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Purification and phytotoxic analysis of ceratoplatanin proteins in Botrytis cinerea

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PURIFICATION AND PHYTOTOXIC ANALYSIS OF CERATOPLATANIN PROTEINS IN *BOTRYTIS CINEREA*

by

SUBODH RATHI

A THESIS

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for the degree of Master of Science
in
The Department of Biological Sciences
to
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We, the undersigned members of the Graduate Faculty of The University of Alabama in Huntsville, certify that we have advised and/or supervised the candidate on the work described in this thesis. We further certify that we have reviewed the thesis manuscript and approve it in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences.

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ABSTRACT
The School of Graduate Studies
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Title Purfication and phytotoxic analysis of Ceratoplatanin proteins in *Botrytis cinerea*

Botrytis cinerea is a necrotrophic fungus infecting over 200 species of plants world-wide. Proteins secreted during *B.cinerea* infection are important for both the initiation and spread of disease. A proteomic analysis using LC-MS identified a number of potential target proteins possibly involved in plant-pathogen interaction; one of which, BcSnod1 is abundantly expressed upon exposure to media containing 4% full red strawberry extract. BcSnod1 (12 kDa) is a member of a larger ceratoplatanin family of small proteins with four conserved cysteine residues, which are involved in inducing local and systemic defense responses in host organisms, human or plants. To determine the role of BcSnod1 protein in pathogenesis, the BcSnod1 gene was cloned and over expressed in *Escherichia coli* and *Pichia pastoris* expression systems. Recombinant protein was purified using Ni-NTA Column chromatography and HPLC respectively. The purified recombinant protein obtained from *Pichai pastoris* when injected in tomato leaves resulted in cell death at the point of infiltration and cell death around the point of infiltration. In tobacco leaves, the cell death is only observed at the point of infiltration. Recombinant protein obtained from *Escherichia coli* has an inconsistent and reduced cell death. This study concludes that BcSnod1 is phytotoxic in nature and may play an important role in initiation and spread of *Botrytis cinerea* infection.

Abstract Approval:

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LIST OF ABBREVIATIONS

µg/mL	microgram(s) per milliliter(s)
µL	microliter(s)
BcSnod1	*Botrytis cinerea* Snod like protein (BC1G_02163.1)
BcSnod2	*Botrytis cinerea* Snod like protein (BC1G_08735.1)
bp	basepair(s)
dpi	days post infiltration
His tag	histidine tag used for purification of protein
IPTG	isopropyl β-D-1-thiogalactopyranoside
kDa	Kilo Dalton(s)
M	Molar
mL	milliliter(s)
mM	millimolar
PCD	Programmed cell death
pETS1	pET28b vector containing BcSnod1 gene
pETS2	pET28b vector containing BcSnod2 gene
pGBcS1	pGemTEasy plasmid containing BcSnod1 gene
pGBcS2	pGemTEasy plasmid containing BcSnod2 gene
pPBcS1	pPic9k vector containing BcSnod1 gene
ROS	Reactive oxygen species
TSL	total soluble lysate

CHAPTER I

INTRODUCTION

A. Introduction to *Botrytis cinerea*

Botrytis cinerea is a necrotrophic plant pathogen, causing damage to almost 235 plant species worldwide (Elad *et al*., 2007). *B. cinerea* is also known as Grey Mold due to its property of producing a large number of spores which are a threat to crops. It can infect vegetables, ornamental plants, fruits and cereals but is noticed for the economic damage caused to grapes. Even though *B. cinerea* grows rapidly in cool summer climatic conditions, it can grow in varied environmental conditions which include dry or humid, cold temperature or heat. *B. cinerea* not only affects the growing crop but can be quiescent in the product and can cause heavy losses during the post harvest period (Brooks and Cooley, 1917). It is proposed that *B. cinerea* enters a host when it is young and remains quiescent and then becomes active when the environment is conducive or plant physiology changes (Adaskaveg *et al*., 2000; Williamson, 1994; Elad, 1997). The complete genome sequence of *B. cinerea* (42.66 Mb) is now available from the Broad Institute and T4 genome database (as of May 2011). The economic losses caused by *B. cinerea* are estimated to be around $2 billion per annum (Elmer and Michailides, 2007). It causes serious damage to mature or senescent leaves in diclotyledons & Noble Rot.

The only economically beneficial outcome of *B. cinerea* is in the wine industry which is known as the noble rot. When the infection of *B. cinerea* is slow on grapes, it causes slow decay of the berries, which results in shrinkage of berries due to sequestering of the natural juices, giving a novel taste to the wine prepared from those grapes. Due to the slow decay induced by *B. cinerea*, phytoalexins are secreted from the grapes at the site of infection and these phytoalexins are responsible for the specific flavor of wine.

B. cinerea belongs to Kingdom Fungi. It has been classified into Phylum Ascomycota based on the characteristic of producing spores in long cells known as ascus. *B. cinerea* is subclassified in subphylum Pezizomycotina, class Leotiomycetes and order Helotiales. It belongs to family Sclerotiniaceae and genus *Botryotinia*. The genus consists of 22 species and is divided into two branches, one of which is strictly pathogenic to dicots while one is pathogenic to both monocots and dicots. *B.cinerea* strain B05.10 is pathogenic for dicots as revealed by a study done by Staats *et al*. (2005).

B. cinerea is dark brown to black in color and hence known as Grey Mold. It has a characteristic conidia and branching tree like conidiophores. *B. cinerea* can reproduce sexually and asexually. Asexual reproduction is mostly common in *B. cinerea* and is known by its anomorphic name while the telomorphic name is *Botryotinia fuckeliana* which is rarely found (Faretra *et al*., 1988). Humidity favors the growth of this fungus. The life cycle starts in spring with wet and humid weather. *B. cinerea* grows on plants, tissues, fruits, etc., of the host and keeps on producing conidia; if by chance the condition becomes unfavorable, then it undergoes formation of a hard rock like structure known as sclerotia which are clumped mycelia which may be observed on dead plant tissue (Coley-Smith, 1980). Sclerotia can survive a variety of the worst environmental conditions and

start to grow again in spring releasing spores. Some precautions like adequate air circulation and overhead watering are practiced to avoid infectious spread of disease.

The cell wall is fundamental for the viability and pathogenecity of fungus. It is important as it determines the fungal morphology, material exchange and host penetration. *B. cinerea* adheres to a host by an extracellular matrix which consists of carbohydrates-20%, proteins-28% and lipids-6% (Doss *et al*., 2003). Proteins present in the extracellular matrix play an important role in the initial establishment of the colony and further spread of diseases. Other than this, melanin is found abundantly in the extracellular matrix which gives *B. cinerea* its grey color.

B. Ecology and Epidemiology

Botrytis cinerea produces various structures of life forms which are mycelium, hyphae, conidia, chlamydospores, sclerotia. These life forms have different viabilities depending on the environmental factors, out of which sclerotia have the highest viability and can be viable for almost 16 months. Primary infection starts from the inoculums from the last session which basically comes from sclerotia in vine and may differ in different crops. *B. cinerea* has three stages for infection which are establishment, containment or arrest phase and resumption of active growth (Wade and Cruickshank, 1992; Latunde-Dada, 2001). The primary stage starts with the infection of host during which *B. cinerea* develops a colony and may start infection in the host if the conditions are favorable or may go in an inactive stage or quiescence (Williamson *et al*., 2007). It can stay in a latent phase in host for a long period and can return to an infectious stage and start to proliferate and produce conidia.

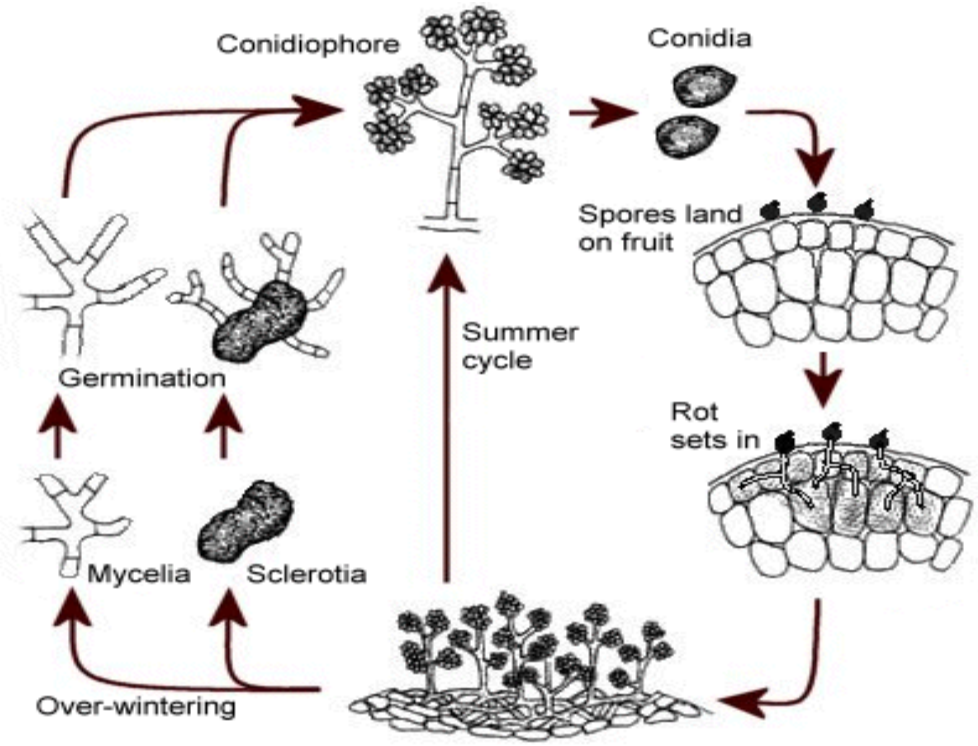


Figure 1.1: Proposed life cycle of *Botrytis cinerea* in a grape vine. Sclerotia germinates initiating the infection in grapes which eventually forms new conidia resulting in outbreak of the disease. Mycelium are generally formed on fallen leaves which may also initiate the infection, forming conidia. (http://www.thewinedoctor.com/author/sweetnoble.shtml)

The epidemiology of *B. cinerea* in vine has been illustrated by Elad, *et al*., in *Botrytis*: Biology, Pathology and Control. Sclerotia which are formed during winter develop conidia upon favorable conditions. These conidia are spread primarily by the wind during the day time, these acts as primary sources of infection in late winter or early spring. Sclerotia develop on fallen fruits or underneath leaf surfaces due to the overwinter session. Once the conidia lands on the host, it starts to develop the appresoria, penetrating the host which may be leaves, fruit, root, tubers, etc. The conidium geminates and establishes itself on the host; it starts the necrotrophic behavior, grows and starts developing new conidia. An epidemic starts with the onset of spring with dry weather conditions which favor the spread of *B. cinerea*. The conidia produced after the establishment of the primary infection act as the source of infection. The extent of infection varies according to the host, its age and part infected. In vine, *B. cinerea* infects the fruits, causing dropping of those grapes. These grapes may be the source for primary infection as these have mycelium growing on them which in adverse environmental conditions will turn into scelortia and be the source of primary infection for the next season. Other than this, insects may act as vectors for the spread of *B. cinerea* infection. Chances of infection increase with wounded plant parts which may be due to natural causes, insects or other pathogens.

C. Role of Secreted Proteins in Pathogen Infection

Secreted proteins from the pathogens play an important role in pathogenesis not only in fungi but also in bacteria. In bacterial infections, avirulence factors from bacteria are released in the plant through the type III secretion system. These proteins help the bacteria to initiate and establish an infection. The secreted proteins consist of proteins

which may degrade the cell wall, kill the plant cell, repress the plant immune response, degrade phytoalexins, stop or interfere with the plant signaling that ultimately leads to the death of the cell.

In fungi, the proteins secreted from the pathogen initially degrade the cell wall of the host making the penetration of appresoria easy for the primary infection. The secretions include polygalacturonases which play an active role in degradation of pectins present in the cell wall of the host, ceratoplatanin like proteins which have phytotoxic activity and glycerol phosphatidyl-inositol transfer protein (PG/PI-TP) with detoxicification activity but no role in pathogenesis (Shah *et al*., 2009). Once the primary infection has been established, the pathogen keeps secreting proteins to spread the infection. These proteins also suppress the plant immune response making the infection more severe. Other than this, there are many other hypothetical proteins with predicted activity or no known activities secreted during pathogenesis but are supposed to help the pathogen in spreading disease.

D. Plant Defense Responses

Hosts may detect the presence of pathogenic protein; once a pathogenic protein is recognized, the host starts the defense response which includes release of phytoalexins, lytic enzymes, reactive oxidizing agents, cell wall lignification and a number of pathogenesis related proteins (Figure 1.2)(Mert-Turk, 2002). In bacterial infections, once the presence of specific bacterial proteins is detected (for example, Avr1 from *Pseudomonas syringae*), the host starts programmed cell death so as to keep the infection localized (Frederick *et al*., 1998). As given in Figure 1.2, the pathway for programmed cell death starts with detection of pathogenic fungi which leads to the release

phytoalexins, ROS or other pathogenesis related proteins which inhibit the growth of pathogen. Other modifications include changes in metabolism, or structural reinforcement like change in cell wall composition making it difficult for the pathogen to enter the cell (Dixon and Lamb, 1999). Following the release of phytoalexins and ROS, the cell starts the programmed cell death.

D.1 Phytoalexins

Phytoalexins are defined as low molecular weight, anti-microbial compounds that are both synthesized and accumulated in plants after exposure to microorganisms or abiotic agents (Paxton, 1980; VanEtten *et al*., 1994). Elicitors are the molecules which signal the synthesis of phytoalexins. Elicitors in plant pathogen interaction may be polysaccharides, glycoproteins, lipids, lipopolysaccharides, oligosaccharides and even enzymes from the pathogen which are recognized by the plant cell leading to synthesis of phytoalexins (Anderson, 1989). Phytoalexins inhibit the growth of fungi by accumulating at the site of infection, thereby defending the plant against fungal infection. The EC50 of phytoalexins for fungi is 10^{-3} or 10^{-5} M. Plants secrete phytoalexins at a lower concentration which makes it difficult for the plant to kill a fungus. But phytoalexin may be one of the many pathways which may be working to help inhibit the infection (Kuc, 1995). In due course of evolution, fungi have evolved with a mechanism of secreting molecules for degrading phytoalexins. Phytoalexins share common backbone chemistry, which is an aromatic ring structure, but are not generally common as different plants synthesize different phytoalexins.

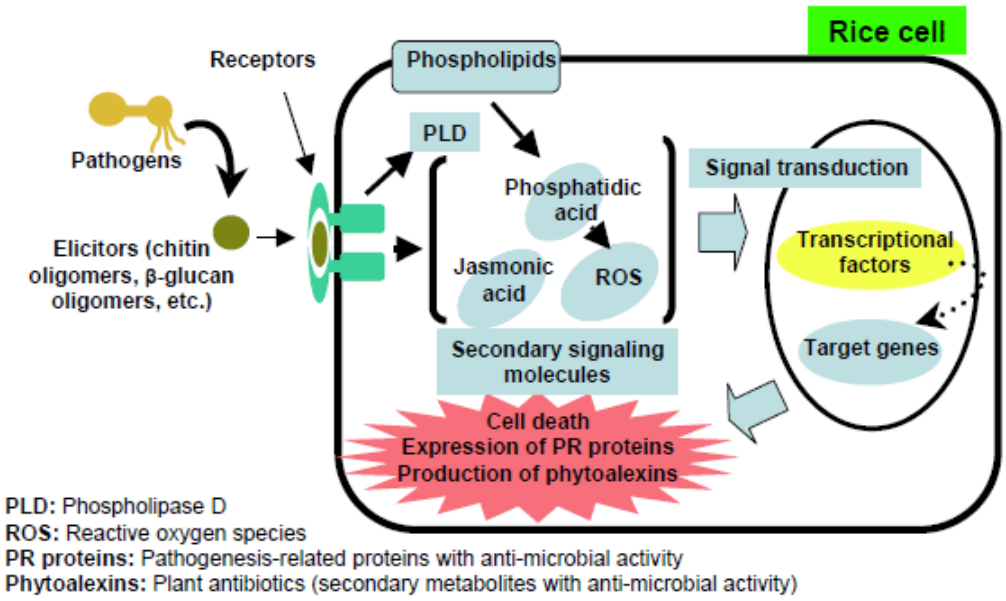


Figure 1.2: Defense responses of a plant cell in rice. After the identification of pathogen receptor, phytoalexins and ROS are released which leads to programmed cell death.

Resveratrol is the most common type of phytoalexin found in vine and other angiosperms. Other than reservatrol, 3-O-β -glucoside of resveratrol and a dimethylated derivative of resveratrol (3,5-dimethoxy-4'-hydroxystilbene) named pterostilbene are found in grapes. High concentrations of reservatrol are toxic for *B. cinerea* growth; not only the mycelium growth is inhibited, but also the conidia are unable to germinate in the presence of resveratrol (Adrian *et al*., 1997). As such, resveratrol displayed no direct toxicity to *B cinerea* which means resveratrol is to be modified for its toxicity which is done by laccase gene. Bclcc2, laccase gene is responsible for transforming resveratrol into fungitoxic substance (Schouten *et al*., 2002).

D.2 Reactive Oxygen Species (ROS)

Reactive Oxygen Species are compounds generally produced during normal metabolism in the plant cell which include superoxide (O^{-2}) or hydroperoxide (HO^{-2}) radicals produced during oxygen reduction. Reactive oxygen species play a role in plant – pathogen defense. The production of this ROS bursts off during pathogen attack; also many bacterial pathogens are unable to grow in the presence of high levels of ROS. The mechanism by which ROS is activated is unknown but due to the activation of ROS, change in calcium flux causes programmed cell death.

E. Ceratoplatanin Family

The ceratoplatanin family consists of secreted fungal proteins found in pathogenic as well as non pathogenic fungi. This family has been classified in NCBI with accession number pfam 07249 under the superfamily clo6331 (Pazzagli *et al*., 2006). According to EMBI-EBI database accession number IPR 10829, there are 124 protein sequences in different fungi of which activity of five of them has been confirmed and is included in the

NCBI database. The members of this family have shown to act as virulence factors, for example, MSP1 from rice blast fungus *Magnaporthe grisea* or as a elicitor of plant defense response in plants such as Sm1 from *Trichoderma virens*. The ceratoplatanin family has also been found in fungi which infect animals such as *Coccidioides immitis* (Pan and Cole, 1995) which secretes antigen CS-AG which has shown allergenic properties. Also, Aspf13 from *Aspergillum fumigatus* induces strong immunological response, and the pathogens are responsible for lung diseases (Nierman *et al*., 2005).

The founder member of the ceratoplatanin family was the protein secreted by *Ceratocystis fimbrata*, having phytotoxic activity named as ceratoplatanin and the causal agent of canker strain disease in *Platanus acerifolia* (Pazzagli *et al*., 1999). Also the localization studies with ceratoplatanin indicated that the protein is located in the cell walls of the ascospores (Scala *et al*., 2004; Boddi *et al*., 2004). Ceratoplatanin is a small protein with 12.4kDa which is phytotoxic and is released at an early stage of liquid culture. It is able to elicit a defense related response in host as well as non host plants. Another character of the ceratoplatanin protein includes two disulphide bonds and a high percentage of hydrophobic amino acids. Based on the above characteristics, a new family of fungal proteins was formed which have the following characteristics:

1. Phytotoxic nature
2. Small secreted proteins (about 150 amino acids long)
3. Four conserve cysteine molecules
4. High percentage of hydrophobic amino acids
5. Elicit plant defense response
6. Secreted during infection

Proteins with similar characteristics were found in other Acsomycetes fungi which include

Snodprot1 (Sp1) from *Phaeospheria nodorum (Stagnospora nodorum)*

Snodprot from *Neurospora crassa*

Aspf13 from *Aspergillus fumigates*

Sp1 from *Leptosphaeria maculans*

MSP1 from *Magnaporthe grisea*

Sm1 from *Trichoderma virens*

Ep1 from *Hypocrea atroviride*

Phaesospheria nodorum (Stagnospora nodorum) is the causal agent of the leaf and glume blotch disease of wheat which secretes a ceratoplatanin protein named Snodprot1 (Sp1). Studies conducted by Hall *et al*. concluded that Sp1 is a small protein of 137 amino acids long and is expressed and secreted during the infection to the wheat leaves. In their study, they concluded that when purified Sp1 was applied to the leaves of wheat, it was not able to cause any damage like necrosis or phytoalexin secretion to the host plant (Hall *et al*., 1999).

MSP1 is another member of ceratoplatanin family secreted by the fungus *Magnaporthe grisea* which is a causal agent for rice blast disease. In the study by Jeong *et al*., MSP1 was proposed to be essential for the virulence of the fungi. In their experiment, *M. grisea* with a deletion in MSP1 was created to characterize the phenotypical as well as the virulent nature of fungi without MSP1. They concluded that *M. grisea* without MSP1 has a reduction of 10 fold in the virulence; the phenotypic features were the same as the wild type. Also, purified MSP1 was not able to produce any

phytotoxic effect on the plant. It has been reported that when MgSM1 was expressed with a low efficiency promoter in *Arabidopsis,* it confers resistance to various fungi (Yang *et al*., 2009).

A nonpathogenic fungus *Trichoderma virens* secretes a protein belonging to the ceratoplatanin family Sm1 (Djonović *et al*., 2006). *Trichoderma* is a biocontrol fungus which enhances the speed of decomposition of biomass. Even though this fungus is non-pathogenic, it secrets Sm1, which is phytotoxic in nature. Studies conducted by Djonović *et al*. concluded that when purified Sm1 was applied on a plant surface, it triggers secretion of reactive oxygen species and also induces genes which are related to plant defense response. The study also concluded that when cotton seeds were pretreated with Sm1, it confers disease resistance against a foliar pathogen named *Colletotrichium*. Results also indicated that Sm1 may be involved in the induction of plant disease resistance by *Trichoderma spp*.

The ceratoplatanin are found in fungi which may be affecting plants or animals. A human pathogenic fungi *Coccidioides immitis* secretes a protein antigen of about 19.8 kDa (Pan and Cole, 1995). This anigen CS-Antigen shares some common characteristics with the ceratoplatanin family which includes hydrophobic amino acids, four cysteine conserved amino acids. Studies by Pan and Cole conclude that CS-Antigen is bioactive exhibiting serine proteinase activity and stimulates antibody formation suggesting a role in the virulence of fungi. Another human pathogen *Aspergillium fumigates* secretes Aspf13 protein which belongs to the ceratoplatanin family and is bioactive and triggers immune response in human beings.

Other than the above, non pathogenic fungus *Hypocrea atroviridis* secretes Ep1, a member of the ceratoplatanin family (Seidl *et al*., 2006). Studies conducted by Seidl *et al*., concluded that this protein is secreted in stress conditions which include limited carbon source and nitrogen as well as changes in osmotic pressure. The activity is yet to be determined, but as it is secreted in large quantities, its bioactivity is predicted. The Sp1 protein of *Leptosphaeria maculans*, the cause of black leg disease of *Brassica napus*, is another member of the ceratoplatanin family and has the conserved cysteine molecules and is reported to be bioactive (Wilson *et al*., 2002).

F. Ceratoplatanins in *Botrytis cinerea*

Proteomic analysis on secreted proteins of *B .cinerea* indicates the presence of two proteins having small molecular weight, four conserve cysteine molecules and sequence similarity with ceratoplatanin. These two proteins were named as BcSnod1 and BcSnod2 and the alignment with other members of the ceratoplatanin family is given in Figure 1.4.

BcSnod1 without the signal peptide is 119 amino acids long. Bioinformatics analysis indicates that the secreted protein is 12.4 kDa in molecular weight. As compared with the characteristics of the ceratoplatanin family, BcSnod1 is a small secreted protein, which has four conserved cystein residues, whose role is yet to be determined. Also QPCR studies indicated that BcSnod1 is secreted by *B.cinerea* after 30 min of being in contact with the host and is one of the earliest secreted proteins (unpublished data). The amino acid sequence of BcSnod1 is given in Table 1.1.

Table 1.1: Amino acid sequences of BcSnod1 and BcSnod2 with accession numbers from Broad Mit. The bold letters indicate the signal peptide for both proteins which are cleaved during secretion.

>BcSnod1 (BC1G_02163.1) hypothetical protein (translated coding sequence) Group 8(1) **MQFPTLATLLTFAVSATA** ITVSYDVGYDDASRSLAVVSCSDGSNGLLTKG YTTQGSLKNFPNIGGASVVAGWNDANCGSCYQLSYGGRSINVLVIDHAGA GFNIGEQALNTLTGGQAAALGRIDASYTQVDKSACGL*
>BcSnod2 (BC1G_08735.1) hypothetical protein (translated coding sequence)Group 8(2) **MQFRNPLSLLTLLLTSTQA** IQVTYDSGYDNAARSLSVVSCSNGPNGLETR FPQYKVQGDLPTFARIGGASTIAGWNSPNCGTCYTLTYQGVSINILAIDT AATGFNIAESAMNTLTNGRAVQLGNIDADWTLVTPEECGLPAEGGGSPCK A*

BcSnod2 has 60 % sequence identity with BcSnod2. BcSnod2 without signal peptide is 132 amino acids while the secreted protein is 13.69 kDa. It has some features of the ceratoplatanin family which include secreted small protein and the conserved cysteine residues described for BcSnod1. The amino acid sequence of BcSnod2 is given in Table 1.1 while the alignment in between BcSnod1 and BcSnod2 is given in Figure 1.3.

G. Sequence Similarity of Ceratoplatanin of *B.cinerea* with other Ceratoplatanins

BcSnod1 shares a 47% amino acid sequence identity with CS-Antigen protein secreted by human pathogenic fungi *Coccidiodes immitis* (Pan and Cole, 1995). BcSnod1 has 38% sequence similarity at the amino acid level with the ceratoplatanin protein from *C. fimbriata* which is phytotoxic in nature. The fungi *C. fimbriata* which is the causal organism for canker strain disease in *Platanus acerifolia* secretes this protein to kill the plant cell (Pazzagli *et al*., 1999). As described earlier, ceratoplatanin members are not only found in pathogenic fungi but also in non pathogenic fungi, for example, Snodprot

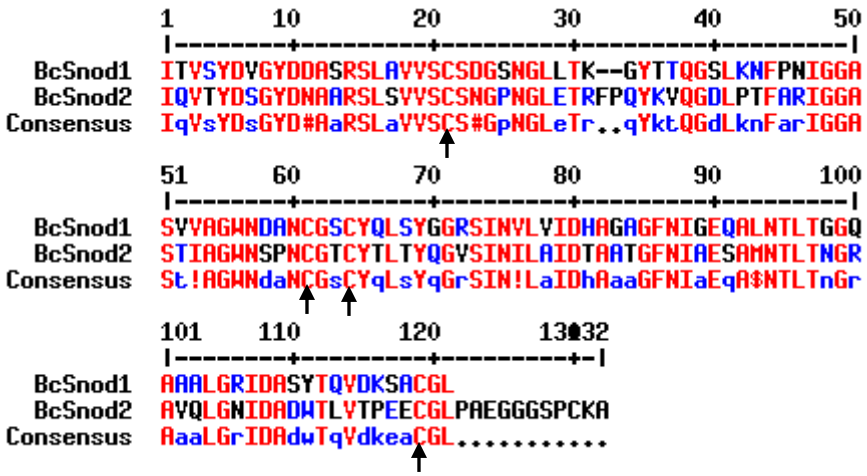


Figure 1.3: Alignment of BcSnod1 and BcSnod2 without signal peptide. The black arrow indicates four conserved cysteine residues which form a disulphide bond

from *Neurospora crassa* has a 58% amino acid sequence similarity with BcSnod1 and 58% with Epl1 protein secreted by *Trichoderma atroviride*. Although, the functions of both proteins are unknown, one cannot tell that whether BcSnod1 is phytotoxic or not just based on sequence similarity. Percents of sequence similarity between ceratoplatanins from several sources compared with *B.cinerea* ceratoplatanins are given in Table 1.2 while the multiple alignments are given in Figure 1.3.

Table 1.2: Percent amino acid sequence identity between Ceratoplatanins from different fungi included in NCBI pfam 02479

Causal Organism	Protein secreted	% Sequence similarity with BcSnod1	% Sequence similarity with BcSnod2
Phaeospheria nodorum	Sp1	61%	56%
Aspergillus fumigates	Asf13	58%	55%
Neurospora crassa	Snodprot	61%	56%
Leptosphaeria maculans	Spl1	54%	54%
Ceratocystis fimbrata	Ceratoplatanin	38%	35%

H. Studying Protein Function

To study the activity of a certain protein, the two most common methods to study are either *in vitro* analysis of the protein activity or knockout the gene. *In vitro* analysis requires the overexpression of the protein using an expression system and purification of the protein to check its activity. While in the case of knockout the gene, elimination by recombination or expression of iRNAs is required to inhibit the expression of the particular gene to determine the changes in the global expression, metabolic and cellular phenotypes. Since, knock out has a very low output in *B. cinerea*, overexpression in *E. coli* was done to check the activity of BcSnod1 and BcSnod2.

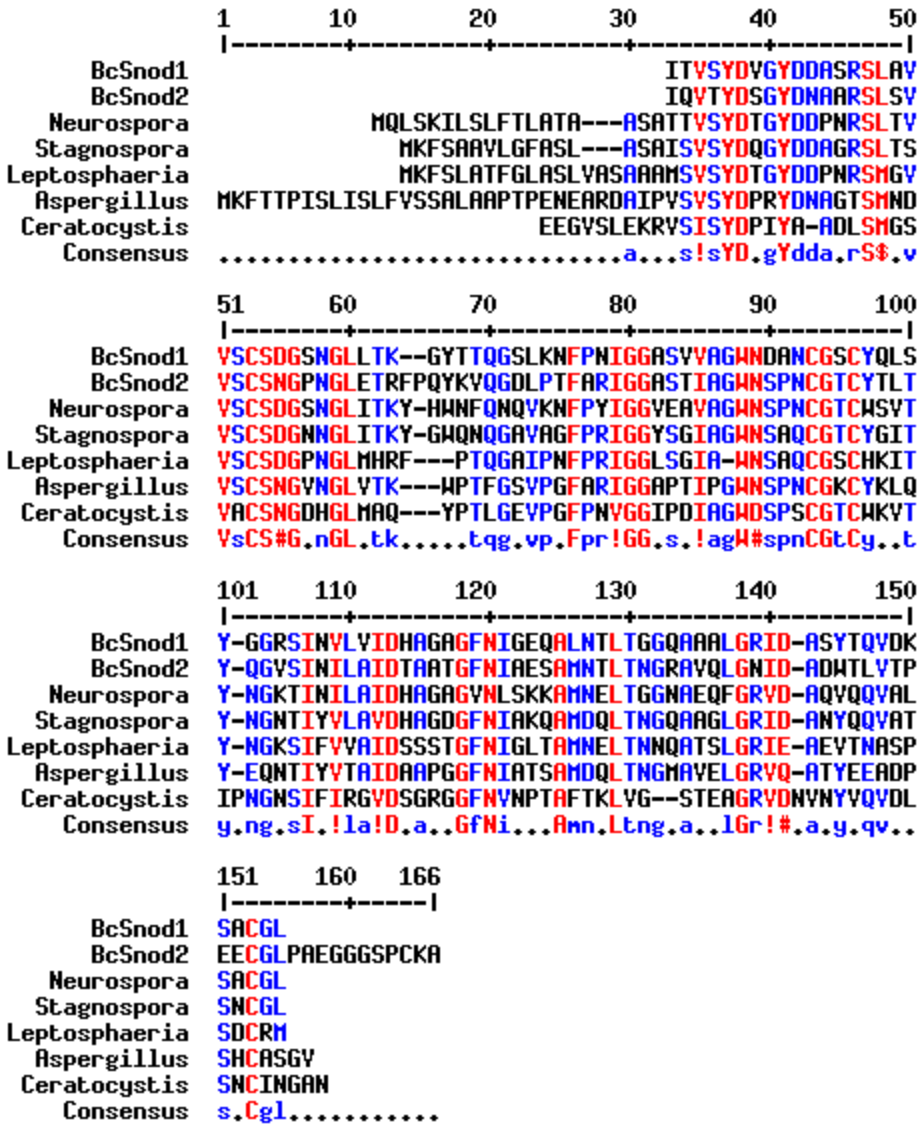


Figure 1.4: Multiple alignment at the amino acid level of Ceratoplatanins from different fungi with known activity with *B. cinerea* ceratoplatanins

I. *E. coli* Expression System

E. coli is the most commonly used expression system for large quantity protein production. *E. coli* has a very fast growth rate, and hence it takes very little time for a biomass to build up before induction of protein. After induction, a good concentration of protein can be obtained within a small period of time. Other than this, a variety of vectors is easily available which have specific tags and promoters, increasing the probability for ease in purification of a protein. Being a prokaryotic system, it doesn't require a large setup for protein production. *E. coli* is easy to screen after transformation for recombinants *E. coli* with the vector and insert of interest. The major disadvantage of using an *E. coli* system is the inability of formation of a correct disulfide bond and any post translational modification specifically when expressing a eukaryotic protein.

J. Yeast Expression System: *Pichia pastoris*

Pichia pastoris is a methalotrophic yeast which is able to grow on single carbon compounds such as methane as a source of carbon and can divide and grow in the presence of methanol. Recently, it has been extensively used for recombinant protein expression. Being a eukaryotic organism, *Pichia* has a proper folding mechanism of protein, posttranslational modification and the gene can be engineered in such a way that respective protein is secreted in the media. Secretion of the required protein in media makes the purification easy. Also, *Pichia* doesn't secrete a large amount of background protein, again helping to purify the protein of interest. *Pichia* is able to grow with high cell density and can also tolerate pH fluctuations not affecting the secretion of the protein. The only disadvantage of using *Pichia* is it takes a long time to grow and a long period of induction is required for a good amount of protein secretion.

The vector used for protein expression production in yeast is pPic9k. The vector has an AOX promoter (Alcohol oxidase) which gets induced in the presence of methanol and is suppressed in the presence of glucose. It has an ampicillin resistance gene which helps for selection of the positive colony during transformation in *E. coli.* Other than this, the most important feature which is present in pPic9k is the ability to secrete the protein of interest in the media. The vector is designed in such a way that the α-factor mating signal sequence from *Sacchromyces cerevisae* is in between the promoter and the multiple cloning sites. The gene of interest is to be designed in such a way that it is in frame with the signal peptide; during translation, the signal peptide is cleaved and the protein of interest is secreted in the media. Also, the gene of interest with the AOX promoter is integrated in the chromosomal DNA by homologous recombination which occurs at the AOX promoter in between the chromosomal DNA and the vector.

K. Statement of Purpose

A proteomic analysis using LC-MS mass spectrometry identified a number of potential proteins possibly involved in infection and spread of the disease (Shah *et al*., 2009). One of which, BcSnod1, was found to be abundantly expressed after exposure of *B. cinerea* to media containing 4% full red strawberry extract. BcSnod2 was found to be 60% similar to BcSnod1 and was identified in the genome of *B. cinerea* by sequence similarity to BcSnod1. Both the protein BcSnod1 (12.08 kd) & BcSnod2 (13.69 kd) belong to the family of small secreted proteins known as ceratoplatanins, recognized as having highly conserved cysteine residues (Pazzagli *et al*., 2006). These small protein sequences which belong to some pathogenic fungi are able to induce local and systemic defense responses in host organisms.

The objective of the study was to find the role of these ceratoplatanin like proteins from *Botrytis cinerea* in pathogenesis as they are secreted early during plant-pathogen interaction.

CHAPTER II

MATERIALS AND METHODS

A. *Botrytis cinerea* Genomic DNA Extraction

B.cinerea B05.10 spores were spread on potato dextrose agar (Difco Cat No.254920) with cellophane overlaid on agar and incubated for 7 days in the dark at room temperature. After 7 days, the mycelia were collected and ground in liquid nitrogen with mortal and pestle. A solution consisting of 500 µl of TES (100mM Tris-HCL,pH 8.0 (Sigma, St Louis, MO, USA), 10 mM EDTA and 2% sodium dodecyl sulfate) was added to the powdered mycelium in an 1.5 mL Eppendorf tube. 100 µg of Proteinase K was added to the tube and incubated for one hour with occasional mixing at 60^{o}C. The concentration of NaCl was brought to 1.4 M with 5 M NaCl (NaCl, Acros Organics, NJ, USA) followed by the addition of 10% acetyl trimethyl ammonium bromide (CTAB, Aldrich Chemical Company, Milwaukee, WI, USA) and incubated at 65^{o}C for 10 min. One volume of SEVAG (chloroform:iso-amyl alcohol 24:1 (Sigma, MO, USA))was mixed and incubated at 0^{o}C for 30 min and centrifuged at maximum speed for 10 min. Finally, 0.55 volumes of isopropanol was added to the supernatant to precipitate DNA and the tube was centrifuge at maximum speed for 5 minutes. The pellet was washed twice with 70% ethanol (AARPER, Shelbyville, KY, USA) and resuspended in 50 µl of sterile deionized water.

B. Cloning and Transformation of BcSnod1 and BnodcS2 in pGemTEasy from *Botrytis cinerea*

Genomic fungal DNA was used for amplifying the BcSnod1 gene; since the BcSnod1 gene didn't have any introns, genomic DNA was directly used for polymerase chain reaction (PCR) amplification. The primers used for PCR are given in Table 2.1.

Table 2.1: Primers used for amplifying BcSnod1 and BcSnod2 from *Botrytis cinerea*.

BcSnod1 Fp	5' GTGCATATGATCACCGTCTCCTACG 3'
BcSnod1 Rp	5' GTGCTCGAGTTACAATCCACAAGCACTCTTGTCG 3'
BcSnod2 Fp	5' GTGCATATGATCCAAGTAACCTACG 3'
BcSnod2 Rp	5' GTGCTCGAGCTAAGCCTTACACGGTGATCC 3'

Amplification components were mixed in a PCR amplification tube which included reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$), 2.5mM $MgCl_2$, 10 pM of forward and reverse primer, Taq polymerase and DNA template. Amplification was conducted with a thermal cycler (Eppendorf) using the following conditions: 1) activation step at 95^{o}C for 5 minutes; 2) amplification step using 30 cycles at 95^{o}C for 30 sec, 54^{o}C for 30 sec and 68^{o}C for 1 min minute, and 3) extension step at 68^{o}C for 5 minutes.

The amplified fragments were run on a 0.8% agarose gel electrophoresis (0.8 gm agarose (Invitrogen) in 100 ml 1X TAE (0.04 M Tris-acetate (Amresco, Ohio), 0.001 M EDTA (Merk, Germany) and the respective bands were excised from the gel. The gel fragments were then cleaned using the Gel Cleaning Kit (Real Biotech

Corporation Cat No. YDF100) and then ligated into the pGemTEasy overnight at 16^{o}C. 30 µL of competent *E. coli* DH10B cells were mixed with 2 µL of vector in cuvette (Labrepco, PA, Cat No. 11608031) and transformed by electroporation (Cell Porator, Gibco BRL, Life Technologies, Inc., 350V, 330 µF, 4 KΩ). Transformed *E. coli* cells were plated on 2xYT + X-Gal (80 µg/mL) + carbenicillin (150 µg/mL) + isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.5 mM) media and incubated for 24 hours at 37^{o}C. Blue white selection was performed to select for positive colonies. Positive colonies were sequenced for confirmation. The plasmid were extracted according to the manual of Qiagen Mini Prep kit (Cat No. 27106).

C. Cloning BcSnod1 Gene in pET28b

pET 28b is an expression vector which has a T7 promoter, Lac operator and has six histidines before multiple cloning sites. The T7 promoter expresses the RNA in high levels while the lac operator can be used to induce the protein expression by supplementing IPTG. Six histidines were used for protein extraction and purification; purification was done by metal affinity chromatography, specifically nickel column.

Plasmids of pGemTEasy + BcSnod1 (pGBcS1), pGemTEasy + BcSnod2 (pGBcS2) and pET28b vector were digested with restriction endonuclease Not1 and Xho1 to cut out the BcSnod1and BcSnod2 coding regions from pGemTEasy for cloning respectively. The digestions were done overnight at 37^{o}C and the following day the fragments were analyzed via 0.8% agarose gel electrophoresis. DNA fragments of interest were excised. The RBC gene clean kit was used to purify both DNA fragments and ligated overnight at 16^{o}C. Electroporation was performed for transformation of

vector with BcSnod1 in *E. coli* DH10B cells as described previously. The resulting plasmids were named pETS1 and pETS2.

For protein expression, the *Rosetta* strain of *E. coli* (Novagen Cat No. 70954-3) was used which is choramphenicol resistant and has a low protein background favoring protein purification. For transformation of pGBcS1 plasmid in Rosetta, plasmid isolation was performed using the Qiagen kit, and the plasmid was transformed in Rosetta using the Calcium Mediated Chemical Transformation protocol. The previously prepared chemically competent cells were thawed on ice for 10 min and 20 ng of plasmid DNA was added to 50 µl of cells and kept on ice for 5 min. A quick heat shock of 30 sec at 42^{o}C follows the incubation, immediately after which the cells were again kept on ice for 10 min. 950 µL of SOC media was added to the cells which were subsequently incubated at 37^{o}C for an hour. Finally, 100 µL of SOC containing cells were spread on 2xYT media containing Kanamycin (70 µg/mL) and chloramphenicol (34 µg/mL). Screening for the presence of the gene of insert was performed by restriction digestion and PCR. The same procedures were performed for transformation of pGBcS2 and pET28b vectors in *E. coli*. For confirmation, sequencing was performed using the primers given in Table 2.2.

Table 2.2: Primers used for sequence confirmation of the BcSnod1 and BcSnod2 genes in the pET28b Plasmid.

T7 Promoter	5’ TAATACGACTCACTATAGGG 3’
T7 Terminator	5’CTAGTTATTGCTCAGCGGT 3’

D. Cloning BcSnod1 in pPic9K

To clone BcSnod1 in pPic9K, the multiple cloning sites EcoR1 and Not1 were selected. The forward and reverse primer used for cloning in pPic9K generated an EcoRI and NotI restriction site, respectively. The primers used are given in Table 2.3 and the constituents were the same as given in Section 2.B.

Table 2.3: Primers Primers used for amplification of BcSnod1 from pTBcS1 to clone into pPic9k.

S1Pic9Fp	5' CACGAATTCATCACCGTCTCCTACGACG 3'
S1Pic9Rp	5' ATAGCGGCCGCTCATTACAATCCACAAGCACTCTTGTC 3'

The amplified fragments were subjected to 0.8% agarose gel electrophoresis and DNA fragments of interest were cut and cleaned using a RBC Gel Cleaning Kit. Before ligation, the gel cleaned fragment and vector pPic9K were digested by EcoR1 and Not1 overnight and gel cleaned. For ligation, fragments were mixed and incubated overnight at 16^{o}C. Electroporation was used to transform competent *E. coli* cells; 50 µL of competent cells were mixed with 2 µL of ligation and electroporated as given in Section 2.B. The electroporated cells were mixed with 950 uL of SOC and incubated for one hour at 37^{o}C. 100 µL of cells were then spread on 2xYT + Carbenicillin (150 µg/mL) and incubated at 37^{o}C for 24 hours for observing transformed individual colonies. Sequencing was performed with the positive colonies for confirmation of insert, the resulting plasmid was named pPBcS1. The presence of insert in frame with the coding region of the vector was confirmed using the primers in Table 2.4.

Table 2.4: Primers used for sequence confirmation of the BcSnod1 gene in the pPic9k plasmid.

AlphaSeq	5' TACTATTGCCAGCATTGCTGC 3’
3’ AOX	5’ GGCAAATGGCATTCTGACATCCTC 3’

E. Expression of BcSnod1 Protein in *E. coli* (Rosetta)

For expression of proteins, a single colony of *E. coli* was inoculated in 3 ml of LB media + 70µg/mL kanamycin + 34 µg/mL chloramphenicol and grown overnight at 37^{o}C. The next day, IPTG (Sigma Aldrich) was added at the concentration of 1.0 mM and the cells were incubated at 37^{o}C for 4 hours. 1 mL cells were pelleted down and lysis buffer 100 µL (Table 2.5) was added to it and kept at -80^{o}C for 10 min and then at 37^{o}C for 10 min; repeat the same procedure three times. The cells were then sonicated using a sonicator (Fisher Scientific, model: 4C15) giving a pulse of 1 sec and a lag of 3 seconds for a minute and centrifuged at 12000 rpm 4^{o}C for 10 min. The supernatant was used for determining the presence of protein. For control, uninduced samples were collected and kept at -80^{o}C and lysed using lysis buffer and sonicated. 16% Tris Tricine SDS-PAGE was performed for analyzing the presence of a protein.

F. Scaling-up for Protein Expression in *E. coli*

For larger quantities of protein, a single colony of *E. coli* was grown overnight in 10 mL of LB + 70µg/mL kanamycin + 34 µg/mL chloramphenicol at 37^{o}C. The culture was then transferred to a beaker containing 1 liter of LB + kanamycin + chloramphenicol and grown overnight till the OD 600 = 0.6. Protein expression was induced by adding IPTG to the culture and then incubated at 37^{o}C for 4 hours. The cells were pelleted down

and lysis buffer (Table 2.5) 100 mL was added to the pellet and incubated at -80^{o}C for 10 min and 37^{o}C for 10 min thrice. The cells were then sonicated and centrifuged.

Table 2.5: Buffer Composition used for protein extraction and purification from *E. coli Rosetta* expressing BcSnod1, BcSnod2. Lysis buffer to lyses the cells while the wash is to remove any protein bound to beads of Nickel other than protein with His tag and Elution buffers to elute the protein.

Buffer	**Composition**
Lysis Buffer	20 mM Tris-HCL, 500 mM NaCl, 8 M Urea, pH=8 (adjusted using HCl)
Wash Buffer	20 mM Tris-HCL, 500 mM NaCl, 8 M Urea, 20 mM Imidazole, pH=8 (adjusted using HCl)
Elution Buffer	20 mM Tris-HCL, 500 mM NaCl, 8 M Urea, 250 mM Imidazole, pH=8 (adjusted using HCl)

G. Purification of BcSnod1 and BcSnod2 from Extract

To the lysate obtained after sonicating *E. coli* cells, 5mL of Ni-NTA beads (Novagen Cat No.70899-3) was added and incubated at 4^{o}C for 1 hour. The mixture was added to a chromatography column (Bio Rad Cat No. 731-1550) in batches. Wash buffer 5 mL (Table 2.5) was added to the column for removing any proteins with His tag. The protein was eluted twice with 1 mL of elution buffer (Table 2.5).

H. Refolding BcSnod1 and BcSnod2

Since the protein was insoluble in the lysis buffer. The protein was denatured in urea and refolded. Refolding includes decreasing the concentration of urea slowly. This

was achieved by using a dialysis cassette (Millipore Cat No. 66333). The elution obtained was injected in the cassette and the cassette was dialyzed at 4^{o}C for 2 hours in a dialysis buffer A (Table 2.6). Following this, the cassette was then dialyzed in a beaker containing dialysis buffer B, C, D, E (Table 2.6) for two hours each. The final dialysis was done in a dialysis buffer with 20 mM Tris-HCl pH=7.0 containing no urea. The soluble protein was then used for injection in plants for activity.

Table 2.6: Composition for dialysis buffer used to dialyze the protein after elution. Dialysis cassettes were kept for 2 hours in each buffer from dialysis buffers A-E and for 12 hours in dialysis buffer F at 4^{o}C.

Dialysis Buffer	Composition
Dialysis Buffer A	20 mM Tris-HCL, 500 mM NaCl, 6 M Urea
Dialysis Buffer B	20 mM Tris-HCL, 500 mM NaCl, 4 M Urea
Dialysis Buffer C	20 mM Tris-HCL, 500 mM NaCl, 2 M Urea
Dialysis Buffer D	20 mM Tris-HCL, 500 mM NaCl, 1 M Urea
Dialysis Buffer E	20mM Tris-HCL, 250 mM NaCl, 0.5 M Urea
Dialysis Buffer F	20 mM Tris-HCL, 250 mM NaCl, 3 mM DTT

I. Transformation of pPic9K in Yeast (*Pichia pastoris*)

For transformation of pPic9K in *Pichia pastoris* (GS115 His^{-}), electrocompetenet cells were made and stored at -80^{o}C (Wu and Letchworth, 2004). A single colony of *Pichia pastoris* was inoculated in 100 ml of Yeast Peptone media until the OD $_{600}$ reaches 2.0 at 30^{o}C. The cells were centrifuged (Eppendorf, Model No.5810R) at 1500g for 5 min and 1 M sorbitol (Fisher Chemicals) was added to make the cell concentration

$8X10^8$ cells/mL (ThermoScientific Cat No.335905). To 8 mL of 100 mM lithium acetate (MP Biomedicals Inc, OH), 10 mM DTT (Fisher Inc.), 0.6 M sorbitol and 10 mM Tris-HCl pH=7.5 to 1 mL of cells ($8X10^8$ cells/mL) were added and incubated at room temperature for 30 min. The cells were centrifuged using a centrifuge at a speed of 1500g for 5 min. To those cells, 1.5 mL of 1.0 M ice cold sorbitol was added and washed thrice using 1 mL of 1.0 M of sorbitol. After the final wash, the electrocompetent cells were stored at -80^{o}C until used in 1.0 M sorbitol making the final cell concentration $1X10^{10}$ cells/mL.

Before electroporation of *Pichia*, pPBcS1 plasmid was linearized by digesting at the BglII site and run on gel. BglII digestion cuts pPic9K in two fragments, approximately 5600 bp and another about 3000 bp. The band with the insert is with higher weight i.e., 5600 + 360 = 5960 bp, and hence was excised and gene clean was performed using a RBC gene clean kit.

For transformation, the electrocompetent cells were thawed on ice for 10 min. 80 µL of electrocompetents cells were mixed with 3 ng of linearized DNA in a fresh eppendorf tube and kept on ice for 5 min. The total mixture was then pipetted into an electroporation cuvette (0.1 cm Invitrogen Cat No.65-0030). The electroporating pulse was applied at 1.5 kV, 25 µF, 200 Ω using Gene Pulser II (Bio Rad). Immediately after pulse, 1 mL of 1.0 M cold sorbitol was added to the cuvette and all the cells were incubated at 30^{o}C for an hour. About 200 µL of electroporated cells was plated in a histidine deficient Regeneration Dextrose (RH) media containing 1 M sorbitol, 2% dextrose, 1.34% YNB, 4×10^{-5} % biotin and 0.005% amino acids without histidine. The plates were incubated at 30^{o}C until colonies grew, which took 6 days.

J. Screening Positive Colonies in *Pichia*

Colonies grown on the histidine deficient plates were once again screened by performing a PCR. For PCR in *Pichia,* a colony was dissolved in 10 µl of sterile distilled water and 2.5 µL of lyticase enzyme (Sigma Aldrich Cat No. L2524) was added to the tube. The tube was kept at 37^{o}C for 10 min and -80^{o}C for 10 min so as to help break the cell membrane. Now 5 µL of lysate was used for PCR for amplification and verification of insert. The primers used for screening are given in Table 2.7 and the details of the reaction mixture and PCR parameters are the same as in Section 2.B.

Table 2.7: Primers used for checking the presence of BcSnod1 in *Pichia pastoris.*

5'AOX	5´ GACTGGTTCCAATTGACAAGC 3´
3'AOX	5' GGCAAATGGCATTCTGACATCCTC 3'

K. Expression Trials in *Pichia*

For expression of the protein BcSnod1, a single colony of *Pichia* with respective inserts was inoculated in 100 mL of minimal glycerol media (1.34% YNB, 1% glycerol, 4 X 10^{-5}% biotin) overnight at 30^{o}C with shaking at 225 rpm; 5 replicates were screened for protein expression in both *Pichia* with the BcSnod1 insert (PBcS1) and *Pichia* with the vector (pPic9K) only. After OD $_{600}$ reaches 2.0, cells were pelleted and resuspended in 10 mL of minimal methanol media (1.34% YNB, 0.5% methanol, 4 X 10^{-5}% biotin) and incubated with shaking at 30^{o}C. Concentrated methanol was added every 12 hours to the media to make the final concentration 0.5%. On the third day, 1 mL sample was withdrawn from both *Pichia* with PBcS1 and pPic9K only from all 5 different cultures,

centrifuged and supernatant frozen at -80^{o}C. The same procedure was repeated on the fifth and the tenth day for all cultures. All the supernatant cultures were tested for the presence of insert by Tris-Tricine SDS gel electrophoresis. For Tris-Tricine gel electrophoresis, 20 µL of supernatant was mixed with 20 µL of loading buffer and boiled for 5 min at 95^{o}C. After cooling, the samples were loaded on solidified gel and run at 70V constant voltage for 1.5 hours. After 1.5 hours, the gel was stained by keeping it in the staining solution (30% Methanol, 10% Glacial Acetic Acid, 1gm of Brilliant Blue dye) for 1 hour and destained using the destaining solution (30% Methanol, 10% Glacial Acetic Acid) for another hour. The gel indicated the presence of protein in the respective supernatant.

L. Large Scale Expression and Extraction of BcSnod1 from *Pichia*

After expression trails, the colony secreting maximum protein was used for further protein production. For large scale protein production, a single colony was grown overnight at 30^{o}C in 10 mL of minimal glycerol media. The next morning, 1000 mL of minimal glycerol media was inoculated with 10 mL from the previous day and was incubated on a shaker with 225 rpm in a 4 liter flask. The cells were pelleted down by spinning at 1500g for 10 min, resuspended in 200 mL of minimal methanol media and incubated by shaking at 30^{o}C. Every 12 hours, 100% methanol was added to make the concentration of methanol 0.5% in media, i.e., 1 mL. Yeast cells were grown up to 10 days under the same conditions, just by adding methanol every 12 hours.

After 10 days, the cells were centrifuged down and the supernatant was concentrated using filters (Amicon, Millipore) having a molecular weight cut off 5 kDa.

About 200 mL of supernatant was concentrated up to 40 times to make a final volume of 5 mL. A small quantity (20 μL) of concentrated supernatent was loaded on Tris Tricine to check the presence of BcSnod1 in the concentrated supernatant. A bradford assay (Bradford, 1976) was conducted to determine the concentration of protein present in the supernatant.

M. HPLC Fractionation of Concentrated Supernatant

The concentrated supernatant was applied to HPLC for fractionation or separation of proteins. A C4 column (Bio-Rad) was used for separation of the protein mixture. Elution was performed using a gradient of 0-90% water/acetonitrile + 0.05% (v:v) trifluroacetic acid. 1 mL fractions were collected and the eluted peaks in the chromatogram were analyzed using Tris Tricine SDS PAGE for the presence of protein in the fraction.

The HPLC fractions with BcSnod1 were dialyzed with 20 mM Tris-HCL pH=7.0 before plant injection. For dialysis, fractions from 41-47 were mixed together and then injected in a slide-a-lyzer cassette which had a MWCO of 10 kDa. The cassette was then suspended in 1000 mL of 20 mM Trsi-HCl and kept at 4^{o}C overnight.

N. Plant Transient Assay

The plants used for transient assay were Tomato (*Solanum lycopersicum*) and tobaccoss (*Nicotiana xanthi*) grown in an environmental chamber; they were two months and four months old respectively. For detach leaf assay, leaves were cut from the plant and kept in a wet box in the environmental chamber.

The protein after dialysis was used for plant transient assay. In the transient assay, *BcSnod1* protein (approx. 15 µg) was infiltrated in the mid vein of the leaves of tomato and tobacco using a syringe and 30.5 gauze needle. In the detached leaf assay, leaves were infiltrated with protein after plugging out of the plant. In another detached leaf assay, 10µL of protein was pipetted on the leaves and kept in a wet box in an environmental chamber. As a control, extracts from *Pichia* with no insert were infiltrated or pipetted on the leaves.

CHAPTER III

RESULTS

A. Cloning and Transformation

PCR amplifications using *B. cinerea* genomic DNA yielded DNA fragments of approx 370 bp for BcSnod1 and 410 bp for BcSnod2 (Figure 3.1a), which correlates with the information on Broad site where both genes do not contain any intron. Amplification of pGBcS1 and pGBcS2 yielded DNA fragments of 370 bp and 410 bp; sequencing by T7 promoter primer indicated that the insertion was correct with no mutation in any of the nucleotide.

For pET28b cloning, pGBcS1 and pGBcS2 were digested with restriction endonuclease Nde1 and Xho1 and ligated, followed by transformation of cells using electroporation. PCR amplification of DNA from positive colonies of *E. coli* having pTBcS1 or pTBcS2 resulted in DNA fragments of 370 bp and 410 bp with primers shown in Table 2.1 (Figure 3.1b). Amplification with T7 promoter and terminator primers results in DNA fragments of approx 700 bp and 740 bp. Sequencing results indicated the presence of His tag before the BcSnod1/2 genes with both open reading frames with the codons that encode the His tag.

Amplification of positive colonies of pPBcS1 resulted in a 370 bp DNA fragment (Figure 3.1b) which concludes the presence of the desired insert. Sequencing results indicated that the open reading was in the frame position required for protein expression.

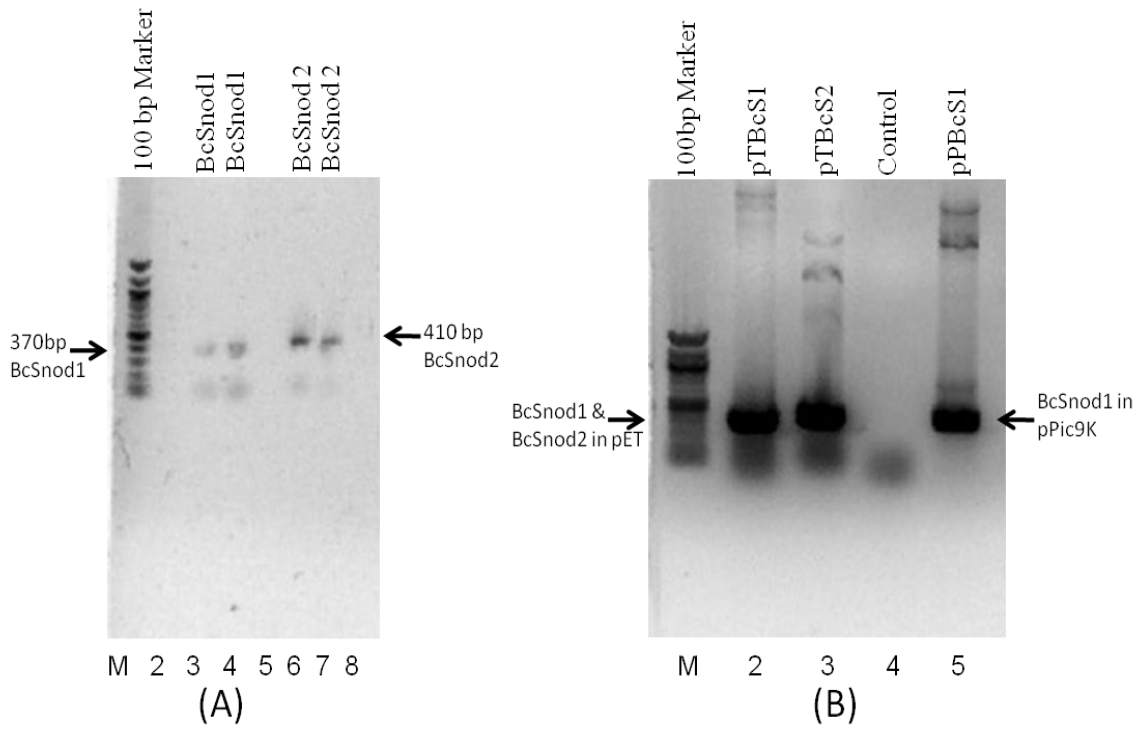


Figure 3.1: A. Amplification of BcSnod1 and BcSnod2 from fungal genomic DNA. The BcSnod1 gene yields a band of 370 bp (lane 3&4) while BcSnod2 gene yields a band of 410 bp (lane 5&6), two separate reactions were employed. B. Amplification of positive *E. coli* colonies containing BcSnod1 and BcSnod2 in pET28 vector which yields bands of 370 bp (lane2) and 410 bp (lane 3) respectively, the pPic9k containing the BcSnod1 gene yields band of 370 bp (lane 5).

B. Expression Trials in *E. coli*

For both BcSnod1 and BcSnod2, protein production was induced by supplementing the media with 1.0 mM IPTG for four hours at 37^0C. A good concentration of protein was obtained within a four hour time frame (Figure 3.2, Lane 2). We obtained proteins with a molecular weight of approx.15 kDa for BcSnod1 and 15.5 kDa for BcSnod2, which correlates with bioinformatics studies which determined that the BcSnod1 protein is 14.70 kDa and the BcSnod2 is 15.20 kDa. The protein obtained was insoluble in the lysis buffer without any urea (Figure 3.2, Lane 3), so two different temperatures, 30^oC and 16^oC, and different time periods for induction which included 12 hours and 16 hours, respectively, were analyzed for soluble protein production. Unfortunately, the products for the new type of induction also were insoluble proteins. To solubilize the proteins, initially we used lysis buffer containing 2 M and 6 M, of urea, but no traces of soluble protein were present and hence lysis buffer containing 8 M urea was used. The soluble proteins are shown in Figure 3.2 (BcSnod1) and Figure 3.3 (BcSnod2).

C. Scale-up Protein Expression in *E. coli* and Refolding of BcSnod1 and BcSnod2

For large quantities of protein, 1 liter of media was grown to 0.6 OD_{600} and then induced with 1.0 mM IPTG for four hours at 37^oC. Cells after centrifugation were resuspended in lysis buffer containing 8 M urea, due to which the protein was solubilized in the buffer and was present in the supernatant after lysing with a sonicator. BcSnod1/2 genes were cloned such that the protein expressed will have six histidine on the N-terminus to help with their purification using Ni-NTA columns. For purification of the

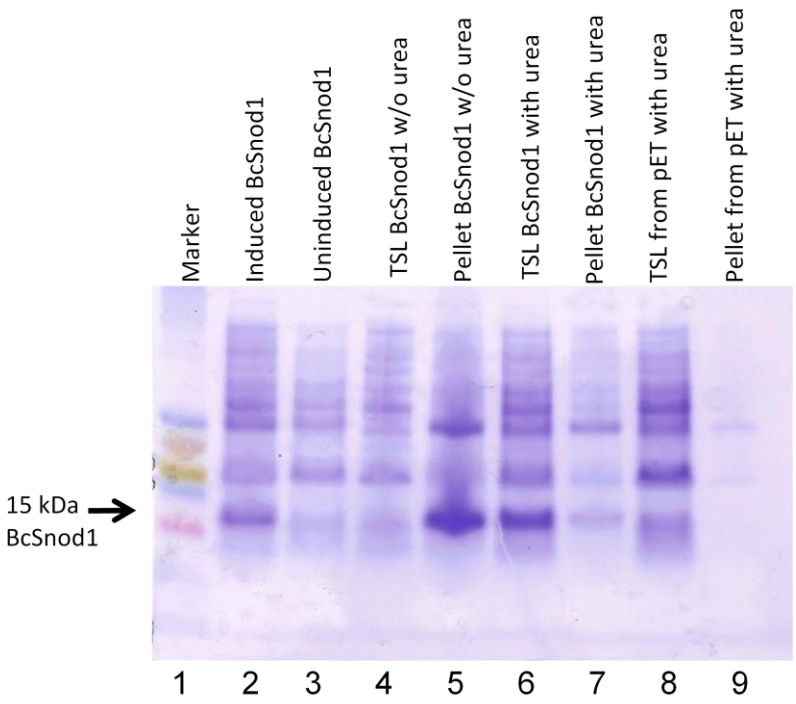


Figure 3.2: Expression trials of the BcSnod1protein from the *E. coli* expression system. Lane 1 is the Marker, lane 2 is the total crude lysate of *E. coli* containing BcSnod1 induced with IPTG having a band at 15 kDa while lane 3 is the uninduced sample. Lane 4 is the total soluble lysate obtained using a lysis buffer containing no urea, lane 5 is the pellet obtained after sonication having precipitated the BcSnod1 protein. Lane 6 is the soluble BcSnod1 protein present into total soluble lysate obtained lysis buffer with 8 M urea while lane 7 is the pellet which has very little BcSnod1. Lanes 8 and 9 are the total soluble lysate and pellet respectively from *E.coli* containing pET in a vector.

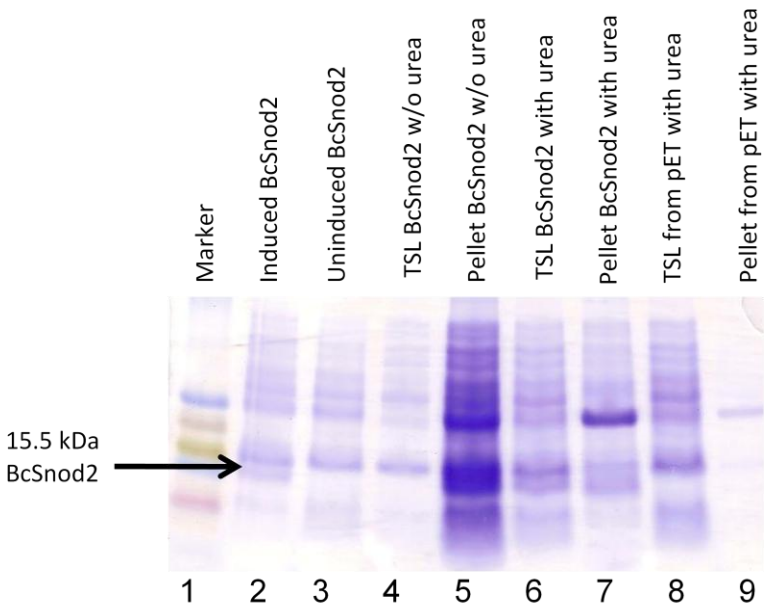


Figure 3.3: Expression trials of protein from the *E. coli* expression system. Lane 1 is the Marker, lane 2 is the total crude lysate of *E. coli* containing BcSnod2 induced with IPTG having a band at 15.5 kDa while lane 3 is the uninduced sample. Lane 4 is the total soluble lysate obtained using the lysis buffer containing no urea, lane 5 is the pellet obtained after sonication having precipitated the BcSnod2 protein. Lane 6 is soluble BcSnod2 protein present in the total soluble lysate obtained using lysis buffer with 8 M urea while lane 7 is the pellet which has very little BcSnod2. Lanes 8 and 9 are the total soluble lysate and pellet respectively from *E.coli* containing pET in a vector

proteins, the extracts (supernatant after lysis) were passed through Ni-NTA columns and the bound proteins were eluted using imidazole. Figure 3.4 indicates total soluble lysate of BcSnod1 and BcSnod2 as compared to purified protein using Ni-NTA chromatography. The eluted protein was then dialyzed as the presence of urea interferes with the protein folding as well as the bioactivity of the protein. We used serial dialysis with gradual reduction of urea concentration for protein refolding. The Bradford assay confirmed about 2 mg of protein was purified from 1 liter of culture.

D. Screening for *Pichia* Transformants

Pichia transformation yielded approximately 3.0 x 10^4 colonies/μg of DNA. 5 transformants were tested using PCR amplification. As given in Figure 3.5, amplification of *Pichia* transformed with pPBcS1 gave only one single band of approximately 900 bp, indicating transformation by BcSnod1 gene; the empty vector was also transformed and amplification yielded a single band of 500 bp.

E. Expression Trials in *Pichia*

Methanol acts as an inducer for protein production in *Pichia* transformants. For BcSnod1 production, initially a colony was grown in 100 mL of minimal glycerol media, so that *Pichia* cells were present in large quantities. The cells were then resuspended in minimal methanol media for inducing protein production. Methanol was added regularly so that the concentration of methanol remained at an inducible level in the media. Five colonies were screened for protein production, on the third day after induction. BcSnod1 protein was secreted by *Pichia* culture in the media. Similar samples were collected on days 5 and 7, all indicated the secretion of protein in media. As a control,

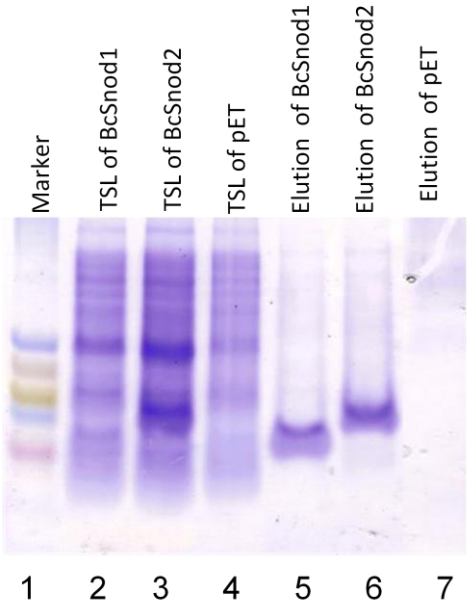


Figure 3.4: Extraction and purification of BcSnod1 and BcSnod2 proteins. Lane 1 Marker, lane 2 total soluble lysate of BcSnod1 protein, Lane 3 total soluble lysate of BcSnod2, lane 4 total soluble lysate of pET (control). Lanes 5, 6 and 7 contain a purified protein sample purified from total soluble lysate of BcSnod1, BcSnod2 and pET respectively.

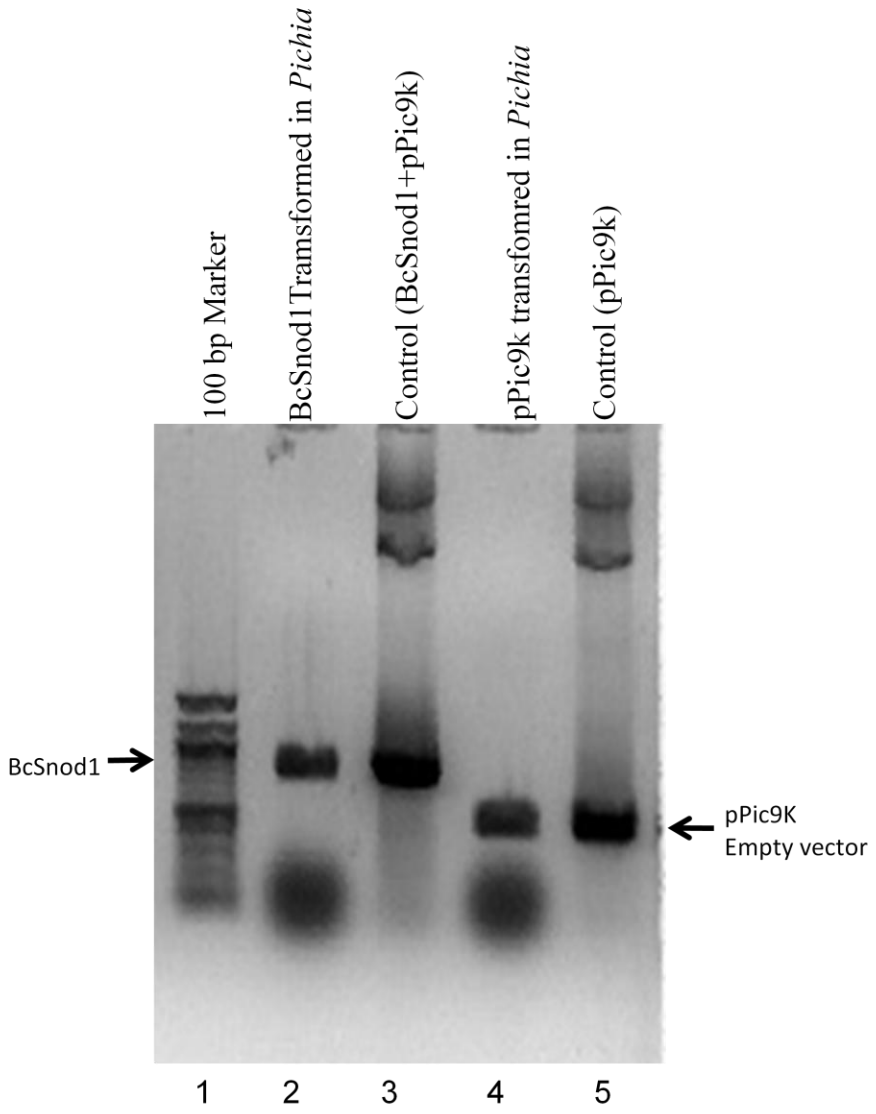


Figure 3.5: Confirmation of transformation in *Pichia.* Lane 1 is 100 bp marker, lane 2 is the fraction amplified from yeast cells, yielding a band at 900 bp indicating the presence of the BcSnod1 gene. Lane 3 is the fraction of amplified plasmid pPic9k with BcSnod1, used as a control. Lanes 4 and Lane 5 are empty vectors in *Pichia* and plasmid from *E. coli*.

Pichia transformed with the empty vector was induced to check for the background protein production in *Pichia* itself. Concentrated methanol was added every day to keep the concentration of methanol to 0.5% in the media for secretion of the BcSnod1 protein, and on the 10^{th} day, *Pichia* cells were separated from total media by centrifugation and the supernatant was used for purification of the protein.

F. Scale up in *Pichia*

A strain producing maximum protein was selected for large quantity protein expression. Initially, 1 liter of culture was grown in minimal glycerol media and then resuspended in 100 mL of induction media, which is minimal methanol media. After 10 days of induction, the supernatant was collected and used for purification of protein. The supernatant was concentrated using filters, making the final volume 5 mL. HPLC was employed in purification of BcSnod1; Figure 3.7 is a chromatogram indicating the peaks obtained during HPLC with concentrated solution from *Pichia* expressing BcSnod1 (red) and empty vector (black). The protein was eluted with 55% acetonitrile + 0.05% trifluroacetic acid in fractions 41, 42, 43, 44, 45, 46, and 47 during HPLC purification. (Figure 3.7).The total amount of BcSnod1 protein obtained from 1 L of minimal glycerol media or 100 mL of minimal methanol media was 6 mg. As a control, *Pichia* with pPic9k was induced and also employed for HPLC purification.

G. *In Planta* Transient Assay

For transient assay, plants grown in a greenhouse were selected. Tomato plants 4 weeks old having good foliage were used for protein bioactivity. Leaves which were not too young or too old were selected. For tobacco, the BcSnod1 protein was infiltrated

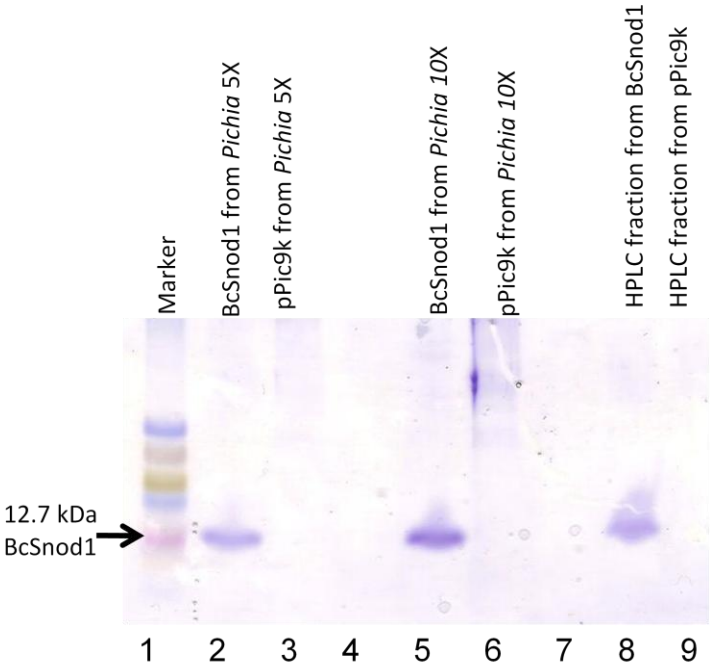


Figure 3.6: Expression and purification of BcSnod1 in *Pichia pastoris*. Tris Tricine SDS PAGE 10% - Lane 1 Marker, lane 2 concentrated supernatant obtained from culture of *Pichia* transformed with BcSnod1, lane 3 contains concentrated protein from *Pichia* with an empty vector to be used as a control. Lanes 5 and 6 are samples concentrated with 10 times of *Pichia* expressing BcSnod1 and pPic9k control. Lanes 8 and 9 are the HPLC fractions (41) of BcSnod1 and pPic9k.

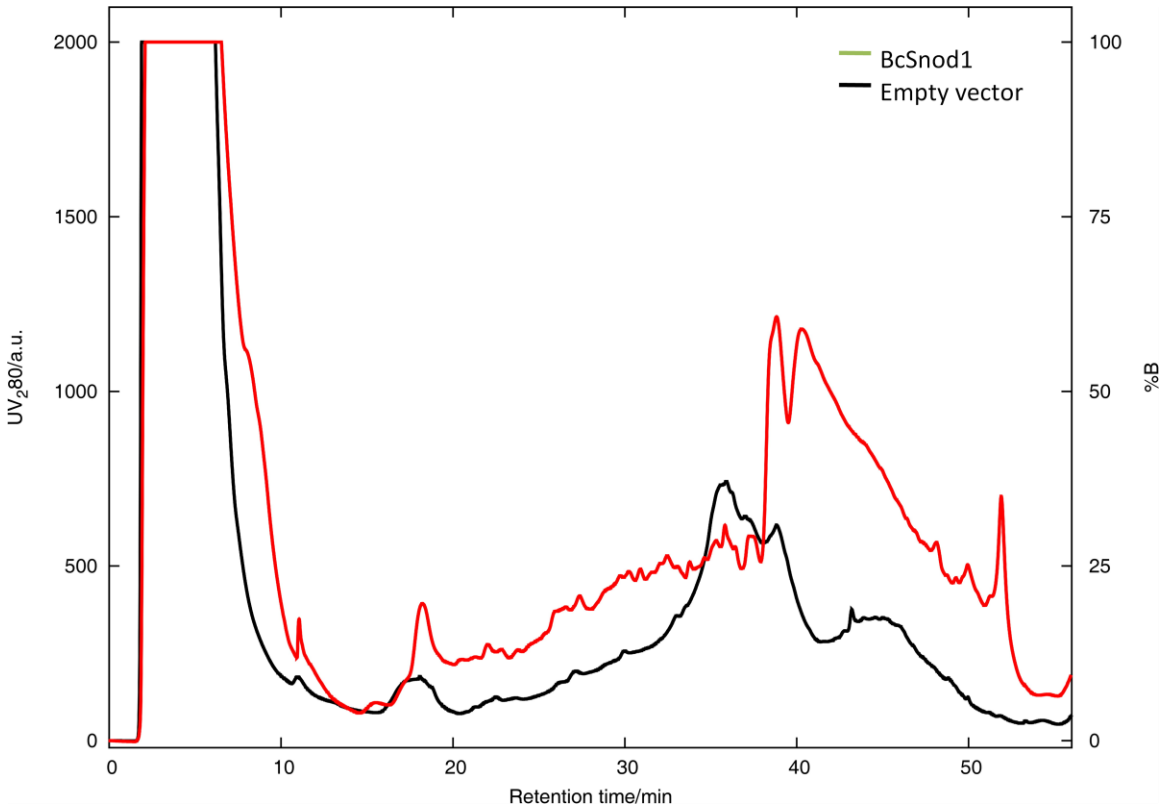
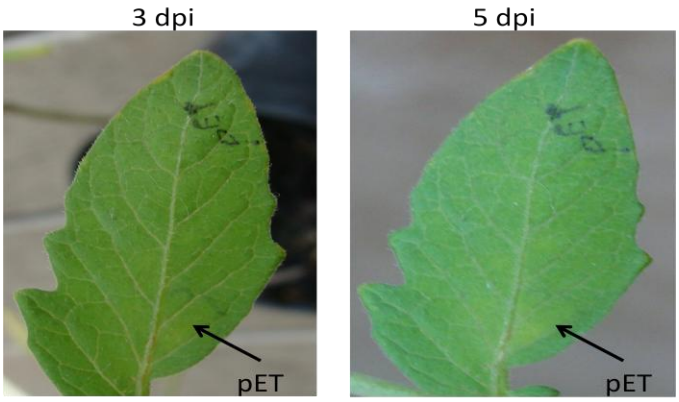


Figure 3.7: Chromatogram of HPLC purification of *Pichia* extract expressing BcSnod1 protein (red) and empty vector (black). The BcSnod1 protein was present in fractions 41-47.

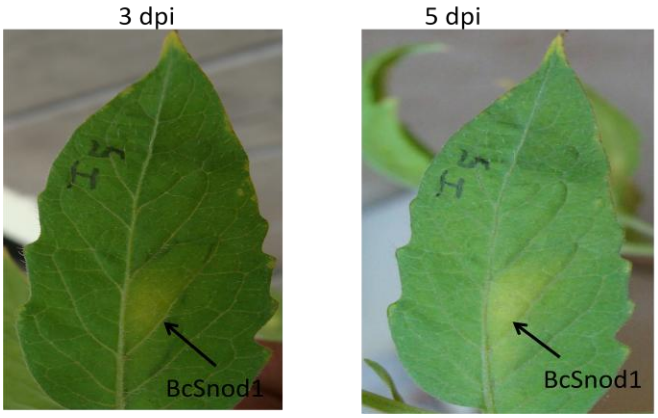
on the same branch of the plant. Tobacco plants which were 8 weeks old were used. Young leaves were used for testing the activity of the protein. BcSnod1 protein and control in the tobacco leaf were infiltrated in the same leaf. During *in planta* infiltration, purified proteins were injected in the mid vein of leaves.

In the tomato, the area injected with BcSnod1 from *E.coli* started to decolorize indicating a yellow spot after 3 days post inoculation (dpi); the same results were obtained with BcSnod2 from *E. coli* where the decolorization was observed after 3 dpi. Elution control from empty vector expression did not change the color on the leaves (Figure 3.8). This decolorization never turned into necrosis, which means cell death even after 10 dpi. 6 such attempts were made in which 4 from BcSnod1 indicated decolorization while 3 with BcSnod2 injection indicated decolorization.

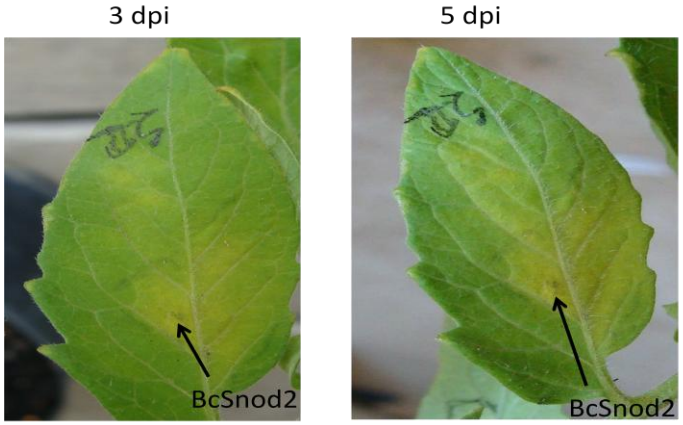
BcSnod1 protein obtained from *Pichia* when injected in tomato leaves starts to decolorize the area of infiltration within 3 dpi; this decolorization starts turning into necrosis indicating cell death after 10 dpi (Figure 3.9) and spreads in the adjoining parts after 15 dpi. Control extracts from *Pichia* expressing the empty pPic9k vector induced yellowing in the region of injection after 10 dpi; this yellowing did not spread in the adjoining regions of the leaves as seen in BcSnod1, even though some necrosis was observed in the area of infiltration. In tobacco leaves, the area of injection showed a hypersentivity response, which is lesion formation after 5 dpi and necrosis at 15 dpi, but this necrosis was limited to the area of injection of the protein and did not spread to any other leaf area even after 15 dpi (Figure 3.10).



A



B



C

Figure 3.8: *In Planta* Transient assay with protein from *E.coli* expression system. A Tomato leaves injected with elute obtained from *E. coli* having an empty vector (Control). B and C Leaves injected with BcSnod1 and BcSnod2 protein respectively indicating signs of discoloration after 3 and 5 dpi without any necrosis.

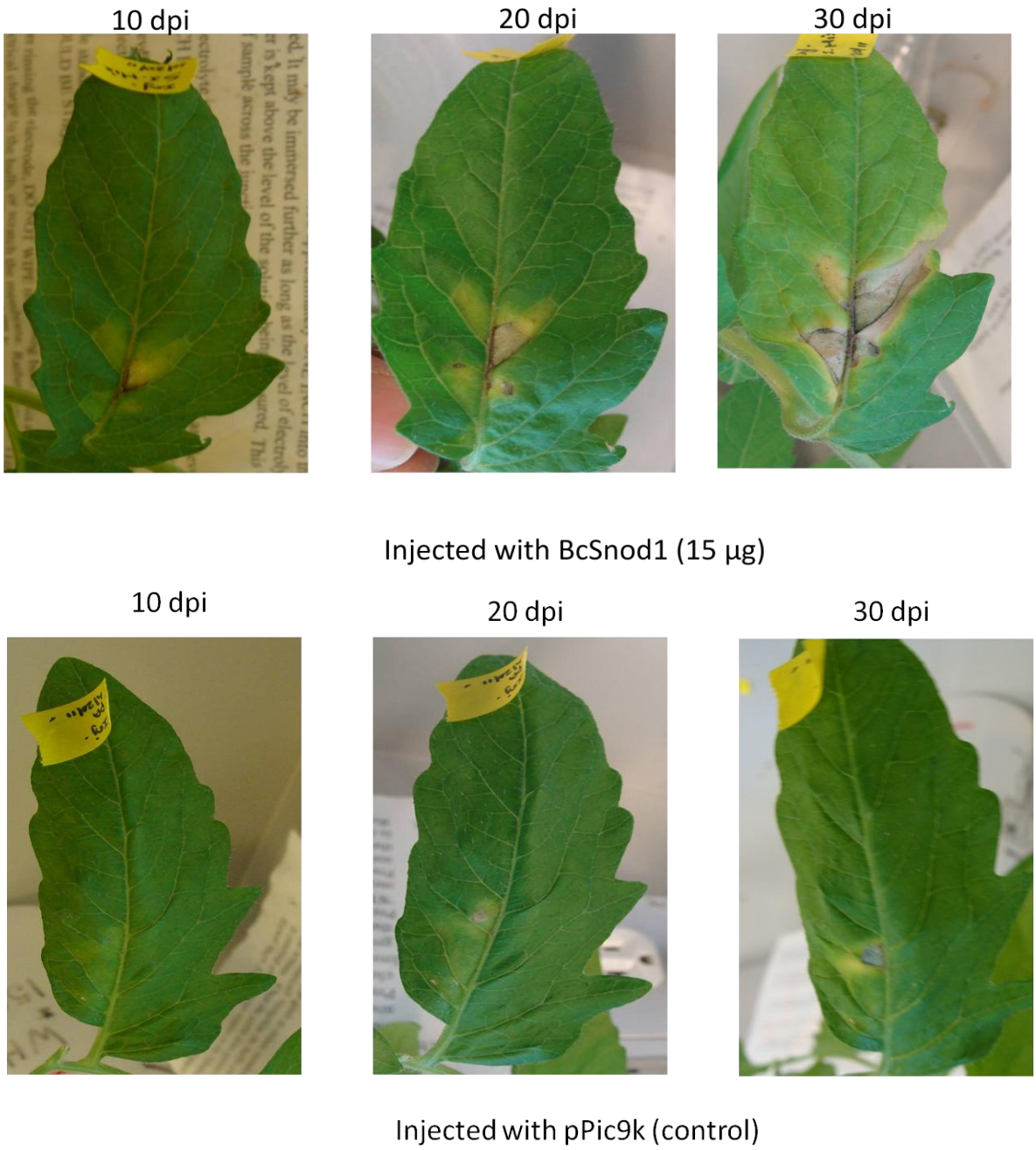


Figure 3.9: Tomato leaves injected with 15µg BcSnod1 protein and as a control the pPic9k elution was injected in same volume. Decoloration starts within 3 days of infection which continues up to 10 days, initiating necrosis in the decolored parts of leaves, leaving the whole injected part necrotized with 15 dpi. After 15 dpi, decoloration can be observed in adjacent parts of the injected area. The lower panel indicates leaves injected with pPic9k (control), where slight decoloration is observed after 15 dpi with some necrosis after 30 dpi. No spread of decoloration in the adjoining regions is observed.

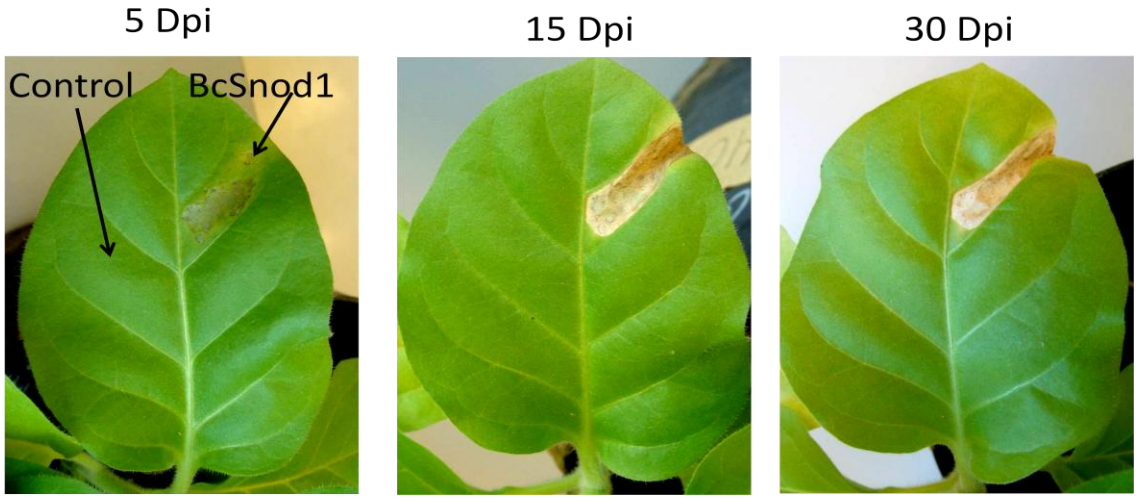


Figure 3.10: Tobacco leaves infiltrated with the BcSnod1 protein obtained from the HPLC fraction of concentrated supernatant from *Pichia* culture. In tobacco, hypersensitivity response can be observed from day 5 onwards while this response turns into necrosis killing plant cells after 15 dpi. But unlike the tomato, in tobacco, necrosis doesn't spread in the adjoining area of protein infiltration.

H. Detached Leaf Assay

Two types of detached leaf assay were performed, one with infiltration of protein in leaves and one with pipetting a drop of protein on the leaves. Healthy tomato leaves were cut off 4 week old plants for the detached leaf assay and placed in a moist box in an environmental chamber. In leaves with infiltration, a hypersensitivity response can be observed starting 1 dpi in the area injected with the BcSnod1 protein (Figure 3.11). Discoloration of the area of infiltration was observed after 3 dpi, which turns to maximum at 10 dpi. Some discoloration was observed in leaves infiltrated by the empty vector after 5 dpi, but the intensity was very low as compared to the BcSnod1 protein.

In detached leaves, a drop of solution containing 7 µg of protein was placed on the upperside of the leaf and showed signs of hypersensitivity response within 3 dpi; no necrosis was identified after 7 dpi (Figure 3.12). No more results were recorded as the leaves died 7-8 days after detaching from the plant.

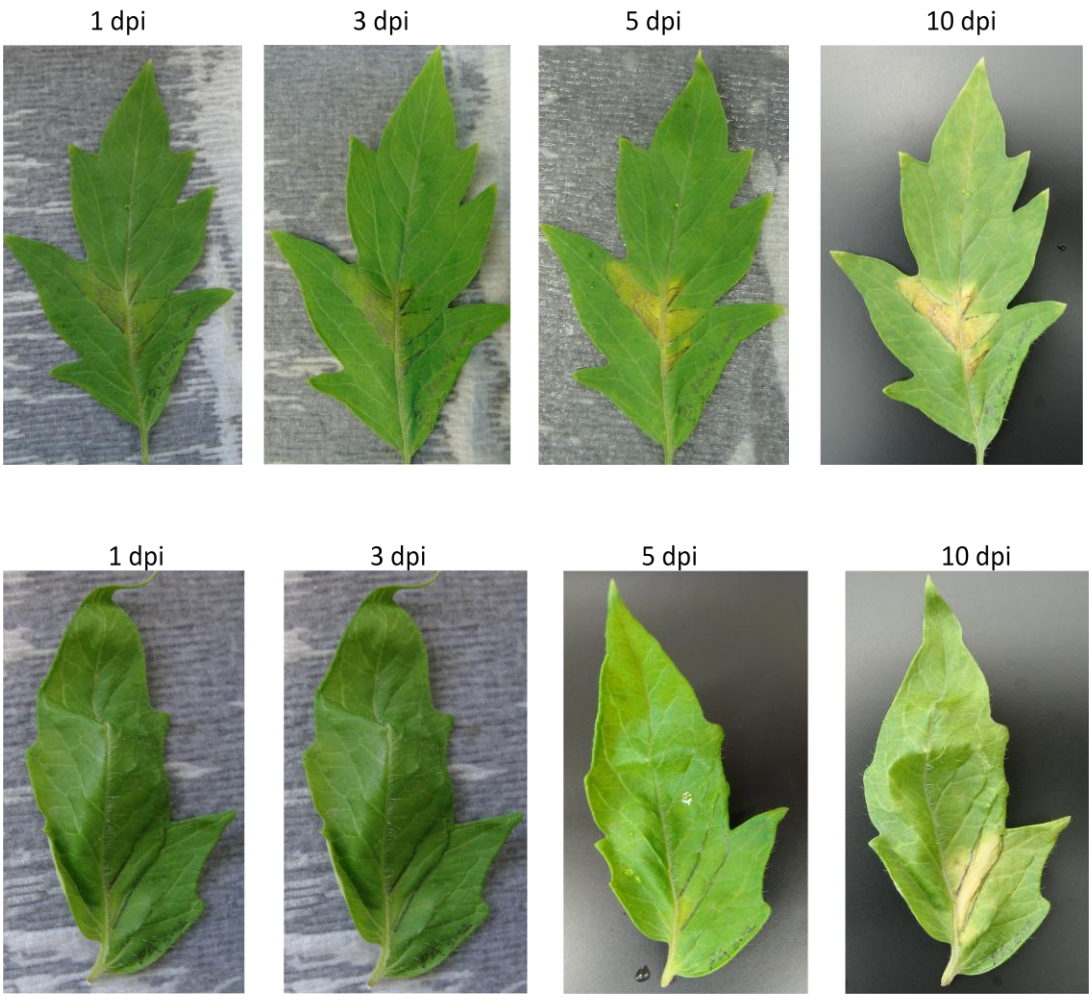


Figure 3.11: Tomato leaves detach leaf transient assay. Tomato leaves were infiltrated with the BcSnod1 protein and kept in a greenhouse. Discoloration in the area of injection can be observed after 1 dpi which increases with the number of days, showing maximum discoloration after 10dpi but no necrosis. The control, which was infiltrated with elution from pPic9k, started discoloration after 5 dpi.

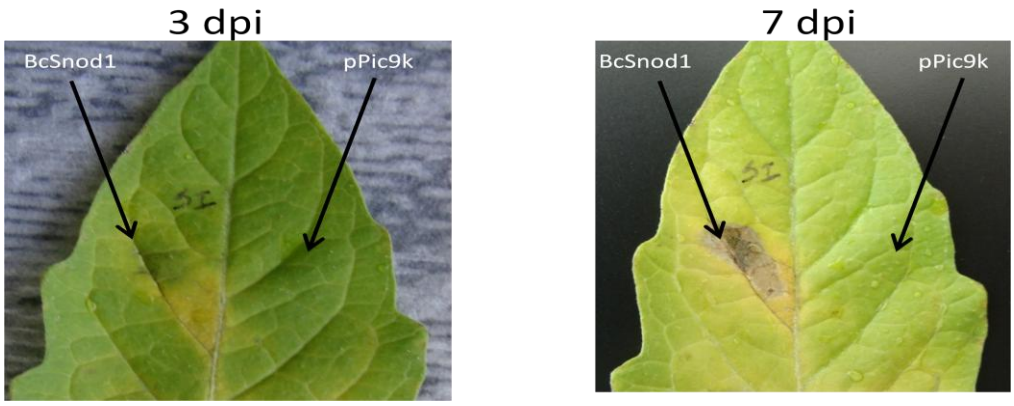


Figure 3.12: Tomato leaves detached leaf assay. 7 µg of BcSnod1 protein from the HPLC fraction was pipetted on upper surface of the leaf. HR starts 3dpi with little discoloration, and necrosis was observed 7 dpi.

Table 3.1: Tomato plant transient Assay. The result observed after treatment of tomato plant with the BcSnod1/2 protein obtained from *E.coli* and *Pichia* as well as control for them. The attempt column indicates the number of times the same procedure was repeated while the number in the result column indicates the times the same result was observed.

Method →	Infiltration (Tomato - *In planta*)			Pipetting (Tomato - *In planta*)		
Protein ↓	Attempt	Results observed		Attempt	Results observed	
E. coli BcSnod1	6	Late discoloration	4	6	Late discoloration	4
		No change	2		No change	2
E. coli BcSnod2	6	Late discoloration	3	6	Late discoloration	3
		No change	3		No change	3
E. coli Control	6	Late discoloration	0	6	Late discoloration	1
		No change	6		No change	5
Pichia BcSnod1	3	Necrosis	3	3	Necrosis	3
		No change	0		No change	0
Pichia Control	3	Late discoloration	1	3	Late discoloration	1
		No change	2		No change	2

Table 3.2: Tobacco plant transient Assay. The result observed after treatment of tobacco leaves with the BcSnod1 protein obtained from *Pichia* as well as control. 3 Attempts were made for each of the experiment; the number in the result column indicates the times the same result was observed.

Method →	Infiltration (Tobacco - *In planta*)			Pipetting (Tobacco - *In planta*)		
Protein ↓	Attempt	Results observed		Attempt	Results observed	
Pichia BcSnod1	3	Necrosis	3	3	Necrosis	3
		No change	0		No change	0
Pichia Control	3	Late discoloration	1	3	Late discoloration	1
		No change	2		No change	2

CHAPTER IV

DISCUSSION

Plant pathogen interaction starts with penetration and establishment of a colony in the host for which the pathogen secretes proteins to degrade the cell wall and cause cell death. Early interaction between the host and pathogen leads to secretion of defense molecules and expression of the genes related to the pathogenesis by the host (Alfano and Collmer, 2004). The pathogen in return secretes molecules or compounds which will disable the plant defense response and help the pathogen to establish an infection. During this course, phytoalexins are secreted by the plant to inhibit the growth of the pathogen as a mode of defense. These phytoalexins are degraded by the pathogen keeping its virulence invariable (Andrain *et al*., 1998). With the increase in virulence of the pathogen, plants may start programmed cell death to keep the infection localized (Greenberg, 1996). Programmed cell death is the result of secretions from the pathogen which causes the host cell to die causing necrosis. Necrosis by definition means the premature death of a cell, which is observed by formation of a brown-black ring around the death cells. Hypersensitivity response is another response observed in plants which are able to keep the pathogen under check causing lesion formation or discoloration at the point of interaction (Mitler *et al*., 2000). In both cases, the pathogen is known to be phytotoxic as it causes damage to the host. BcSnod1 causes necrosis in tobacco and tomato plants, making it phytotoxic in nature (Pontier *et al*., 1998).

A. Protein Expression

Bcsnod1 and BcSnod2 were expressed in *E. coli.* These proteins after extraction and purification had delayed HR response and no necrosis was observed. The delayed activity may be due to a misfolded protein as eukaryotic proteins were expressed in a prokaryotic system. The posttranslational modification gets affected as prokaryotes do not have mechanisms to make those changes. BcSnod1 and BcSnod2 proteins obtained from *E. coli* were insoluble in non denaturing lysis buffer and a denaturation procedure was used to solubilize the protein. The probability of correct disulphide formation during dialysis or renaturation is very low which may result in delayed activity.

BcSnod1 expressed in *Pichia pastoris* was active and able to induce cell death by necrosis of the area infiltrated concluding that BcSnod1 is phytotoxic. Similar phytotoxicity was observed when tobacco plants were treated with the ceratoplatanin protein from *Ceratocytis fimbrata* to confirm the activity of protein. *Pichia* is an eukaryotic expression system; the proteins are secreted through the secretory pathway. The vector used for cloning the BcSnod1 gene has a signal peptide which follows the secretory pathway and is secreted in the media. The amount of protein obtained by *Pichia* was six times greater than *E. coli*. The protein obtained was soluble in media.

B. Activity of Protein

BcSnod1 and BcSNod2 obtained from *E.coli* and *Pichia* had varied intensity of activity on tomato leaves. Proteins obtained from *E. coli* were able to decolorize the tissue but were not able to cause any necrosis. It took 5 dpi to observe the activity in the *E. coli* protein, but in contrast, the protein from *Pichia* was observed in 3 dpi. Comparing

the activity of other members of the ceratoplatanin family which take 24 hours to exhibit activity, BcSnod1 and BcSnod2 from *E. coli* took more time to exhibit any activity and it was not consistent (Pazzagli *et al*., 1999). The inconsistency in exhibiting the activity leads to hypothesize that the protein obtained from *E. coli* may not be modified properly.

The activity of BcSnod1 differed on different plant hosts as well as if the assay was performed *in planta* or on detached leaves as given in Table 3.1. In the tomato, the area where the protein was infiltrated initially decolorized and then necrotized with dead cells. This necrosis then started spreading in the adjoining regions which caused cell death in the adjoining region of the region of infiltration. The tobacco plant is different in that the necrosis or cell death can only be observed in the area of infiltration and does not spread in the adjoining region. For explaining these differences in the cell death in different plants, we may hypothesize that the tomato is the host of *B. cinerea* and hence the protein BcSnod1 may signal programmed cell death. This signal is carried out in the adjoining region causing cell death in areas other than the area of infiltration making the tomato more susceptible to BcSnod1. In the case of tobacco, it is a non-host of *B. cinerea* and the signal for programmed cell death may not be carried all the way to other region of leaves and it only affects the area of injection causing cell death in that specified area only.

Detached leaf assay in the tomato indicates that BcSnod1 is able to induce cell death in the detached leaves. The detached leaves exhibit cell death much early than the attached leaves. Since the leaves which are attached to the plants get a continuous supply of nutrient and food material, they are able to synthesize molecules necessary for defense response. The deatched leaves do not get any nutrients or food and hence the ability to

produce any defense related molecules is impaired which may lead to early cell death as compared to the attached leaves.

C. Possible Role of Ceratoplatanin in Plant-Pathogen Interaction

Ceratoplatanins are secreted early during plant-pathogen interaction. The possible role with necrotrophic fungi may be to initiate or signal programmed cell death (Fontana *et al*., 2006). During early interaction of plant and pathogen, the plant starts to defend itself by secreting some chemical compounds making the pathogen unable to grow. If the pathogen is still active, programmed cell death is initiated with the aim of keeping the infection localized and the pathogen under check.

In the *B. cinerea* infection, if the plant starts programmed cell death due to the presence of certaoplatanins, it may help the *B. cinerea* in spreading infection (Greenberg and Yao, 2004). Being a necrotrophic fungi, it needs the host cell to die before it can utilize all the macromolecules present in those cells; secreting ceratoplatanins might be the mode of action to kill the plant cells and initiate pathogenesis. Once pathogenesis is initiated, then BcSnod1 may be involved in necrotizing the tissue, expanding the area of infection by *B. cinerea* causing spread of grey mold.

D. Possible Outcomes of Ceratoplatanin Treatment on Host

The foremost defense mechanism of the plant defense system after pathogen recognition starts with the expression of pathogenesis related (PR) defense genes (Fontana *et al*., 2006). PR gene expression is accompanied by secretion of phytoalexins to inhibit the growth of the pathogen. Pathogens have evolved mechanisms to degrade the plant phytoalexins, so the host moves to the second phase of defense which is secretion of

reactive oxidative species (ROS). Release of ROS causes a change in the Calcium flux in the host which ultimately leads to programmed cell death as a final mode of defense to prevent the spread of infection. After initiation of pathogenesis, there are many other genes which are expressed to prevent the initiation and spread of disease.

E. Future Studies

Future studies may involve elucidating the pathway by which the host leads to PCD. This may include confirming the elicitation of phytoalexins due to the BcSnod1 protein. ROS detection will confirm the usual pathway for PCD which includes the secretion of phytoalexins, followed by ROS secretion leading to calcium flux, resulting in programmed cell death. As we have concluded that BcSnod1 is phytotoxic in nature, it is possible that it has a certain role in pathogenecity or may be one of the compounds that help the pathogen in pathogenecity. Knocking out experiments can be carried out so as to determine the role and vitality of BcSnod1 in the pathogenesis of *B.cinerea* (Jeong *et al.,* 2007). Other than this, structural studies can be useful to know the characteristics of the protein. Plants expressing ceratoplatanins at very low levels are resistant to some other fungi, BcSnod1 gene can also be expressed in a tomato plant at very low levels and its resistance to *Botrytis* or other pathogenic fungi can be determined (Yang *et al*., 2006).

We were able to clone BcSnod1 in pPic9k with a His tag on the N-terminal so as to carry the pull down assay. The his pull down assay may help to find the interacting protein in the host, thereby providing an idea about the mechanism of recognition of fungal protein by the plant.

CHAPTER V

CONCLUSIONS

Leaf transient assay by purified BcSnod1 protein proved that BcSnod1 is phytotoxic in nature like other members of the ceratoplatanin family. *B.cinerea* secretes two certoplatanin proteins, out of which BcSnod1 (BC1G_02163.1) has the phytotoxic activity while the activity of BcSnod2 still remains questionable as BcSnod2 (Bc1G_08735.1) purified from *E.coli* did not have consistent phytotoxic activity. BcSnod1 purified from *E.coli* had a delayed activity and was unable to cause necrosis while BcSnod1 from *Pichia* exhibited early cell death and was able to cause cell necrosis. BcSnod1 activity varies with host in regards to spread of necrosis in the area of infiltration and adjoining regions. Tomato is more susceptible to BcSnod1 as compared to tobacco. The ability of the host to secrete phytoalexins and ROS leading to PCD due to the BcSnod1 protein may be a possibilty as ceratoplatanins from *Ceratocystis fimbrata* are able to elicit the secretion of phytoalexins.

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