Gp_dh_N, GapA
elongation factor 1 beta subunit	BC1G_03337.1	cd03181	GST_C_EFB1gamma
fructose-bisphosphate aldolase	BC1G_04836.1	cd00946	FBP_aldolase_IIA
dnaK-type molecular chaperone BiP	BC1G_04390.1	pfam00012	HSP70

Table 3.3: Domains of Secreted Proteins Identified upon Exposure of *B. cinerea* to 4% pulped Full Red Strawberry in NY media.

	***Botrytis cinerea* Gene Number**	**Domain Motif Code**	**Common Domain Name**
disulfide bond formation	BC1G_02223.1	cd02995, cd02961, cd02982, cd02981	PDI_a_PDI_a'_C, PDI_a, PDI_b', PDI_b
Peptidyl-prolyl cis-trans isomerase (cyclophilin)	BC1G_01740.1	cd01926	cyclophilin_ABH_like
peptidyl-prolyl cis-trans isomerase (FK506 binding protein)	BC1G_03430.1	pfam00254	FKBP_C
unknown no match	BC1G_04945.1	smart00321	Domain in WSC proteins
unknown	BC1G_13938.1	smart00602	VPS10 domain
unknown	BC1G_07825.1	COG1359	Uncharacterized Conserved Domain
unknown	BC1G_05980.1	cd00237	
unknown	BC1G_10301.1	pfam02018	CBM_4_9
unknown	BC1G_00769.1	smart00360, smart00517	RRM, PolyA - C terminal domain
related to Plant protein of unknown function (DUF946)	BC1G_02930.1	pfam06101	DUF946
unknown	BC1G_10587.1	cd02859	AMFKbeta_GBD_like
unknown	BC1G_13428.1	cd02396	PCBP_like_KH Domain
unknown	BC1G_05132.1	smart00360	RRM
unknown	BC1G_07482.1	pfam01565, COG0277	FAD Binding Domain

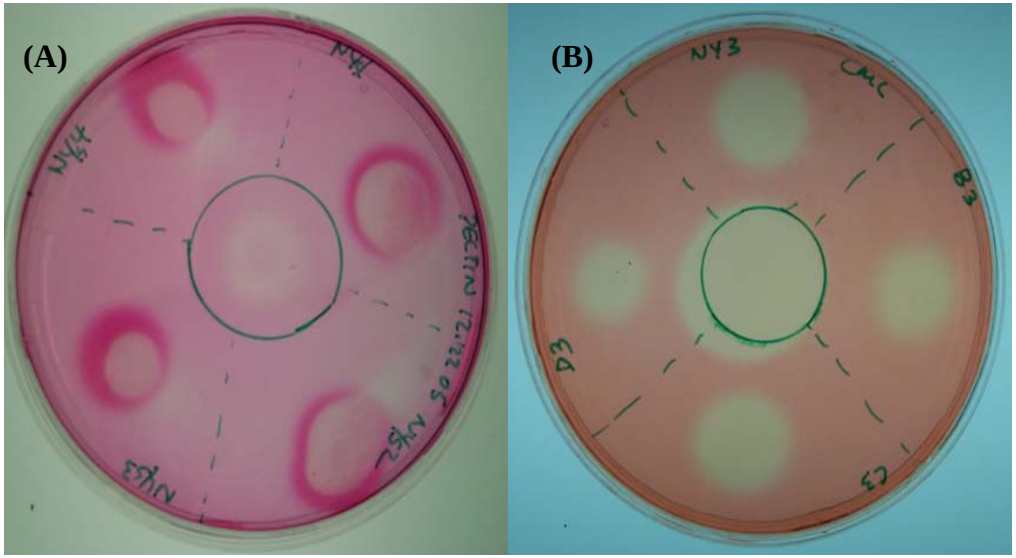

Figure 3.2: Plate assays for the qualitative detection of pectin (2a) and carboxymethycellulose (2b) degrading enzymes found in collected secretions. The center sphere is the applied control for each assay, 0.09 U (or 0.039mg) of *Aspergillus niger* and 40ug of *Trichoderma reesei* cellulase for the pectin and carboxymethylcellulose assay plates, respectively. Surrounding spots are various secretion replicates that were collected.

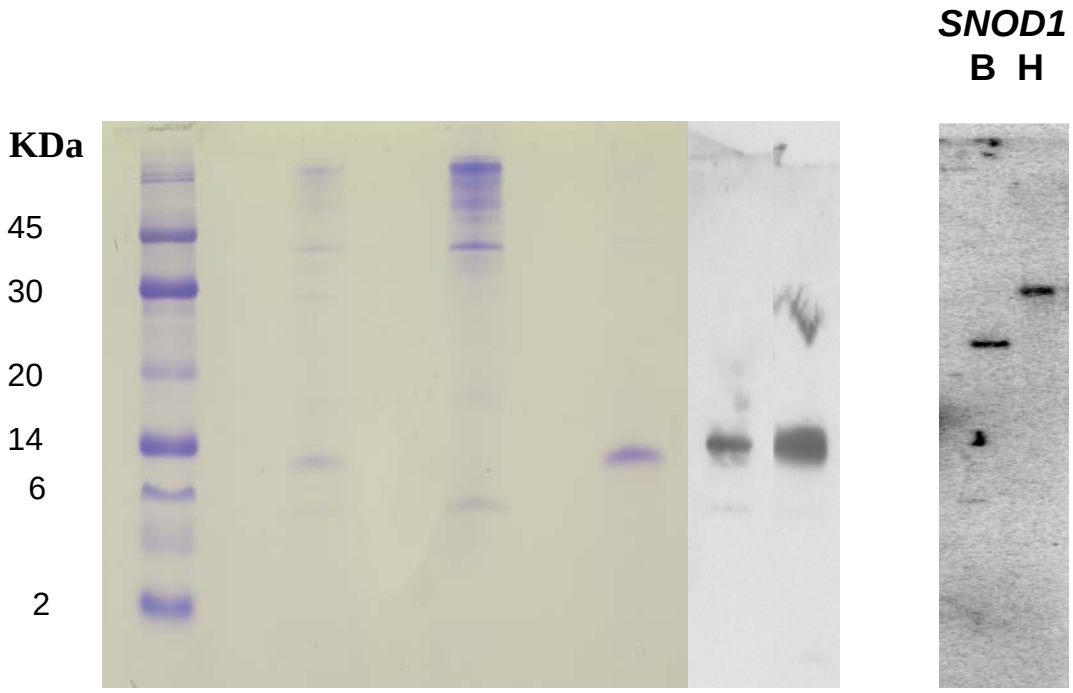

Figure 3.3 A: 16.5% SDS denaturing tricine gel of unpurified secretion and collected peak 1 and peak 2 after fractionated on a HiTrap Q-column: Lane 1, MWM; Lane 2, 100 µg/lane crude sample; Lane 4, 10 µg/lane peak 1; Lane 6, 10 µg/lane peak 2. Western: Left to right, 100 µg of crude sample and 10 µg of peak 2, respectively. Probed with antibody made to antigenic peptide of SNOD1. B: The genomic complexity of SNOD1 in the *B. cinerea* BO5.10 genome. B-BamHI and H-HindIII digests of 30 µgms of genomic DNA per lane.

CHAPTER 4

COMPARATIVE PROTEOMIC ANALYSIS OF *BOTRYTIS CINEREA* SECRETEOME[1]

[1] Punit Shah, Hind El Mubarek, James A. Atwood III, Gopi K. Podila and Maria R. Davis, Ron Orlando submitted to *Journal of Proteome Research* 04/18/2008

ABSTRACT

Botrytis cinerea is a filamentous fungus infecting more than 200 plant species, causing significant economic losses worldwide. Secreted proteins are released as an initial response of the fungus to its plant host. We report the use of a high throughput LC-MS/MS approach to analyze *B. cinerea* BO5.10 secreted proteins. Secretions were collected from fungus grown on a solid substrate of cellophane membrane while mock infecting media supplemented with the extract of full red tomato, ripened strawberry or Arabidopsis leaf extract. Overall 89 *B. cinerea* proteins were identified from all growth conditions. Sixty proteins were predicted to contain a SignalP motif indicating the extracellular location of the proteins. Seven proteins were observed in all the growth conditions implying a constitutive nature of their secretion. Differences in secretion were observed when *B. cinerea* was exposed to fruit extract compared to Arabidopsis leaf extract. Identified in the secretions were transport proteins, proteins well characterized for carbohydrate metabolism, peptidases, oxidation/reduction, and pathogenicity factors that provide important insights into how *B. cinerea* may use secreted proteins for plant infection and colonization.

INTRODUCTION

Botrytis cinerea is a broad host pathogen that derives its nourishment from dead or necrotic plants. It is ubiquitous in its range of occurrence and can easily be found anywhere in the world. Besides being an opportunistic fungus at ambient temperature (23ºC), it thrives well at 4ºC where most fungi either die or become dormant. It is able to accelerate its putrefaction of post-harvest plants at refrigerated temperatures, which greatly reduces the shelf-life of valuable produce such as strawberries and tomatoes.

B. cinerea, like many other filamentous fungi, secretes an arsenal of extracellular enzymes to break down plant cell wall polymers for pathogen penetration and nutrient consumption. To date, these enzymes have predominantly been studied on a protein-by-protein basis using molecular genetics, biochemical techniques and recently, whole organism proteome analysis[1,2]. Several enzymes secreted by *B. cinerea* have been thoroughly studied including; aspartic proteases[3], pectin methyl esterases (PMEs)[4] and polygalacturonases (PGs)[5]. Previously six different *B. cinerea* polygalacturonase (BCPG) genes have been identified[5,6,7]. Targeted inactivation of polygalacturonase 2 (BCPG2) has resulted in *B. cinerea* mutants with reduced virulence on multiple plant hosts[5,6,7]. Five of the 6 *BCPG*s were successfully expressed in *Pichia pastoris* and the purified proteins tested individually on tomato, broad bean and *Arabidopsis thaliana* leaves resulting in various levels of necrotizing activity on these plants[7]. These results imply an important role for polygalacturonases as secreted enzymes in pathogenicity.

The ability of *B. cinerea* to infect a broad range of plants suggests that the fungus secretes a variable profile of enzymes and other proteins, in addition to those previously identified, to “fine-tune” its mechanism of attack in different cellular environments. Secreted proteins are of particular relevance when studying fungal pathogenicity since these molecules

trigger the on-going interaction with the plant host. Thus, the identification and study of the complete pool of secreted proteins (the secretome) may divulge unique infection mechanisms that could lead to new control measures for fungal diseases.

Recent advances in mass spectrometry, high pressure liquid chromatography, bioinformatics, and the availability of a multitude of genome sequences has for the first time made it possible to study the secretome of fungi in a high throughput manner. The secretome of several filamentous fungi have been analyzed, including; *Fusarium graminearum, Aspergillus flavus, Aspergillus oryzae, Pleurotus spadius* and *Phanerochate chrysosporium*[8-13]. A number of studies have pointed out that the expression of fungal secreted proteins is, in part, dependent upon the growth conditions, including liquid versus solid media, or in the presence of different plant substrates[8, 12, 14].

Here we use liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) to investigate the secretome produced by *B. cinerea* in response to mock interactions using the extract either of fully ripe strawberry, fully ripe tomato fruit or Arabidopsis plants, and employing growth on a solid surface. Arabidopsis, although not a native host, is a widely used as a plant model, and a number of studies have investigated the responses of Arabidopsis to *Botrytis cinerea* infection[15-17]. We expect that the identification of the secretomes produced on different fruit cultures will provide a better understanding of the correlation of secreted enzymes to a host plant during pathogen attack. This information will be useful for developing novel defense mechanisms to safeguard plants against *B. cinerea* infection.

MATERIALS AND METHODS

Microorganisms

B. cinerea strain BO5.10 was obtained from Muriel Viaud (INRA, Versailles, FR). Stock spores were obtained after a single culturing of the primary culture on potato dextrose broth (Difco, Detroit, MI) for several weeks. Conidia were collected by gentle mixing with 0.05% Tween 80 in sterile water on the surface of the mature conidiophores. Frozen stocks of isolated spores at 10^6 spores/ml were prepared with sterile glycerol (15% final concentration) and stored at -80^{o}C in multiple aliquots.

Inoculation of cellophane membranes with B. cinerea spores

Cellophane membranes (Spontex, Belgium) were used to immobilize the *B. cinerea* spores. Membranes cut to 100mm circular diameters were treated by boiling in 1mM EDTA (Mallinckrodt, Phillipsburg, NJ) for 10min, drained and rinsed thoroughly in distilled water, and autoclaved to sterilize. The treated cellophane membranes were then blotted briefly to remove excess liquid and overlaid on agar plates. *B. cinerea* BO5.10 was inoculated on the sterile cellophane membranes by streaking 1 x 10^4 spores gently over the surface of the membranes overlaid on NY agar (2g/L malt extract, 2g/L yeast extract, 15g/L agar) containing either cheesecloth filtered pulp of full red strawberries, red tomato fruit (4% pulp in NY, 96% NY media, 15 g/L agar) or mature *Arabidopsis* whole plants at 1% pulp in 96% NY media, and 15g/L agar. The pH of all the groth media, after addition of the pulp was 4.0-4.5. The spores were allowed to germinate for 5-8 days at 22ºC. Controls were treated identically but contained only NY agar without any added plant material.

Collection of secreted proteins

To collect the secreted proteins the membranes containing the germinated fungal spores were removed from the agar plate, floated onto a plate containing 35ml of 37.5 sodium acetate buffer at pH 4.4, and incubated in the dark for 10 days. Since media carry-over was not observed, rinsing the membranes prior to transfer was avoided in order to minimize the potential loss of fungal spores or disturbing fungal mycelium. The resulting solutions, containing the *B. cinerea* secretomes, were collected in 50ml conical tubes for further analysis.

One Dimensional Electrophoresis

One ml of each sample containing secreted proteins was frozen and lyophilized to approximately 30µL then mixed with 10µL of SDS sample buffer (Invitrogen, Carlsbad, CA). The sample was boiled for 10min, allowed to cool to room temperature then 20µl was loaded onto a NuPAGE 12% Bis-Tris precast gel (Invitrogen, Carlsbad, CA). Separation was carried out by applying a constant voltage of 150V. The gel was then stained with silver[18].

Proteolytic digestion

One ml of the each sample was reduced by incubating with 1mM dithiothreitol at 55ºC for two hours followed by carboxyamidomethylation with 10mM iodoacetamide at room temperature in the dark for 30min. The solution was adjusted to a pH of 8 using 100mM ammonium bicarbonate solution. The protein mixtures were then enzymatically digested with sequencing grade porcine trypsin (Promega, Madison, WI) using a ratio of 1:50 enzyme to substrate by incubation overnight at 37ºC. The samples were then spin-filtered through a 30,000 molecular weight cutoff filter (YM-30, Millipore), dried by vacuum centrifugation and reconstituted in 100µL of 0.1% formic acid.

LC-MS/MS analysis

Trypsin digested secreted proteins were analyzed in triplicate for the strawberry and tomato fruit samples and in duplicate for both the control and *Arabidopsis* samples. An Agilent 1100 capillary LC (Palo Alto, CA) was attached to a T splitter to deliver nL flow rates into the mass spectrometer. Five μm diameter C_{18} beads (Rainin, Woburn, MA) were packed into a pulled fused silica capillary (10.5cm x 100μm ID) under 1,000 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 mins, 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 an LTQ linear ion trap mass spectrometer (Thermo Fisher, San Jose, CA) at 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. Dynamic exclusion was enabled for 160secs. Raw tandem mass spectra were converted into a peak list using ReAdW followed by the mzMXL2Other algorithm[19]. The peak lists were then searched using Mascot 1.9 (Matrix Science, Boston, MA).

Database searching and protein identification

A target database was created by combining annotated proteins from *Botrytis cinerea BO5.10* genes (Broad Institute, MA) with protein sequences from *Arabidopsis* (Col-0), tomato and strawberry obtained from the National Center for Biotechnology Information (www.ncbi.nih.gov). The plant sequences were used to filter out spectra derived from contaminating plant proteins. A decoy database (decoy) 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) full tryptic cleavage with 2 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 false discovery rate (FDR) using the ProValT algorithm. [20] At 1% FDR, the mascot ion score thresholds for identification of a protein with 3 or more peptides was >28, with 2 peptides >39 and with a single peptide >46 for strawberry mock infections. Minimum Mascot ion score thresholds for protein identification with three or more peptides was 27, 26 and 35, with 2 peptides 44, 51 and 37, and for single peptides 44, 51 and 50 for the control, *Arabidopsis* and tomato mock infections, respectively. After a list of proteins was created, spectral counts (i.e. the number of MS/MS spectra identifying each protein) were determined.

Protein functional annotation

For proteins with no assigned function, 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^{-30}. The mechanism of secretion was predicted using SignalP and SecretomeP to identify classical and nonclassical signal motifs, respectively, or to predict if neither motif was represented[21, 22].

RESULTS AND DISCUSSION

Growth conditions and secreted protein isolation

B. cinerea is an economically important pathogenic fungus, however only a handful of proteomic studies have been performed to date on this organism. Previous studies in other fungi have demonstrated that sufficient quantities of secreted proteins can be obtained for proteomic studies when the fungus is grown in the presence of various host substrates[8-13]. We therefore adopted a similar approach to profile the secreted proteome of *B. cinerea* in the presence of three different plant substrates. However, unlike previous studies, we employed a solid surface for fungal growth rather than a liquid media in order to better reflect the conditions under which *B. cinerea* infections of plants naturally occur. *B. cinerea* BO5.10 spores were streaked onto cellophane membranes that were then floated on top of NY agar media supplemented with either the pulped fruit of strawberry, tomatoes or the leaf extract of *A. thaliana*. A media containing only NY agar (control) was also inoculated and used as a control. Secreted proteins were then collected by floating the membrane in an acetate solution.

This approach is dependent on minimal carryover of plant proteins into the acetate solution and the fungus maintaining its normal secretion pattern in the absence of plant substrate post-growth. (Figure 4.1) is an image of the silver stained one dimensional gel of the secreted proteins collected from the three growth conditions and the NY agar control. It is well known that *B. cinerea* is able to infect strawberry and tomato fruit much more efficiently than *Arabidopsis* leaves. Thus the rate of growth on these differing substrates is reflected in the abundance of secreted proteins collected from each culture condition, indicating that the growth conditions using our solid support approach mimics what occurs in nature [14].

To determine if plant protein carryover was occurring the proteomic data generated in these analyses were searched against all the available protein sequences from strawberry, tomato, and *Arabidopsis* as described above. The absence of any statistically significant plant-specific protein identifications indicated that carryover from the plant extract did not occur at a detectable level.

Proteomics approach

The proteome of *B. cinerea* has been previously studied using two dimensional gel electrophoresis $(2\text{-D})^2$, resolving between 300 and 400 spots and identifying a total of 28 proteins. While the 2-D gel method has been implemented for secreted proteome studies in other fungi such as *Sclerotinia sclerotiorum*[23], we adopted an LC-MS/MS approach to obtain both qualitative and semi-quantitative data on the secreted proteome of *B. cinerea*.

The starting protein concentration is normalized prior to proteomics analysis for many comparative/quantitative proteomics studies, in an attempt to account for differences in efficiency in protein extraction from the different samples and to allow comparison of the protein expression levels between sample sets. However, most comparative/quantitative studies are performed on samples that differ only slightly in their proteome profiles and the relative amount/number of proteins does not differ dramatically between samples. In our case, major differences in the relative abundance of secreted proteins were expected between the growth conditions (Figure 4.1) and thus normalizing the samples by starting protein concentration was not practical. Instead we simply started each proteome analysis with an equal volume of acetate solution containing the secreted proteins as described above. By relying on sample volume rather than protein concentration we assumed that in nature, equal amounts of proteins are not secreted by the fungus when grown under different nutritional conditions.

This disproportional secreted protein expression, as shown in (Figure 4.1), is further reflected in the fact that over 70% of the collected MS/MS spectra resulted from the strawberry and tomato culture conditions (Supplemental Table 1). A total of 5,695 MS/MS spectra were confidently assigned to 89 unique proteins at a protein false discovery rate of less than 1% across all culture conditions. The secreted proteins assigned to the strawberry and tomato preparations accounted for 88.2% or 5,023 assigned MS/MS spectra with an average of 60 proteins being identified in these two conditions while an average of 20 proteins were observed in *Arabidopsis* and control media (Supplemental Tables 2-6). The relatively low number of MS/MS spectra and peptides identified in *Arabidopsis* supplemented and control media as compared to strawberry and tomato supplemented media indicated lowered levels of secretion by the fungus under non-native host conditions. The number of identified proteins from spectra and the relative protein abundances observed from the gel indicate that the fruit extract supplemented media growth conditions are nutritionally favorable.

Assigning protein function and secretion

Sixty proteins out of 89 were predicted to have signal peptide sequences (Table 4.1, Supplemental Table 2). However, twenty nine of the identified proteins lacked a predicted N-terminal signal peptide sequence. The presence of a predicted cleavage site within the N-terminal region of the gene encoding for the protein served an important role in supporting that the protein was secreted. The absence of such a site can possibly indicate that the extracellular location of the protein is a result of cell lysis. Another plausible explanation is that proteins lacking a signal peptide are in fact secreted through a non-classical secretory mechanism, which has recently been demonstrated in yeast[24, 25]. The Cu/Zn superoxide dismutase of *B. cinerea,* which plays an important role in French bean virulence, has been confirmed to be secreted by a non-classical

pathway in some organisms like *Aspergillus fumigates* and *Claviceps purpurea*[26]. Our identification in this analysis of superoxide dismutase (BC1G_00558.1) leads us to believe that a non-classical pathway of secretion is present in *B. cinerea*.

An attempt was made to identify those proteins with non-classical targeting signals by using the SecretomeP prediction software[22]. Nineteen of the twenty nine proteins lacking a signal peptide sequence were predicted by SecretomeP to have a non-classical targeting signal sequence indicating a possible secretion through a non-classical pathway (Supplemental Table 2). The function of some of these 19 proteins, including two pectin methyl esterases (BC1G_06840.1, BC1G_00617.1) and superoxide dismutase (BC1G_00558.1) are consistent with secretion, as they are involved in plant cell wall degradation, host defense or counter-defense by the pathogen[4, 26]. The presence of 10 identified proteins with no classical or non classical predicted signal sequences such as ubiquitin, phosphogluconolactonase, and flavin-nucleotide binding protein could be explained by cell lysis[27-31]. However, it is possible that these proteins could be present as truly secreted multifunctional proteins - a class of proteins that exhibit different functionality if expressed inside versus outside the cell. Such dual functions of proteins have been confirmed in other organisms [32].

The *B. cinerea BO5.10* genome is not completely annotated, thus 70 of the 89 identified proteins were assigned as either “hypothetical” or “protein coding” giving no indication of putative function. In an attempt to assign putative function to the identified secreted proteins, BLAST homology searches were performed. For cases in which significant homology was observed, the putative protein name and function were assigned (Table 1, Supplemental table 2). The identified proteins with significant homology to annotated proteins in other organisms were then classified into the following categories: carbohydrate metabolism and transport, peptidases,

oxidation/reduction, and pathogenicity factors. In total, 54 of the 70 proteins with unknown function yielded significant homology and could be assigned a putative function (supplemental Table 2).

Substrate independent protein secretion

Seven proteins were present in all four growth conditions, consistent with their secretion by the fungus during the process of growth and development and thus important for infection (supplemental table 2). These included two pectin methyl esterases (BC1G_00617.1, BC1G_06840.1); glucan, 1-4, alpha glycosidase (BC1G_04151.1); beta-glucanase like protein (BC1G_10221.1); ceratoplatanin (BC1G_02163.1) and two hypothetical proteins (BC1G_12374.1 and BC1G_00198.1) (Figure 4.2). All of these proteins except BC1G_06840.1 contained a classical signal peptide. Four out of the 7 proteins were classified as cell wall degrading enzymes, two as hypothetical, and one as a putative pathogenicity factor.

Of important note was the identification of ceratoplatanin (BC1G_02163.1) in each of the 4 growth conditions. Almost one quarter of the spectra identified belong to BC1G_02163.1 indicating that this protein is very highly expressed in a substrate independent fashion. Ceratoplatanin is a member of the fungal specific small protein family. Members of this family have been reported to be secreted proteins that act as elicitors and in some cases pathogenicity factors[33]. Genes similar to ceratoplatanin have previously been identified in other fungi[34]. In *B. cinerea*, the snodprot gene (*bcspl1*), with a sequence highly homologous to BC1G_02163.1 has been reported to be induced *in planta* by ethylene in the early phase of interaction [34]. It has also been suggested that *bcspl1* acts as an elicitor of plant defense in the early phase of infection but can act as a virulence factor during the later stages of the *B. cinerea* infection process[34]. Studies have also shown that in *Magnaportha grisea* (rice blast fungus) deletion mutants of the snod prot

genes greatly reduced the ability of the fungus to induce disease, whereas deletion mutants of the snod prot gene from *L. maculans* did not result in a reduction of virulence[35]. These studies combined with the high levels of secretion of BC1G_02163.1 observed in this study further suggest that this protein plays an important role in host-pathogen interactions and is substrate independent.

Secretome in non nutritional media

Seventeen proteins were identified in the control which omitted any plant material and contained only NY media (supplemental table 3). The existence of some fungal growth and the minor degradation of the cellophane membrane observed in the control confirm the ability of the fungus to grow on a cellophane membrane in contact with NY media. There were 21 *B. cinerea* proteins identified when the fungus was grown on *Arabidopsis* supplemented NY media. Eleven *B. cinerea* proteins were identified in both the *Arabidopsis* and the control growth conditions. Seven out of these 11 proteins were substrate independent proteins, meaning they were observed in all growth conditions while the remaining four were identified in both the *Arabidopsis* and control but not under nutritionally favorable growth conditions. These included two peptidases BC1G_02944.1 and BC1G_12776.1, an aspartic protease (BC1G_03070.1), and an exo polygalacturonase (BC1G_01617.1). An additional six proteins were detected in either the *Arabidopsis* or control conditions and not observed in either of the nutritionally favorable growth conditions. In the *Arabidopsis* supplemented media, one inositolpolyphosphate phosphatase (BC1G_11835.1), one glycoamylase precursor (BC1G_02333.1), and an extracellular phytase enzyme (BC1G_02314.1) were uniquely identified while xyloglucan specific endo β ,1-4 glucanase (BC1G_00594.1), endo14 xylanase precursor (BC1G_01778.1), and acid phosphatase (BC1G_02965.1) were observed only in the control media. The identification of similar enzymes

in the strawberry and tomato growth conditions may imply that the unique identification of these enzymes in the non nutritional media is a result of dynamic range limitations imposed by the mass spectrometer rather than true differential expression. Thus the inability to identify these enzymes in the nutritionally favorable conditions of full red tomato extract and strawberry extract is more likely a result of the increased total secretion of other proteins in the nutritionally favorable growth conditions.

Notable differences were evident in the secretomes of fungi grown on the Arabidopsis supplemented vs control media. While *Arabidopsis* is not a nutritionally favorable growth condition for *B. cinerea*, an increase in the fungal growth rate and a nearly 2 fold increase in the total spectral count for all identified proteins was observed when the fungus was grown in the presence of *Arabidopsis* extract as compared to control media alone (Supplemental Table 1). The presence of plant proteins and cell wall carbohydrates in the *Arabidopsis* supplemented media was inferred by an increase in the spectral count and total protein score for enzymes associated with protein and carbohydrate degradation. For example, BC1G_04151.1, a glucan 1,4-alpha glucosidase, an enzyme that cleaves the 1,4 linkage between glucose residues found in plant cell wall structures was identified by 61 average spectral counts in the *Arabidopsis* supplemented media versus 30 in the control (Figure 4.3). BC1G_02944.1 S53 Protease, a serine protease was also upregulated with a spectral count of 16 in the *Arabidopsis* supplemental media versus 8 in the control (Figure 4.3).

Secretome in nutritional media

When *B. cinerea* was grown on strawberry and tomato extract supplemented media, 65 (Supplement Table 4) and 56 (Supplement Table 5) proteins were identified, respectively, compared to 21 (Supplement Table 6) and 17 proteins identified for NY media supplemented

with *Arabidopsis* whole plants and the NY media only. Forty two proteins were common between the tomato and strawberry supplemented growth conditions, of which 17 are involved in carbohydrate metabolism or protein transport, 2 were peptidases, 8 were hypothetical and one was a pathogenicity factor. A large set of common *B. cinerea* proteins was observed between the nutritionally favorable growth conditions, indicating that the fungus secretes an array of enzymes to degrade and feed on fruit as a food source. Cell wall degradation is the primary mode of host penetration and nutrient production by *B. cinerea* and hence an increase in the number of identified secreted proteins involved in carbohydrate metabolism and transport is in agreement with the presence of a more exploitable substrate.

When the fungus was grown in the presence of a nutritional plant substrate a dramatic increase in the number of spectral counts associated with the hypothetical enzyme (BC1G_12374.1), and ceratoplatanin BC1G_02163.1 was observed. For example hypothetical protein (BC1G_12374.1) was identified with 26 and 54 average spectral counts in the tomato and strawberry preparation but only 4 and 4 in the *Arabidopsis* and control media (Figure 4.3). Among the 79 proteins identified in the strawberry and tomato preparations, 29 proteins were related to carbohydrate transport and metabolism, while 7 proteins were proteases, as compared to 12 and 4 in the non nutritional media. An increase was also observed in the number of spectra (a measure of protein relative abundance) observed for these proteins. The presence of a large number of common enzymes secreted in the nutritionally favorable growth conditions might be key to understanding *B. cinerea*'s ability to infect a large number of plant species.

Differences within nutritional rich media

Overall more enzymes and spectra were observed from the strawberry supplemented media indicating that strawberry is a more favorable growth condition for *B. cinerea*. However,

there exists a relatively small difference between the tomato and strawberry supplemented growth conditions in terms of the enzymes observed. One of these differences was the identification of cellobiohydrolase (BC1G_10880.1) which is unique to the strawberry system with 64 average spectral counts. Cellobiohydrolase generates cellobiose from cellulose. One explanation for the presence of cellobiohydrolase is that the enzyme is secreted to degrade the cellulose component of the cellophane membrane. However the absence of this protein in any other growth condition leads us to believe that this enzyme was secreted for degradation of the cellulose component of the strawberry cell wall or strawberry pulp. Three hypothetical proteins BC1G_00448.1, BC1G_00863.1, and BC1G_08615.1 were also uniquely identified in strawberry supplemented media with a higher number of spectral counts than any other growth condition.

In the tomato supplemented media, 1-4 beta xylanase (BC1G_00576.1) was identified with a average spectral count of 14 compared to 3 in strawberry supplemented media. It has been reported previously that 1-4 beta xylanase degrades the xylan backbone of the hemicelluloses part of the plant cell wall and is required for pathogenesis of *B. cinerea*[36]. While they were identified with relatively low numbers of spectral counts and fewer than two peptides, alpha amylase (BC1G_02623.1), pectin lyase (BC1G_12017.1), and cutinase (BC1G_08314.1) were all uniquely assigned to the tomato supplemented media.

CONCLUSION

B. cinerea was shown to secrete a wide array of enzymes to infect a variety of hosts under mock growth conditions. The data provided here indicates that there are significant changes in the relative abundance and composition of the secreted enzymes in a substrate dependent manner. In the presence of more favorable food sources such as full red strawberry extract and ripe tomato

extract; we observed that *Botrytis cinerea* increases protein secretion when compared to either *Arabidopsis* leaf extract or NY media. This study has also provided a pool of potentially important secreted enzymes and proteins with unknown functions which require further biological study.

ACKNOWLEDGMENTS

This work was supported in part by the University of Alabama in Huntsville start-up and Faculty Minigrant Award, BARD award #_IS-3947-06, NSF SGER award #0634703 and NSF MCB award #0641820 to MRD. Additional funding was also provided by the NIH Integrated Technology Resource for Biomedical Glycomics, grant P41RR018502 (RO).

REFERENCES

1. Elad, Y., *Botrytis: Biology, Pathology and Control*. Springer: 2004.
2. Fernández-Acero, F. J.; Jorge, I.; Calvo, E.; Vallejo, I.; Carbú, M.; Camafeita, E.; Garrido, C.; López, J. A.; Jorrin, J.; Cantoral, J. M., Proteomic analysis of phytopathogenic fungus *Botrytis cinerea* as a potential tool for identifying pathogenicity factors, therapeutic targets and for basic research. *Archives of Microbiology* 2007, 187, (3), 207-215.
3. ten Have, A.; Dekkers, E.; Kay, J.; Phylip, L. H.; van Kan, J. A. L., An aspartic proteinase gene family in the filamentous fungus *Botrytis cinerea* contains members with novel features. *Microbiology*: 2004; Vol. 150, 2475-2489.
4. Valette-Collet, O.; Cimerman, A.; Reignault, P.; Levis, C.; Boccara, M., Disruption of *Botrytis cinerea* pectin methylesterase gene Bcpme 1 reduces virulence on several host plants. *Molecular Plant-Microbe Interactions* 2003, 16, (4), 360-367.
5. Wubben, J. P.; Mulder, W.; ten Have, A.; van Kan, J. A. L.; Visser, J., Cloning and partial characterization of endopolygalacturonase genes from *Botrytis cinerea*. *Applied and Environmental Microbiology* 1999, 65, (4), 1596-1602.
6. ten Have, A.; Mulder, W.; Visser, J.; van Kan, J. A. L., The endopolygalacturonase gene Bcpg1 is required for full virulence of *Botrytis cinerea*. *Mol. Plant-Microbe Interact* 1998, 11, 1009-1016.
7. Kars, I.; Krooshof, G. H.; Wagemakers, L.; Joosten, R.; Benen, J. A. E.; van Kan, J. A. L., Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. *The Plant Journal* 2005, 43, (2), 213-225.

8. Oda, K.; Kakizono, D.; Yamada, O.; Iefuji, H.; Akita, O.; Iwashita, K., Proteomic analysis of extracellular proteins from *Aspergillus oryzae* grown under submerged and solid-state culture conditions. *Appl. Environ. Microbiol.* 2006, 72, (5), 3448-3457.

9. Kim, Y.; Nandakumar, M. P.; Marten, M. R., Proteomics of filamentous fungi. *Trends in Biotechnology* 2007, 25, (9), 395-400.

10. Paper, J. M.; Scott-Craig, J. S.; Adhikari, N. D.; Cuomo, C. A.; Walton, J. D., Comparative proteomics of extracellular proteins in vitro and in planta from the pathogenic fungus *Fusarium graminearum*. *Proteomics* 2007, 7, (17), 3171-3183.

11. Medina, M. L.; Haynes, P. A.; Breci, L.; Francisco, W. A., Analysis of secreted proteins from *Aspergillus flavus*. *Proteomics* 2005, 5, (12), 3153-3161.

12. Zorn, H.; Peters, T.; Nimtz, M.; Berger, R. G., The secretome of *Pleurotus sapidus*. *Proteomics* 2005, 5, (18), 4832-4838.

13. Wymelenberg, A. V.; Sabat, G.; Martinez, D.; Rajangam, A. S.; Teeri, T. T.; Gaskell, J.; Kersten, P. J.; Cullen, D., The *Phanerochaete chrysosporium* secretome: Database predictions and initial mass spectrometry peptide identifications in cellulose-grown medium. *Journal of Biotechnology* 2005, 118, (1), 17-34.

14. Viniegra-González, G.; Favela-Torres, E.; Aguilar, C. N.; Rómero-Gomez, S. J.; Díaz-Godínez, G.; Augur, C., Advantages of fungal enzyme production in solid state over liquid fermentation systems. *Biochemical Engineering Journal* 2003, 13, (2-3), 157-167.

15. Ferrari, S.; Plotnikova, J. M.; De Lorenzo, G.; Ausubel, F. M., Arabidopsis local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4. *The Plant Journal* 2003, 35, (2), 193-205.

16. Thomma, B.; Eggermont, K.; Tierens, K.; Broekaert, W. F., Requirement of functional ethylene-insensitive 2 gene for efficient resistance of Arabidopsis to infection by *Botrytis cinerea*. *Plant Physiology* 1999, 121, (4), 1093-1101.

17. Govrin, E. M.; Levine, A., Infection of Arabidopsis with a necrotrophic pathogen, *Botrytis cinerea*, elicits various defense responses but does not induce systemic acquired resistance (SAR). *Plant Molecular Biology* 2002, 48, (3), 267-276.

18. Blum, H.; Beier, H.; Gross, H. J., Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* 1987, 8, (2), 93-99.

19. Pedrioli, P. G. A.; Eng, J. K.; Hubley, R.; Vogelzang, M.; Deutsch, E. W.; Raught, B.; Pratt, B.; Nilsson, E.; Angeletti, R. H.; Apweiler, R.; Costello, C. E.; Hermjakob, H.; Huang, S.; Julian, R. K.; Kapp, E.; McComb, M. E.; Oliver, S. G.; Omenn, G.; Paton, N. W.; Simpson, R.; Smith, R.; Taylor, C. F.; Zhu, W.; Aebersold, R., A common open representation of mass spectrometry data and its application to proteomics research. *Nature Biotechnology* 2004, 22, 1459-1466.

20. Weatherly, D. B.; Atwood, J. A.; Minning, T. A.; Cavola, C.; Tarleton, R. L.; Orlando, R., A heuristic method for assigning a false-discovery rate for protein identifications from Mascot database search results. *Molecular & Cellular Proteomics* 2005, 4, (6), 762-772.

21. Bendtsen, J.D.; Nielsen, H.; Heijne, G.v.; Brunak, S., Improved prediction of signal peptides: SignalP 3.0. *Journal of Molecular Biology* 2004, 340, (4), 783-795.

22. Bendtsen, J. D.; Jensen, L. J.; Blom, N.; von Heijne, G.; Brunak, S., Feature-based prediction of non-classical and leaderless protein secretion. *Protein Engineering Design and Selection* 2004; Vol. 17, (4), 349-356.

23. Yajima, W.; Kav, N. N., The proteome of the phytopathogenic fungus *Sclerotinia sclerotiorum*. *Proteomics* 2006, 6, (22), 5995-6007.

24. Nombela, C.; Gil, C.; Chaffin, W. L., Non-conventional protein secretion in yeast. *Trends in Microbiology* 2006, 14, (1), 15-21.

25. Bendtsen, J. D.; Kiemer, L.; Fausboll, A.; Brunak, S., Non-classical protein secretion in bacteria. *BMC Microbiology* 2005, 5, (1), 58.

26. Rolke, Y.; Liu, S.; Quidde, T.; Williamson, B.; Schouten, A.; Weltring, K. M.; Siewers, V.; Tenberge, K. B.; Tudzynski, B.; Tudzynski, P., Functional analysis of H_2O_2-generating systems in Botrytis cinerea: the major Cu-Zn-superoxide dismutase(BCSOD 1) contributes to virulence on French bean, whereas a glucose oxidase(BCGOD 1) is dispensable. *Molecular Plant Pathology* 2004, 5, (1), 17-27.

27. Ciechanover A., Intracellular protein degradation: from a vague idea thru the lysosome and the ubiquitin-proteasome system and into human disease and drug targeting. *Cell Death and Differentiation* 2005 12: 1178-1190.

28. Carvalho P.; Goder V.; Rapoport T., Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. *Cell*. 2006 126, 361-373.

29. Miclet, E,; Stoven V.; Michels, P.A.; Opperdoes, F.R.; Lallem, J.Y.; Duffieux, F., NMR spectroscopic analysis of the first two steps of the pentose-phosphate pathway elucidates the role of 6-phosphogluconolactonase. *J Biol Chem* 2001 14: 34840-34846.

30. Clarke J.L.; Scopes D.A.; Sodeinde O.; Mason P.J., Glucose-6-phosphate dehydrogenase-6-phosphogluconolactonase. A novel bifunctional enzyme in malaria parasites. *Eur J Biochem.* 2001, 268, 2013-2019.

31. Surolia N.; Krishnamurthy K.; Adiga P.R., Enzymic basis of deranged foetal flavin-nucleotide metabolism consequent on immunoneutralization of maternal riboflavin carrier protein in the pregnant rat. *Biochem. J.*1985 230, 363–367.

32. McGoldrick, S.; O'Sullivan, S. M.; Sheehan, D., Glutathione transferase-like proteins encoded in genomes of yeasts and fungi: insights into evolution of a multifunctional protein superfamily. *FEMS Microbiol. Lett* 2005, 242, 1-12.

33. Pazzagli, L.; Cappugi, G.; Manao, G.; Camici, G.; Santini, A.; Scala, A., Purification, characterization, and amino acid sequence of cerato-platanin, a new phytotoxic protein from *Ceratocystis fimbriata* f. sp. platani. *Journal of Biological Chemistry* 1999, 274, (35), 24959-24964.

34. Chagué, V.; Danit, L. V.; Siewers, V.; Gronover, C. S.; Tudzynski, P.; Tudzynski, B.; Sharon, A., Ethylene sensing and gene activation in *Botrytis cinerea*: A missing link in ethylene regulation of fungus-plant interactions? *Mol Plant Microbe Interact* 2006, 19, (1), 33-42.

35. Jeong, J. S.; Mitchell, T. K; Dean, R. A., The *Magnaporthe grisea* snodprot1 homolog, MSP1, is required for virulence. *FEMS Microbiology Letters* 2007, 273, 157–165

36. Brito, N.; Espino, J. J.; Gonzalez, C., The endo-ß-1, 4-xylanase Xyn11A is required for virulence in Botrytis cinerea. *Mol. Plant-Microbe Interact* 2006, 19, 25-32.

Table 4.1: Proteins identified from *B. cinerea* that have a predicted signal peptide when grown with the four different nutritional sources.

			Tomato		Blank		Strawberry		Arabidopsis	
Gene ID[a]	Protein Name/Function[b]	Signal P	Score[c]	%Cov[d]	Score[c]	%Cov[d]	Score[c]	%Cov[d]	Score[c]	%Cov[d]
Carbohydrate metabolism and transport										
BC1G_04151.1	Glucan 1,4-alpha-glucosidase	0.912 Y	435	12	575	17	346	8	960	25
BC1G_01617.1	Exo-polygalacturonase	0.996 Y	-	-	182	7	-	-	212	4
BC1G_01778.1	Endo 1-4 xylanase-D precursor	0.966 Y	-	-	167	6	-	-	-	-
BC1G_00594.1	Xyloglucan specific endo beta-1,4 glucanase	0.999 Y	-	-	158	9	-	-	-	-
BC1G_00617.1	Pectin methyl esterase	1.000 Y	156	4	138	4	152	4	186	4
BC1G_10221.1	Beta glucanase-like protein	0.816 Y	97	2	91	1	181	4	314	7
BC1G_11143.1	Endopolygalacturonase 1	1.000 Y	590	27	85	10	735	40	-	-
BC1G_12859.1	Alpha-glucosidase	1.000 Y	304	4	-	-	494	8	143	3
BC1G_08314.1	Cutinase	1.000 Y	275	17	-	-	-	-	-	-
BC1G_14030.1	Beta-1,3glucanosyltransferase	1.000 Y	144	3	-	-	202	5	57	2
BC1G_02333.1	Glucoamylase precursor	1.000 Y	-	-	-	-	-	-	69	3
BC1G_02936.1	Cutinase	1.000 Y	163	22	-	-	65	10	-	-
BC1G_09079.1	Beta-1,3 endoglucanase	0.999 Y	928	26	-	-	891	23	-	-
BC1G_07215.1	Family 20 chitobiase	0.999 Y	157	3	-	-	110	5	-	-
BC1G_13862.1	Glycosyl hydrolase family 45	0.999 Y	-	-	-	-	123	4	-	-
BC1G_11018.1	Alpha-1,6-mannanases	0.999 Y	-	-	-	-	159	8	-	-
BC1G_12017.1	Pectin lyase precursor	0.998 Y	176	4	-	-	-	-	-	-
BC1G_10455.1	1,3-beta-glucanosyltransferase Glycolipid anchored surface protein	0.998 Y	161	7	-	-	-	-	-	-
BC1G_00455.1	Alpha-mannosidase	0.998 Y	67	2	-	-	66	2	-	-
BC1G_08755.1	Glucan 1,4-alpha-glucosidase	0.997 Y	1004	22	-	-	1178	22	-	-
BC1G_02623.1	Alpha-amylase	0.994 Y	73	3	-	-	-	-	120	5
BC1G_10880.1	Cellobiohydrolase	0.992 Y	-	-	-	-	799	13	-	-
BC1G_02364.1	Glycosyl hydrolase family 32	0.991 Y	59	2	-	-	256	4	-	-
BC1G_07768.1	Acyl xylan esterase	0.963 Y	78	5	-	-	-	-	-	-
BC1G_00576.1	Endo-1,4-Beta-D-Xylanase.	1.000 Y	853	18	-	-	363	18	-	-
BC1G_07073.1	Malate Dehydrogenase	1.000 Y	-	-	-	-	98	8	-	-
BC1G_06035.1	Exocellulase precursor	0.999 Y	236	6	-	-	263	5	164	6
BC1G_11898.1	Glucosidase	0.999 Y	169	8	-	-	221	10	-	-
BC1G_02314.1	Extracellular phyate	0.995 Y	-	-	-	-	-	-	55	3
BC1G_02714.1	Glycosyl hydrolase Family 25 protein	0.992 Y	114	5	-	-	-	-	-	-
Miscellaneous										
BC1G_00198.1	Twin Arginine Translocation Pathway signal precursor	0.999 Y	88	6	258	8	190	8	262	8
BC1G_02965.1	Acid phosphatase	0.996Y	-	-	47	2	-	-	-	-
BC1G_04390.1	DnaK-type molecular chaperone BiP	0.982 Y	72	2	-	-	-	-	-	-
BC1G_02986.1	Phosphatidylglycerol / phosphatidylinositol transfer protein	0.999 Y	95	13	-	-	189	13	-	-
BC1G_02223.1	disulfide bond formation	0.999 Y	72	2	-	-	68	2	-	-
BC1G_11835.1	Multiple inositol polyphosphate phosphatase	0.978 Y	-	-	-	-	-	-	419	13
BC1G_08016.1	Ribosomal protein P2	0.919 Y	81	11	-	-	118	11	-	-
BC1G_07409.1	Malate dehydrogenase mitochondrial	0.569 Y	59	4	-	-	-	-	-	-
Peptidase										
BC1G_09564.1	Serine Carboxypeptidase	1.000 Y	-	-	340	9	130	4	247	8
BC1G_03070.1	Family Aspartyl protease	0.999 Y	-	-	197	12	-	-	55	3
BC1G_02944.1	Family S53 protease	0.999 Y	-	-	158	6	-	-	343	7
BC1G_12776.1	Fused 3 Protease precursor	1.000 Y	-	-	125	2	-	-	76	2
BC1G_06836.1	Proteases - Subtilase Family	0.999 Y	-	-	-	-	130	4	-	-
BC1G_01026.1	Tripeptidyl petidase A	0.975 Y	102	3	-	-	-	-	-	-
BC1G_03976.1	Peptidase	0.959 Y	51	2	-	-	-	-	-	-
BC1G_00978.1	Fused 3 Protease precursor	0.999 Y	-	-	-	-	100	2	-	-

Continuation of Table 4.1

			Tomato		Blank		Strawberry		Arabidopsis	
Gene ID[a]	Protein Name/Function[b]	Signal P	Score[c]	%Cov[d]	Score[c]	%Cov[d]	Score[c]	%Cov[d]	Score[c]	%Cov[d]
Pathogenecity factor										
BC1G_02163.1	Ceratoplatanin (domain)	0.996 Y	678	44	307	37	819	37	772	44
Hypothetical Proteins with unknown function										
BC1G_12374.1	Hypothetical protein	0.999 Y	274	15	107	6	117	6	262	15
BC1G_13335.1	Hypothetical protein	1.000 Y	158	8	-	-	93	5	-	-
BC1G_13938.1	Hypothetical protein	1.000 Y	-	-	-	-	82	8	-	-
BC1G_00896.1	Hypothetical protein	1.000 Y	194	20	-	-	194	23	-	-
BC1G_09892.1	Hypothetical protein	0.999Y	-	-	-	-	46	4	-	-
BC1G_14136.1	Hypothetical protein	0.999 Y	-	-	-	-	52	15	-	-
BC1G_02060.1	Hypothetical protein	0.999 Y	76	7	-	-	89	7	-	-
BC1G_08635.1	Hypothetical protein	0.999 Y	-	-	-	-	362	17	-	-
BC1G_15542.1	Hypothetical protein	0.999 Y	-	-	-	-	185	26	-	-
BC1G_05033.1	Hypothetical protein	0.998 Y	249	17	-	-	318	17	-	-
BC1G_11950.1	Hypothetical protein	0.997 Y	60	5	-	-	68	5	-	-
BC1G_06019.1	Hypothetical protein	0.762 Y	53	12	-	-	-	-	-	-
BC1G_08615.1	Hypothetical protein	0.962 Y	-	-	-	-	307	7	-	-

a Protein identification number provided by the broad institute Botrytis cinerea BO5.10 database

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

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

d Percentage ratio of all ammino acid from valid peptides matched to the total number of ammino acids in the protein

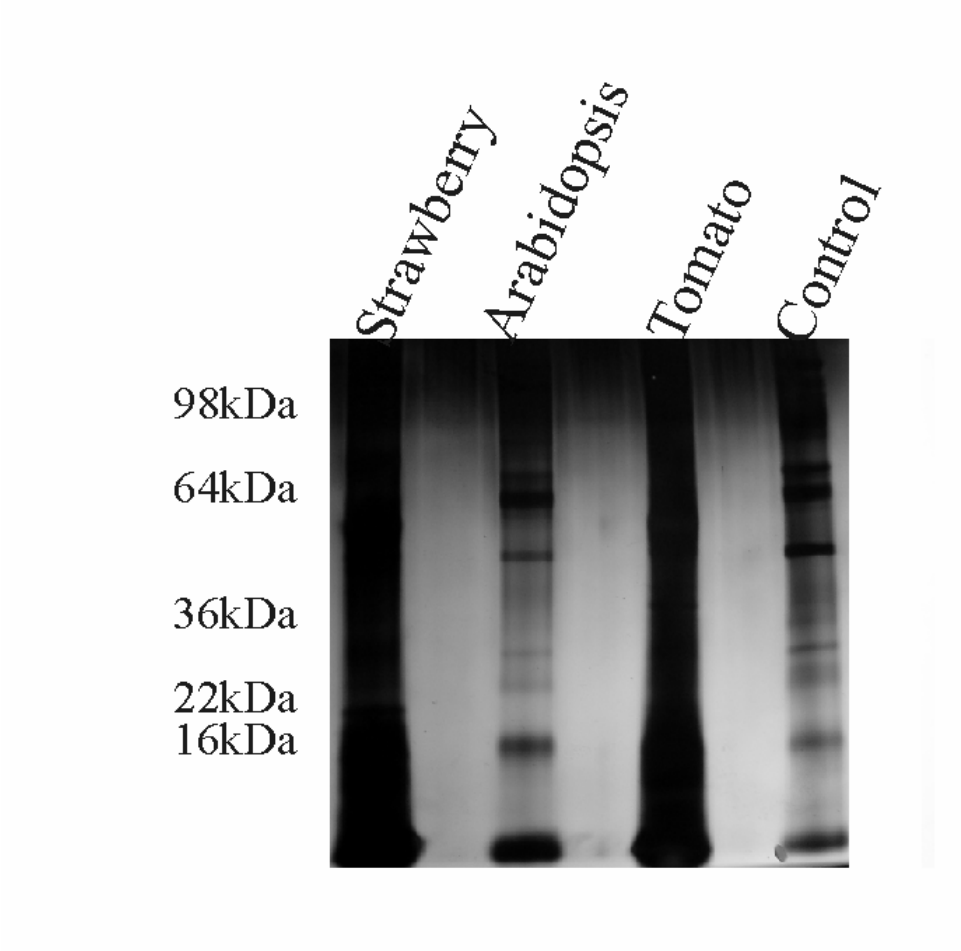

Figure 4.1: Silver stained one dimensional 4-12% gradient polyacrylamide SDS-PAGE analysis of the secreted proteins from *B. cinerea* when grown on cellophane membrane in contact with NY media supplemented with strawberry, Arabidopsis, tomato and non supplemented media. Secreted proteins were collected by floating the cellophane membrane in an acetate buffer solution. The equivalent of 1mL of collection buffer was concentrated and was loaded on to the gel in a total volume of 20 μL. Relative molecular weights from standards are given on the left side of the gel.

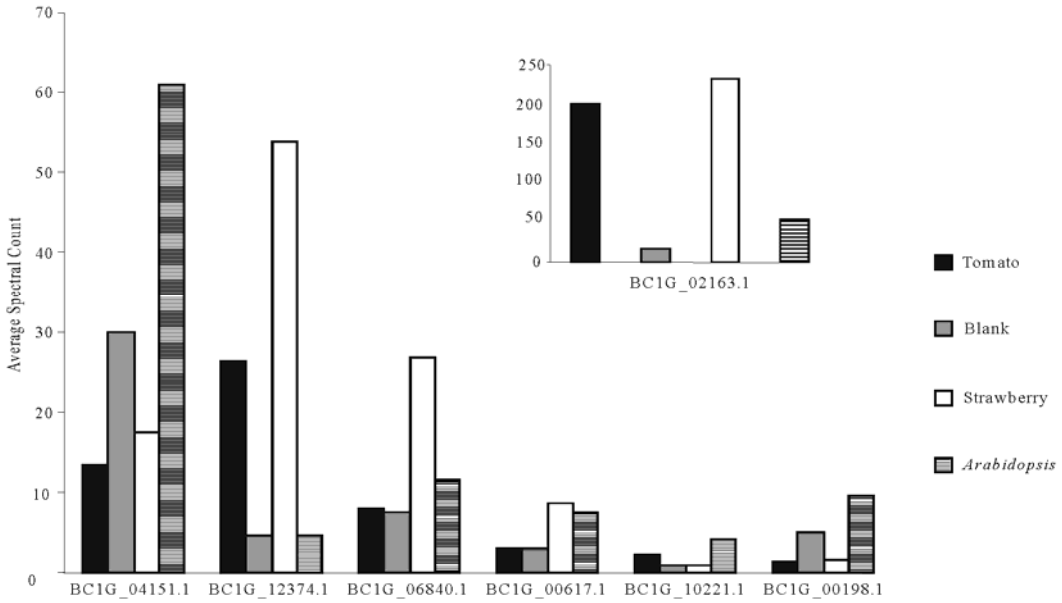

Figure 4.2: Graph showing the average spectral count for *B. cinerea* proteins identified in all four growth conditions (Arabidopsis leaf extract, strawberry fruit extract, tomato fruit extract and non supplemented media). Spectral counts were determined for proteins identified at 1 % False Discovery rate by using ProValT algorithm. Proteins from left to right, (BC1G_04151.1) putative glucan, 1-4, alpha glycosidase, (BC1G_12374.1) hypothetical protein, (BC1G_06840.1) putative pectin methyl esterase, (BC1G_00617.1) putative pectin methyl esterase, (BC1G_10221.1) putative beta-glucanase like protein, (BC1G_00198.1) hypothetical protein and on the top right corner (BC1G_02163.1) ceratoplatanin.

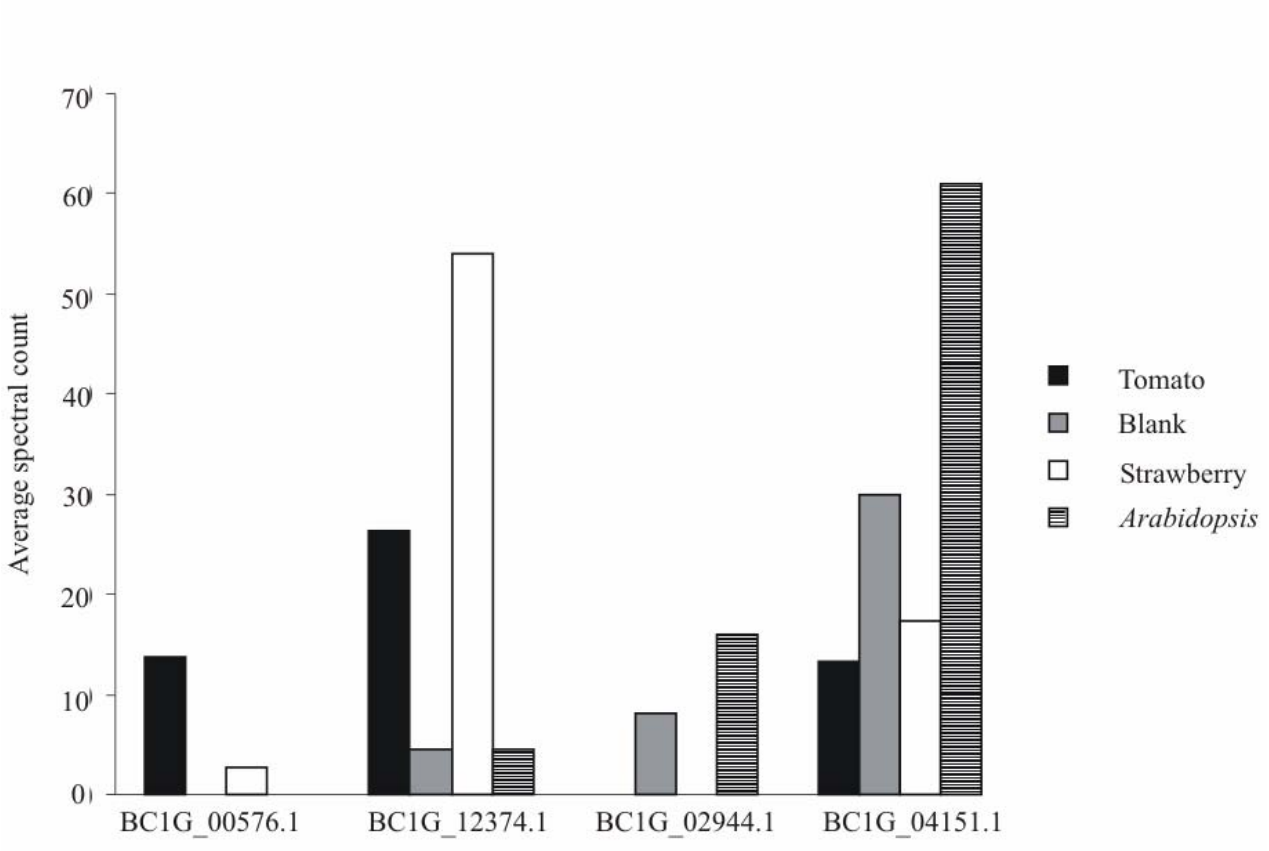

Figure 4.3: Graph showing the average spectral count for four *B. cinerea* proteins detected in different growth conditions (Arabidopsis leaf extract, strawberry fruit extract, tomato fruit extract or non supplemented media). Spectral counts were determined for proteins identified at 1 % False Discovery rate by using ProValT algorithm. Proteins from left to right (BC1G_00576.1) putative 1-4,beta xylanase, (BC1G_12374.1) hypothetical protein, (BC1G_02944.1) putative peptidase, (BC1G_04151.1) putative glucan,1-4,alpha glycosidase.

CHAPTER 5

A PROTEOMIC STUDY OF PECTIN DEGRADING ENZYMES SECRETED FROM *BOTRYIS CINEREA* GROWN IN LIQUID CULTURE[1]

[1] Punit Shah, Gerardo Gutierrez-Sanchez, Ron Orlando and Carl Bergmann. To be submitted to *Molecular Plant-Microbe Interactions.*

ABSTRACT

The enzymes secreted by *Botrytis cinerea* play an important role in the successful colonization of a host plant by this fungal plant pathogen. Some of the secreted enzymes are involved in the degradation of pectin, a major component of the plant cell wall. In this study, a total of 124 proteins secreted by *B. cinerea* were identified by growing the fungus on highly or partially esterified pectin, or in sucrose in liquid culture. Sixty-seven common proteins were identified in each of the growth conditions, out of which 50 proteins exhibited a Signal P motif. Thirteen *B. cinerea* enzymes with functions related to pectin degradation were identified in both pectin growth conditions. Only four *B. cinerea* pectin degradation enzymes were identified in sucrose. Identifying the reaction of the fungus to various nutritional sources is expected to help elucidate the mechanism by which the fungus is able to infect more than 200 different plant species.

INTRODUCTION

Botrytis 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 (Williamson *et al.*, 2007). On certain fruit hosts it has been reported that *B. cinerea* initially infects while the fruits are green and remains dormant (Prusky, 1996; Prins *et al.*, 2000; Adaskaveg *et al.*, 2000). This quiescent infection is superceded by a resumption of fungal growth activity once the fruit ripens (Adaskaveg *et al.*, 2000; Prins *et al.*, 2000; Prusky, 1996). 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-esterification and depolymerization of the cell wall components, resulting in softening of the fruit (Brownleader *et al.*, 1999; Prasanna, 2007). 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 (Willats *et al.*, 2001). The galacturonic acid is highly esterified in the pectin of unripened fruit (Steele *et al.*, 1997). 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 (Prasanna, 2007; Reeve, 1959; Steele *et al.*, 1997; Wakabayashi *et al.*, 2003). *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, exopolygalacturonases, endopolygalacturonases, pectate and pectin lyases, and rhamnogalacturonan lyase and hydrolase (Chen *et al.*, 1997; Kapat *et al.*, 1998; Schols *et al.*, 1990; Wubben *et al.*, 2000). The genome of *B. cinerea* has multiple isoforms for most of the above enzymes. Six isoforms for endopolygalacturonase have been previously reported (Wubben *et al.*, 1999). During plant pathogen interaction, oligogalacturonides are produced from pectin which acts as defense elicitors. In *Fragaria vesca,* a partial degree of demethylation of oligogalacturonides is required for eliciting defense responses to *B. cinera* infection (Osorio *et al.*, 2008). 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 (Lionetti *et al.*, 2007). To better understand at the molecular level the complex interaction between plant 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. 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 on 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.

EXPERIMENTAL PROCEDURES

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), $(NH_4)_2SO4_2$ (1.20 g), NaCl (0.15 g), $CaCl_2{\cdot}H_2O$ (0.40 g), $MgSO_4$ (0.150 g), $FeSO_4{\cdot}7H_2O$ (0.015 mg), $ZnSO_4{\cdot}7H_2O$ (0.015 g), and $MnSO_4{\cdot}H_2O$ (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 1-D 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 95ºC 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 (1x) 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 (1x1 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^{o}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^{o}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 (10.5cm x 100μm 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. Dynamic exclusion was enabled for 160 sec. Raw tandem mass spectra were converted into a peak list using ReAdW followed by mzMXL2Other algorithms (Pedrioli *et al.*, 2004). 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 (Weatherly *et al.*, 2005).

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 (Bendtsen *et al.*, 2004).

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. 1D SDS-PAGE was used for the separation of the *B. cinerea* secreted proteins. Analysis of the 1D SDS-PAGE revealed the presence of numerous proteins (Figure 5.1). 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 in *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

A comprehensive study of the proteins secreted by *B. cinerea* was performed using shotgun proteomics. Three segments from each lane of the 1D SDS-PAGE collected from each culture were digested in-gel and peptides were subjected to LC-MSMS analysis. Each fraction

was analyzed in duplicate. At a 1% false discovery rate (FDR), a total of 124 protein groups were identified (Supplementary Information Table 1), indicating that at least 124 *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 1% FDR, *B. cinerea* grown on PE pectin produced 104 *B. cinerea* proteins identified by 822 MS/MS spectra, while *B. cinerea* grown on HE pectin or on sucrose produced 97 and 88 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. The putative function was used to classify the proteins into different categories: carbohydrate metabolism and transport, peptidases, hypothetical and others. More than half of the identified proteins belonged to the category of carbohydrate metabolism and transport. Thirty-two identified proteins did not match with any annotated protein with sufficient homology to assign a putative function and were classified as hypothetical proteins.

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 86 proteins were secreted out of the 124 (Supplementary Information Table 1) total identified proteins. Of the 67 common proteins identified in all three growth conditions (Table 5.1), 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 5.2). The pectinases included three PMEs, three PLs (define), two endo PGs, two exo PGs, and two PGs with unknown specificity (could be either endo or exo). 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 on either pectin source but not when grown on 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. The three pectin degrading enzymes identified in the sucrose growth medium following *B. cinerea* growth 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 (Old *et al.,* 2005). Only one *B. cinerea* PME was identified as secreted when *B. cinerea* was grown on sucrose and it had a similar number of spectral counts compared to the cultures grown on 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.

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 on potato dextrose in liquid culture (Medina *et al.*, 2005). Although fruit, which serves as a natural host for *B. cinerea,* is a much more complex environment than a sole 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 this case, we have been studying secreted proteins, which change both in terms of number and in overall concentration of secreted proteins depending on the growth condition. 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. The dynamic range of the mass spectrometer may play an important role in such a method. If the protein secreted by the fungus in different systems results in too large a change in concentration, then 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.

Fortunately, 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 124 *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, 30 of these were classified as carbohydrate metabolism and transport proteins, 11 as hypothetical proteins, two as peptidases, and seven others.

Pectin degrading enzymes

Pectinases play an important role in cell wall degradation and successful invasion. Endopolygalacturonases (EPGs) are one of the most widely studied classes of pectin degrading enzymes. EPGs hydrolyze the internal (1-4) linkage between D-galacturonic acid units of pectin (Valette-Collet *et al.*, 2003). Previously, six EPGs have been identified from *B.* cinerea (van Kan, 2006). However, it has been suggested that *B. cinerea* can secrete up to 13 EPG isoenzymes (ten Have *et al.*, 2001). The secretion of different isoforms can be explained by differences in substrate, pH, and environmental conditions (Wubben et al., 2000). The deletion mutants of both BcPG1 and BcPG2 showed reduced virulence on multiple hosts (van Kan, 2006). In this study we identified endoPG 2 (BC1G_13667.1) and endoPG 6 (BC1G_08033.1) when the fungus was grown on either HE or PE pectin as a carbon source in liquid culture. The absence of all EPG isoforms following growth in sucrose indicates the secretion of EPGs is carbon source dependant. This is consistent with previous work demonstrating that the expression of the endo PG gene family has a differential pattern and depends on host tissue, stage of infection and temperature (ten Have *et al.*, 1998). There was no significant change in the secretion of EPGs 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 (Wubben et al., 2000). 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 (Xie *et al.*, 2005), 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 (De Lorenzo *et al.*, 2001, Johnston *et al. 1994*).

Pectin methylesterases (PMEs) de-esterify pectin, releasing methanol and polygalacturonic acid. This de-esterification facilitates the subsequent action of polygalacturonases and pectate lyases (Valette-Collet et al., 2003). On certain hosts, BCPME1 has been previously shown to be an essential determinant of *B. cinerea* virulence (Valette-Collet et al., 2003, van Kan, 2006). 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.

Pectate lyase cleaves polygalacturonic acid into oligogalacturonides via beta-elimination (Valette-Collet et al., 2003), 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 in 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 exopolygalacturonases, 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 on both PE and HE pectin. Two other polygalacturonases, 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 (Chen et al., 1997).

When grown in the sucrose media, *B. cinerea* produced 11 proteins which were not observed in the other media. Of these 11 proteins, five (BC1G_00882.1, BC1G_14570.1, BC1G_05168.1, BC1G_10120.1 and BC1G_15832.1) were classified as hypothetical proteins. However, of the 11 proteins, two were alpha amylases (BC1G_02623.1 and BC1G_02333.1), 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 a catalase (BC1G_12856.1), and one was a Cu/Zn superoxide dismutase (BC1G_00558.1). Cu/Zn superoxide dismutase and catalase are involved in oxidation and reduction.

The secretome of *B. cinerea* in sucrose growth medium has similar characteristics compared to secretomes in pectin media, considering that almost three-quarters of proteins secreted by *B. cinerea* in sucrose are common with proteins found in the pectin growth media. However, in sucrose, 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 124 proteins identified in culture filtrates following fungal growth, only 85 proteins had a signal peptide motif according to the signalP algorithm (Bendtsen et al., 2004b). The predicted N-terminal motif indicating that the "protein is secreted" is frequently used to confirm the extracellular nature of the proteins identified. The absence of the predicted N-terminal motif in 39 proteins indicates that these might be intercellular proteins resulting from cell lysis. The identification of internal proteins in secretome studies due to cell lysis is an inherent feature of secretome proteomics, although 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 signal peptide and hence predicted not to be secreted by the algorithm. 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* (Nombela *et al.*, 2006, Bendtsen *et al.*, 2005).

Although most of the proteins identified from the *B. cinerea* B05.10 database are hypothetical proteins with unknown function, 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 124 *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 does not depend only on the changes in the 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.

ACKNOWLEDGEMENTS

This work was supported in part by NSF “Systems Biology Approach to Tomato Fruit Susceptibility to a Necrotrophic Fungus” (IOB-0544504), DOE “Structures and Functions of Oligosaccharins" (DE-FG02-96ER20221), the Center for Plant and Microbial Complex Carbohydrates (DEFG0593ER20097), and the NIH/NCRR Integrated Technology Resource for Biomedical Glycomics (P41 RR018502).

REFRENCES

1. Adaskaveg, J.E., Foerster, H., and Thompson, D.F. (2000) Identification and etiology of visible quiescent infections of *Monilinia fructicola* and *Botrytis cinerea* in sweet cherry fruit. *Plant Dis* 84: 328-333.

2. Bendtsen, J.D., Kiemer, L., Fausboll, A., and Brunak S. (2005) Non-classical protein secretion in bacteria. *BMC Microbiol* 5: 58.

3. Bendtsen, J.D., Nielsen, H., von Heijne, G., and Brunak, S. (2004) Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* 340: 783-795.

4. Brownleader, M., Jackson, P., Mobasheri, A., Pantelides A, Sumar S., Trevan, M., and Dey, P. (1999) Molecular aspects of cell wall modifications during fruit ripening. *Crit Rev Food Sci Nutr* 39: 149-164.

5. Chen, H.J., Smith, D.L., Starrett, D.A., Zhou, D., Tucker, M.L., Solomos, T., and Gross, K.C. (1997) Cloning and characterization of a rhamnogalacturonan hydrolase gene from *Botrytis cinerea*. *IUBMB Life* 43: 823-838.

6. De Lorenzo, G., D'Ovidio, R., and Cervone, F. (2001) The role of polygalacturonase-1 inhibiting proteins (PGIPs) in defense against pathogenic fungi. *Ann Rev Phytopathol* 39: 313-335.

7. Johnston, D.J., Williamson, D., and McMillan, G.P. (1994) The interaction *in planta* of polygalacturonases from *Botrytis cinerea* with a cell wall-bound polygalacturonase-inhibiting protein (PGIP) in raspberry fruits. *J Exp Botany* 45: 1837-1843.

8. Kapat, A., Zimand, G., and Elad, Y. (1998) Biosynthesis of pathogenicity hydrolytic enzymes by *Botrytis cinerea* during infection of bean leaves and *in vitro*. *Mycol Res* 102: 1017-1024.

9. Lionetti, V., Raiola, A., Camardella L., Giovane, A., Obel, N., Pauly, M., Favaron, F., Cervone, F., and Bellincampi, D. (2007) Overexpression of pectin methylesterase inhibitors in Arabidopsis restricts fungal infection by *Botrytis cinerea*. *Plant Physiol* 143: 1871-1880.

10. Medina, M.L., Haynes, P.A., Breci, L., and Francisco, W.A. (2005) Analysis of secreted proteins from *Aspergillus flavus*. *Proteomics* 5: 3153-3161.

11. Nombela, C., Gil, C., and Chaffin, L.J. (2006) Non-conventional protein secretionin yeast. *Trends Microbiol* 14: 15-21.

12. Old, W.M., Meyer-Arendt, K., Aveline-Wolf, L., Pierce, K.G., Mendoza, A., Sevinsky, J.R., Resing, K.A., and Ahn, N.G. (2005) Comparison of label-free methods for quantifying human proteins by shotgun proteomics *Mol Cell Proteomics* 4: 1487-1502.

13. Osorio, S., Castillejo, C., Quesada, M.A., Medina-Escobar, N., Brownsey, G.J., Suau, R., Heredia, A., Botella, M.A., and Valpuesta, V. (2008) Partial demethylation of oligogalacturonides by pectin methyl esterase 1 is required for eliciting defence responses in wild strawberry (*Fragaria vesca*). *Plant J* 54: 43-55.

14. Pedrioli, P.G.A., Eng, J.K., Hubley, R., Vogelzang, M., Deutsch, E.W., Raught, B., Pratt, B., Nilsson, E., Angeletti, R.H., and Apweiler, R. (2004) A common open representation of mass spectrometry data and its application to proteomics research. *Nature Biotechnol* 22: 1459-1466.

15. Prasanna, V. (2007) Fruit ripening phenomena–An overview. *Crit Rev Food Sci Nutr* 47: 1-19.

16. Prins, T.W., Tudzynski, P., Von, A., ten Have, M.E.H., Tenberge, K., Al Van Kan, J.A.N. (2000) Infection stratergies of *Botrytis cinerea* and related necrotrophic pathogens. *Fungal Pathol* VOL: page #s.

17. Prusky, D. (1996) Pathogens quisescence in postharvest diseases. *Ann Rev Phytopathol* 34: 413-434.

18. Reeve, R.M. (1959) Histological and histochemical changes in developing and ripening peaches. II. The cell walls and pectins. *Am J Bot* 46: 241-248.

19. Schols, H.A., Geraeds, C., Searle-Van Leeuwen, M.F., Kormelink, F.J.M, and Voragen, A.G.J. (1990) Rhamnogalacturonase: a novel enzyme that degrades the hairy regions of pectins. *Carbohydr Res* 206: 105-115.

20. Steele, N.M., McCann, M.C., and Roberts, K. (1997) Pectin modification in cell walls of ripening tomatoes occurs in distinct domains. *Plant Physiol* 114: 373-381.

21. ten Have, A., Breuil, W.O., Wubben, J.P., Visser, J., and van Kan, J.A.L. (2001) *Botrytis cinerea* endopolygalacturonase genes Are differentially expressed in various plant tissues. *Fungal Gen Biol* 33: 97-105.

22. ten Have, A., Mulder, W., Visser, J., and van Kan, J.A.L. (1998) The endopolygalacturonase gene Bcpg1 is required for full virulence of *Botrytis cinerea*. *Mol Plant-Microbe Interact* 11: 1009-1016.

23. Valette-Collet, O., Cimerman, A., Reignault, P., Levis, C., Boccara, M. (2003) Disruption of *Botrytis cinerea* pectin methylesterase gene Bcpme 1 reduces virulence on several host plants. *Mol Plant-Microbe Interact* 16: 360-367.

24. van Kan, J.A.L. (2006) Licensed to kill: the lifestyle of a necrotrophic plant pathogen. *Trends Plant Sci* 11: 247-253.

25. Wakabayashi, K., Hoson, T., Huber, D.J. (2003) Methyl de-esterification as a major factor regulating the extent of pectin depolymerization during fruit ripening: a comparison of the action of avocado (*Persea americana*) and tomato (*Lycopersicon esculentum*) polygalacturonases. *J Plant Physiol* 160: 667-673.

26. Weatherly, D. B., Atwood, J.A., Minning, T.A., Cavola, C., Tarleton, R.L., and Orlando, R. (2005) A heuristic method for assigning a false-discovery rate for protein identifications from Mascot database search results. *Mol Cell Proteomics* 4: 762-772.

27. Willats, W.G.T., McCartney, L., Mackie, W., and Knox, J.P. (2001) Pectin: cell biology and prospects for functional analysis. *Plant Mol Biol* 47: 9-27.

28. Williamson, B., Tudzynski, B., Tudzynski, P., Van, K.A.N., and Jan, A.L. (2007) *Botrytis cinerea*: the cause of grey mould disease. *Mol Plant Pathol* 8: 561-580.

29. Wubben, J.P., Mulder, W., ten Have, A., van Kan, J.A.L., and Visser, J. (1999) Cloning and partial characterization of endopolygalacturonase genes from *Botrytis cinerea*. *App Environ Microbiol* 65: 1596-1602.

30. Wubben, J.P., ten Have, A., van Kan, J.A.L., and Visser, J. (2000) Regulation of endopolygalacturonase gene expression in *Botrytis cinerea* by galacturonic acid, ambient pH and carbon catabolite repression. *Curr Gen* 37: 152-157.

31. Xie, M., Krooshof, G.H., Benen, J.A.E., Atwood III, J.A., King, D., Bergmann, C., and Orlando, R. (2005) Post-translational modifications of recombinant *B. cinerea* EPG 6. *Rapid Commun Mass Spectrom* 19: 3389-3397.

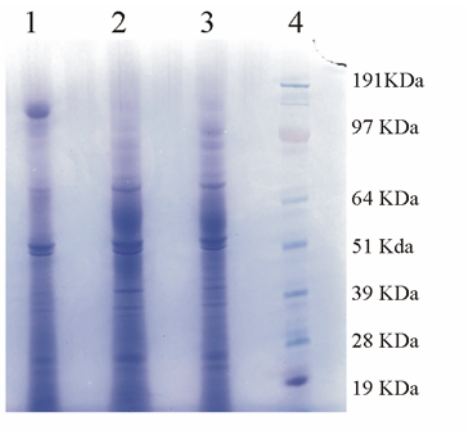

Figure 5.1: Commassie Blue stained 1-D SDS-PAGE analysis of the secreted proteins from *B. cinerea* when grown on liquid cultures with HE pectin, PE pectin and sucrose as the carbon source. Proteins *B. cinerea* secretes when grown on sucrose (Lane 1), on HE pectin, (Lane 2), and on PE pectin (Lane 3). Molecular weight standards are shown in Lane 4.

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[c]	Spectra[e]	Score[c]	Spectra[e]
BC1G_13137.1	Polygalacturonase	0.986Y	492.13	12	791.28	16	73.04	1
BC1G_00617.1	Pectin methylesterase	1.000Y	247.49	6	296.65	11	122.37	2
BC1G_06546.1	Alpha-galactosidase precursor (Melibiase)	0.999Y	247.44	7	47.29	1	129.34	2
BC1G_07622.1	beta-glucosidase	0.995Y	523.25	13	396.44	5	461.52	11
BC1G_07319.1	1,3-beta glucanase	0.999Y	792.22	16	808.75	17	532.25	9
BC1G_10455.1	1,3-beta-glucanosyltransferase Gel1	0.998Y	254.54	4	172.44	3	263.19	10
BC1G_12859.1	alpha-glucosidase precursor	1.000Y	443.98	10	559.85	10	552.39	19
BC1G_04994.1	alpha-L-arabinofuranosidase	0.999Y	816.83	15	460.12	10	50.56	2
BC1G_08372.1	alpha-L-arabinofuranosidase A	0.995Y	100.63	2	336.39	4	102.4	1
BC1G_06328.1	alpha-L-rhamnosidase	0.992Y	218.77	6	265.45	5	231.9	6
BC1G_14030.1	beta-1-3-glucanosyltransferase	1.000Y	409.21	12	525.03	10	420.58	9
BC1G_10247.1	beta-fructofuranosidase	1.000Y	518.21	11	331.52	6	57.64	2
BC1G_03567.1	beta-galactosidase	0.998Y	792.51	17	309.73	6	266.3	6
BC1G_02364.1	beta-glucosidase	0.991Y	608.27	16	602.61	9	273.37	5
BC1G_10221.1	beta-glucosidase 1 precursor	0.816Y	1203.2	29	651.09	13	1544.3	48
BC1G_13690.1	Cell wall glycosyl hydrolase YteR, putative	0.998Y	243.78	4	495.08	13	110.69	3
BC1G_06509.1	Chitin binding protein	1.000Y	307.19	5	308.5	4	572.64	11
BC1G_00594.1	Endoglucanase	0.999Y	586.93	28	516.98	26	290.77	9
BC1G_13938.1	Exo-arabinanase	1.000Y	396.29	10	604.94	15	92.18	2
BC1G_01033.1	Exo-beta-1,3-glucanase	0.929Y	69.41	1	137.36	3	67.03	1
BC1G_01617.1	Exo-polygalacturonase	0.996Y	1099	29	833.38	33	116.36	2
BC1G_07215.1	Family 20 chitobiase	0.999Y	170.5	3	99.4	2	141.94	3
BC1G_11018.1	Family of alpha-1,6-mannanases	0.999Y	480.62	7	411.05	9	631.97	11
BC1G_00409.1	Glcosyl transferase / cell wall glucanase	1.000Y	347.29	6	271.42	7	246.29	4
BC1G_08755.1	Glucoamylase P precursor	0.997Y	359.15	7	126.38	2	366.87	6
BC1G_11898.1	Glucosidase	0.999Y	507.88	12	767.12	17	748.96	12
BC1G_12132.1	Glycosyl hydrolase family 65 protein	0.997Y	152.95	4	186.69	4	286.58	5
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	1016.6	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	1004.4	21	736.07	15	1005.6	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

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

Table 2. Results of the pectinases identified in the current study.

Gene Id[a]	Putative function[b]	Signal P[c]	LE Pectin		HE Pectin		Sucrose	
			Score[d]	Spectra[e]	Score[d]	Spectra[e]	Score[c]	Spectra[e]
BC1G_00617.1	Pectin methylesterase	1.000Y	247.49	6	296.7	11	122.4	2
BC1G_03464.1	Rhamnogalacturonase	1.000Y	59.08	1	142.6	8	-	-
BC1G_13367.1	Endopolygalacturonase 2	1.000Y	179.84	6	53.75	2	-	-
BC1G_09000.1	Pectate lyase	1.000Y	141.08	3	160.5	2	-	-
BC1G_12517.1	Pectate lyase, putative	1.000Y	44.42	1	55.61	1	-	-
BC1G_01617.1	Exo-polygalacturonase	0.996Y	1099	29	833.4	33	116.4	2
BC1G_07052.1	Pectate lyase	0.997Y	-	-	384.5	9	-	-
BC1G_00240.1	Similar to exopolygalacturonase	0.990Y	389.71	5	143.4	2	-	-
BC1G_13137.1	Polygalacturonase	0.986Y	492.13	12	791.3	16	73.04	1
BC1G_06840.1	Pectin methyl esterase	0.232N	332.97	8	436.9	13	363.3	7
BC1G_07496.1	Polygalacturonase	0.000N	106.26	2	230.5	5	-	-
BC1G_00799.1	Pectin methylesterase	0.002N	204.22	6	225.5	5	-	-
BC1G_08033.1	Polygalacturonase 6	0.999Y	712.92	23	564.8	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

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

CHAPTER 6

CONCLUSIONS

The overall purpose of this study was to identify many *B. cinerea* secreted proteins with a combination of mass spectrometry and bioinformatic softwares. The conclusion for Chapters 3, 4 and 5 are the following:

Chapter 3: The secretome of *Botrytis cinerea* was determined with mock infection of full red strawberry extract. *B. cinerea* was grown for 4-5 days on a nylon film overlaid on Petri dishes containing growth media and 4% red strawberry pulp. Afterwards, the membranes were transferred to Petri dishes containing acetate buffer for collection of secreted proteins. Each sample was trypsin digested and analyzed using Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS). A total of 148 unique proteins were identified. Fifty-nine proteins had predicted classical signal-P motif and 38 had non-classical signals. Twenty-seven proteins were catabolic, 32 hypothetical and 10 involved in proteolysis. *To date*, this is the first reported application of LC-MS/MS to perform a direct analysis of protein secretions from the filamentous fungi *B. cinerea.*

Chapter 4: The method developed to study the *B. cinerea* secretome used in Chapter 3 was applied to determine changes in the *B. cinerea* secretome from mock interaction with different host extracts of strawberry fruit, tomato fruit and *Arabidopsis* leaves. In the presence rich culture conditions such as those present on red strawberry extract and ripe tomato extract; it was observed that *B. cinerea* increases protein secretion when compared to *Arabidopsis* leaves extract. This study has also provided a pool of potentially important proteins with unknown functions which require further biological study.

Chapter 5: *B. cinerea* was grown on liquid cultures with highly esterified pectin, partially esterified pectin and sucrose as sole carbon sources. A total of 124 *B. cinerea* proteins were identified in three culture conditions which differed by the carbon nutrients. Sixty-seven proteins

were observed to be common in all three growth conditions. Among these proteins, 50 had an N-terminal signal peptide motif, confirming their secretion. The growth of *B. cinerea* and the secretion of proteins were similar in cultures containing differently esterified pectins but differed in sucrose medium growth conditions. The secretion of most of the pectinases depended on the carbon substrate used by the fungus for growth.

A total of 278 *B. cinerea* proteins were identified in the current study. Ten *B. cinerea* proteins were identified to be secreted constitutively in nutritionally rich growth conditions.