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I am submitting herewith a thesis written by Nicole A. Cardwell entitled "Comparison and Characterization of Chitinase in *Cornus* Species." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Ornamental Horticulture and Landscape Design.

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COMPARISON AND CHARACTERIZATION OF CHITINASE IN *CORNUS* SPECIES

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Nicole A. Cardwell

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Abstract

This research has demonstrated that there is a significant difference between a Dogwood Anthracnose resistant *Cornus* species than a susceptible species in chitinase activity following inoculation with *Discula*. *Cornus mas* (resistant) and *Cornus florida* (susceptible) were inoculated with *Discula destructiva*, (cause of Dogwood Anthracnose disease) and leaves were collected over a 12 day period. Protein from the inoculated leaves was extracted and assayed colorimetrically and enzymatically for chitinase in this investigation. Chitinase characterization was accomplished through the use of the Phastsystem to determine the iso-electric points and SDS-polyacrylamide gel electrophoresis for the molecular weight determinations.

Colorimetric assay indicates chitinase activity in *Cornus mas* is expressed earlier than *Cornus florida* following infection. The enzymatic assay indicates the presence of activity over all days post-inoculation in both *Cornus mas* and *Cornus florida*. Two iso-electric points were determined for *Cornus florida* and three determined for *Cornus mas*. By using SDS-polyacrylamide gel electrophoresis, the molecular weights were identified as 65 +/- 5 kDa and 21 kDa in for both *Cornus* species. The iso-electric points were determined to be 5.6, 6.8, and 8.9 for *Cornus mas* and 5.6 and 6.8 for *Cornus florida*.

Table of Contents

Chapter		Page
I.	Introduction	1
II.	Literature Review	3
	Discula destructiva Disease	3
	Plant Response to Fungal Infection	5
	Chitinase	6
	Glucanase	9
	Characterization of Chitinase	10
	Implications	11
III.	Materials and Methods	13
	Inoculum Production	13
	Plant Culture	13
	Inoculation Procedure	14
	Protein Extraction Using Ammonium Sulfate	15
	Protein Extraction Without Ammonium Sulfate	16
	Chitinase Assay	16
	Electrophoresis	17
	Iso-electric Focusing	18
	Preliminary Test of Chitinase With *Discula destructiva*	18
IV.	Results	19
	Colorimetric Chitinase Assay	19

Continued Table of Contents

Chitinase Using Overlay Polyacrylamide Gel.....21

Preliminary Test of Chitinase With *Discula destructiva*.....29

V. Discussion.....30

References.....37

Appendix.....49

Vita.....59

List of Figures

Figure Page

1. Detection of chitinase activity in ammonium sulfate precipitated proteins at days 4, 6, 8, 10, and 12 after inoculation in *Cornus mas*.....20
2. 7.5% glycol chitin polyacrylamide gel of *Cornus mas*.....22
3. 7.5% glycol chitin polyacrylamide gel of *Cornus florida*.....23
4. 7.5% glycol chitin overlay gel of a 15% polyacrylamide gel with *Cornus mas* and *C. florida*.....25
5. 7.5% glycol chitin overlay gel of 10% polyacrylamide gel with boiled and non-boiled samples *Cornus mas*.....26
6. 7.5% glycol chitin overlay gel of a 10% polyacrylamide gel of *Cornus mas* and *Cornus florida* boiled and non-boiled samples.....27
7. Phastsystem glycol chitin overlay gel containing *Cornus mas* and *Cornus florida*.....28

Chapter 1

Introduction

The flowering dogwood, [*Cornus florida* (L.)] is a valuable native species and is considered one of the most highly prized ornamental flowering tree in the United States. The tree can be found throughout the eastern United States from Maine to Georgia and is an important horticultural crop.

In the last 20 years, several *Cornus* species have been diagnosed with a devastating pathogen known as *Discula destructiva* (Redlin), cause of the Dogwood Anthracnose disease. This host-pathogen relationship usually cause an hypersensitive response in resistant species and death in susceptible species.

Since the seventies, research has emphasized epidemiological control strategies, by studying the dissemination and ecology of the pathogen. These types of studies have been useful, but now emphasis must be placed on the characterization of genetic resistance in dogwood and on understanding pathogenesis-related proteins that are implicated in this process.

The study of pathogenesis-related proteins may provide important information on resistant versus susceptible *Cornus* species, such as *Cornus mas* (L.) and *Cornus florida*. These proteins have been demonstrated to have antifungal properties in other host pathogen interactions. The

potential role of pathogenesis-related proteins has not been examined in *Cornus* species. *Cornus mas* was chosen for this study because it is considered a dogwood anthracnose resistant species. *Cornus florida* was chosen because it is a susceptible species and has the greatest commercial landscape value. Therefore, the objectives of this study were to identify and characterize pathogenesis-related chitinases in *Cornus mas* and *Cornus florida*.

Chapter II

Literature Review

This research was conducted to compare the chitinase enzymes produced by *Cornus* species that are either resistant or susceptible to *Discula destructiva*, cause of the Dogwood Anthracnose. The first section discusses the disease of *Cornus* species. Some properties of pathogen-related proteins (PR Proteins), with an emphasis on chitinases and how they are involved in plant defense against disease will be addressed. This review concludes with a discussion of experimental approaches, results, and prospects.

***Discula destructiva* Disease:** For the past two decades many *Cornus* species have suffered from a devastating pathogen known as *Discula destructiva* (Redlin and Scott, 1991). *Discula* sp. was initially diagnosed in Vancouver in 1976 (Daughtrey and Hibben, 1994). By 1991, dogwood trees in the US and Canada were already suffering from this pathogen for about 10 to 15 years (Redlin, 1991). The disease has spread to the north and south and has been reported in Oregon, British Columbia, northern Idaho and even northern California. The pathogen has also taken a devastating path through New York, Connecticut, Pennsylvania, New Jersey and 60 counties of eastern states (Daughtrey and Hibben, 1994). *Discula destructiva* originated from infected plants brought

into New York harbors by ships from the West Coast. The pathogen (*Discula destructiva*) causes necrosis, leaf spot, blight and twig dieback and eventual death of infected dogwoods (Hibben and Daughtrey, 1988). The first symptoms of *Discula* can be identified on the leaves of a *Cornus* species. The leaf symptoms appear within four weeks following flowering in *Cornus florida* (Daughtrey and Hibben, 1994; Hibben and Daughtrey, 1988) and include visually, from necrosis at tips of newly formed leaves to angular leaf spots (Daughtrey et al. 1996) surrounded by reddish-to-purple brown zones (Daughtrey and Hibben, 1994) that are necrotic at the tip or along the blade periphery (Daughtrey and Hibben, 1994). These symptoms also include blight, where the entire leaf quickly becomes necrotic or chlorotic (Daughtrey et al., 1996).

Discula is considered a persistent pathogen because of its diverse infection sites and abundant inoculum (Hibben and Daughtrey, 1988). The inoculum of *Discula* remains on the leaf and overwinters to provide new infection cycles the following spring (Daughtrey et al., 1996). The *Discula* spores (conidia) can be dispersed by rain, beetles (Colby et al., 1995) and birds (Daughtrey et al., 1996).

The genus *Cornus* contains three species that are most susceptible to dogwood anthracnose: *Cornus florida*, *C. nuttallii* (Audubon) (Daughtrey and Hibben, 1994), *C. stolonifera* (Michx.) and some genotypes of *C. kousa* (Hance)

(Windham et al., 1996). Cornus sericea (L.), C. controversa (Hemsl.), C. kousa var *chinensis* (Osborn) are susceptible when exposed to high inoculum conditions and high humidity. *Cornus mas*, *C. amomum* (Mill) and *C. alternifolia* (L. f.) are reported resistant to the disease (Daughtrey and Hibben, 1994).

There have been several impact studies reported on *Cornus florida*, with tree mortality progression 33% in 1984 to 79% in 1988 in a study of Catoctin Mountain National Park in Maryland. Tree mortality has increased and was estimated at 0.2 million in 1988 (0.5 million acres) to 7.0 million in 1993 (17.3 million acres) (Daughtrey et al., 1996) estimated from plots located in Virginia, North Carolina, South Carolina, Georgia, Kentucky, Alabama and Tennessee. Tree mortality has increased 23% over this period (Anderson, 1991; Knighten and Anderson, 1991; Daughtrey et al., 1996).

Plant Response to Fungal Infection: Infection of plants by a fungal pathogen leads to a host response that varies from mild to extreme (Linthorst, 1991). A mild response does not lead to a high levels of gene expression, but a severe response leads to massive production of pathogenesis-related proteins, chitinase and β-1,3-glucanase.

The proteins chitinase and β-1,3-glucanase are two possible proteins that could offer genetic resistance

against *Discula destructiva* infection. Both proteins have been implicated in the disease response in other host-pathogen interactions. The enzymatic product of the expressed genes can degrade an invading hyphal strand when penetration occurs in the host tissue (Cutt and Klessig, 1992). To enhance resistance, chitinase and β-1,3-glucanase genes have both been transferred into non-resistant *Nicotiana* tissue and have provided some resistance to an invading pathogen (Broglie et al. 1994).

Chitinase: Chitinase (poly(1,4-(N-acetyl-β-D-glucosaminide)) glycanohydrolase, EC 3.2.1.14) has been implicated in plant defense against fungal invasion. Chitinases are believed to be an important defense of plants against some fungal pathogens (Boller, 1987, Abeles, 1970) and are found in a large number of seed-producing plants (Graham and Sticklen, 1993).

The physiological function of chitinase in plants is unknown but there is sufficient evidence for them to be considered defense proteins (Graham and Sticklen, 1993). Since chitin, a polymer of N-acetyl-glucosamine (NAG), is the substrate for chitinase, it is postulated that this enzyme has an antifungal function (Boller, 1987). Chitin is found in the cell walls of many fungal pathogen (Mauch et al. 1988) and purified forms of chitinase have been found to partially digest cell walls of invading pathogenic

fungi (Boller, 1987). Chitinase activity can be induced by wounding, bacterial or fungal pathogens (Majeau et al. 1990; Roby et al. 1990; Graham and Sticklen, 1993), ethylene (Roby et al. 1985, 1991; Graham and Sticklen, 1993), viral infection and toxic chemicals (Graham and Sticklen, 1993).

Plant chitinases isolated so far have been mostly endochitinases such as in *Cucurbita* sp. (pumpkin) (Esaka et al., 1990), *Glycine max* (L.) (soybean) (Wadsworth and Zikakis, 1984) and *Nicotiana tabacum* (L. Hengh.) (tobacco) (Legrand et al., 1987) Endochitinases are enzymes that hydrolytically degrade chitin within the polymeric sugar backbone while exochitinases degrade the chitin from its termini (Graham and Sticklen, 1993). There have been reports of exochitinases found in *Cucumis melo* (L.) (melon) (Roby and Esquerre'-Tugaye', 1987), *Beta vulgaris* (L.) (sugarbeet) (Nielson et al., 1993) and *Daucus carota* (L.) (carrot) (Kurosaki et al., 1989). Exochitinases are not as abundant as endochitinases but, appear to have chitinolytic activity. (Graham and Sticklen, 1993).

Chitinases are expressed constitutively in healthy tissue that has not been challenged by a pathogen but its expression increases with fungal attack (Graham and Sticklen, 1993; Boller, 1987). Some chitinases are only produced constitutively (Beerhues et al. 1990) but many are inducible (Graham and Sticklen, 1993). Induction of

chitinase results in an increase of mRNA and is regulated at the level of transcription (Boller, 1987, 1988; Broglie et al. 1986; Hedrick et al. 1988; Herget et al. 1990; Nishizawa and Hibi, 1991; Graham and Sticklen, 1993). Chitinase accumulates within the vacuoles and extracellular space (Broglie et al., 1994; Bol et al., 1990; Fink et al., 1988; Parent and Asselin, 1984).

Chitinases have been reported to fall into 3 to possibly 4 classes to date (Graham and Sticklen, 1993; Shinshi et al., 1990):

Class 1 chitinase has been purified from leaves, stems and fruits of many plants such as *Phaseolus vulgaris* (L.) (bean leaves) (Boller et al., 1983; Graham and Sticklen, 1993), *Nicotiana* (tobacco) (Legrand et al. 1987; Graham and Sticklen, 1993), and *Pisum sativum* (L.) (pea) pods (Mauch et al., 1988a, 1988b; Graham and Sticklen, 1993). Usually class 1 chitinase is found in the vacuole (Dore et al., 1991; Keefe et al., 1990; Mauch and Stahelin, 1989; Graham and Sticklen, 1993) but some are also located extracellularly such as in *Hordeum vulgare* (L.) (barley) (Swegle et al., 1989) and pea (Vad et al., 1991)

This first class of chitinase accounts for the majority of the chitinolytic activity in the host tissue in which acidic and basic isoforms exist (Graham and Sticklen, 1993). The basic chitinase isoform is reported to accumulate in the vacuole of bean (Boller and Vogeli, 1984;

Dore et al., 1991; Mauch and Stahelin, 1989; Graham and Sticklen, 1993) and tobacco cells (Keefe et al., 1990; Graham and Sticklen, 1993). The literature suggests that chitinase is located in the vacuoles because cells accumulate chitinase as a result of an infection response in a large quantity and are able to release this chitinase if the cell is lysed (Graham and Sticklen, 1993; Cutt and Klessig, 1992).

Class II chitinases are reported to be acidic and located extracellularly (Graham and Sticklen, 1993; Benhamou et al., 1991). This class of chitinase appears to be involved in first defense, releasing elicitors from an invading pathogen (Mauch and Stahelin, 1989) and acting as a signal to elicit class 1 chitinase (Graham and Sticklen, 1989).

Class III chitinases accumulate in the host leaf fluid (Graham and Sticklen, 1993) and have been located in *Arabidopsis, Vigna angularis* (Endl.) (Azuki bean plant) and *Cucumis sativus* (cucumber). Class IV chitinases appear to be located extracellularly such as in *Beta vulgaris* (L.) (sugarbeet) (Rasmussen et al., 1992) and appears to have an antifungal role (Graham and Sticklen, 1993), much as class I chitinases.

Glucanase: β-1,3-glucanase (EC 3.2.1.39, glucanase) was one of the first enzymes described to be a pathogenesis-

related protein (Cutt and Klessig, 1992). It has been proposed that glucanases release β-1,3-glucan fragments from an invading hyphal strand which in turn elicit chitinase production (Mauch and Stahelin, 1989; Keen and Yoshikawa, 1983; Hahn et al., 1989; Takeuchi et al., 1990).

There are two types of glucanases found in plant tissue, acidic and basic. Acidic glucanase accumulates in the vacuoles of leaves (Bol et al., 1990; Fink et al., 1988; Parent and Asselin, 1984). The onset and level of glucanase is positively correlated to the level of pathogen resistance. For example, muskmelon and tomatoes infected with *Fusarium* exhibit a more rapid expression of glucanase in resistant cultivars, showing that glucanase has an obvious role in achieving defense (Linthorst, 1991).

Characterization of Chitinase: Most research on chitinases have been enzymatic studies with recent work dealing with transgenic plants. Characterization of the enzymes has been accomplished through the use of SDS-PAGE, chromatofocusing, and iso-electric focusing. This type of research has identified molecular weights (MW) and iso-electric points (pI) of various chitinases from different plant species.

Chitinase has been identified to have a MW range of 20-45 kDa and pI in the acidic and basic range. For example, the molecular weight of a chitinase from

Arabidopsis thaliana was identified to be 26 kDa (Verburg and Huynh, 1991), and of *Hordeum vulgare* (L.) (Barley) to be 26 kDa (Leah et al, 1991; Jacobson et al., 1991). The molecular weight of chitinases from *Pisum sativum* (pea) leaves; was 34 and 25 kDa (Vad et al., 1991), pods; 33.1, 36.2, 39.0 kDa (Mauch et al., 1988a). In *Avena sativa* (L.) (oat) the molecular weight was 29.8 kDa (Fink et al., 1988). Chitinases from all of the above plants have been identified as class I, except for oat, where it was identified as a class II (Graham and Sticklen, 1993).

To determine whether chitinases are acidic or basic, ion exchange chromatography (IEC) has been used. The following are examples of IEC, pI determinations on various plants: *Hordeum vulgare* (Barley), 9.3. 9.2, 8.7 (Ishige et al., 1991); pea pods, 9.1, 8.9, 9.3 (Mauch et al., 1988a). *Beta vulagaris* (sugarbeet) chitinases were identified as a class III with two isoforms with a pI of 3.0 (Neilson et al., 1993; Graham and Sticklen, 1993).

Implications: Chitinase and glucanase, when induced by various pathogens or environmental stresses, appear to be involved in a number of defense reactions, including the direct attack on the invading pathogen (Boller et al., 1990). Chitinase and glucanase are constitutively expressed in host tissue, and both are usually required for inhibition of fungal growth (Linthorst, 1991, Cabello et

al., 1994). Tests with pea chitinase and glucanase reveals that a combination of both enzymes are required for inhibition of fungal growth (Mauch et al., 1988). This requirement of both chitinase and glucanase indicates that the fungal hyphal cell wall contains β-1,3-glucan polymers and chitin (Mauch et al., 1988). The following scheme applies: β-1,3-glucanase degrades β-1,3-glucans to expose the chitin cell wall, then the chitinases degrade the chitin, thereby destroying the hyphal tips.

Chapter III

Materials and Methods

Inoculum Production: An isolate of *Discula destructiva* Georgia-1 (GA-1) obtained from Dr. Robert Trigiano (The University of Tennessee, Knoxville) was used throughout these experiments. Plugs, approximately 1/4" in diameter, of the GA-1 were transferred onto potato-dextrose V-8 juice agar (PDV8) plates (Dhingra and Sinclair, 1985). An autoclaved dogwood leaf was located on the center of each plate which was to receive a *Discula* plug. The dogwood leaves had been collected from *Cornus mas*, placed in a 3" X 3" X 3" magenta GA-7-3 vessel (Sigma-Aldich, St. Louis, MO) and autoclaved for twenty minutes over two consecutive days. Plates were sealed with parafilm and incubated at 16 hour light and 8 hour dark at 17°C.

Discula sporulation was induced by transferring fungal plugs a second time onto potato-dextrose agar (PDA) in magenta boxes which contained autoclaved *Cornus mas* twigs. The dogwood twigs were autoclaved as with the dogwood leaves, then placed on the PDA before solidification.

Plant Culture: Twenty-five one-year-old *Cornus mas* and *Cornus florida* seedlings, provided by Dr. Willard Witte, (The University of Tennessee, Knoxville) were planted in 100% pine bark in two gallon pots on February 1, 1996.

After one month, they were placed in a greenhouse with short days and long nights. The greenhouse was held at 21°C day and night. Plants were fertilized 4 times with Peter's 20-20-20 soluble fertilizer with (20 N, 8.8 P, 16.6 K) with 300 ppm nitrogen over the entire period just after the plants broke dormancy.

Following five weeks of leaf development, 12 *Cornus mas* and *Cornus florida* were moved to a polyethylene plastic enclosed bench in a greenhouse. The greenhouse bench was covered with a 75% shade cloth and contained two humidifiers and an air conditioner. The inoculation bed kept a temperature of 20-30°C and high humidity conditions. All of the dogwoods were placed in the bench for 24 hours.

Inoculation Procedure: Leaves of *Cornus mas* and *Cornus florida* were wounded with a floral needlepoint holder (floral frog). The floral frog was surface-sterilized before wounding leaves. All leaves were wounded and a 1/4-inch borer was used to remove plugs of the *Discula* (GA-1) for use in the inoculation of the dogwood leaves. Plugs were applied to the leaf surfaces by applying gentle pressure and rubbing the fungi into the leaf surface. Leaves were then enclosed with plastic sandwich-size bags (16.51 cm X 14.92 cm). The plastic bags remained on the leaves during the entire experiment to create high humidity conditions. Control plant leaves were wounded with the

floral frog and inoculated with ddH20 and PDA. These control leaves were enclosed in plastic bags and remained attached during the entire experiment.

Approximately 5-10 leaves of inoculated and non-inoculated were collected at 12 hours, 1, 2, 4, 6, 8, 10, and 12 days following inoculation. Samples were placed in a plastic bag and immediately placed in liquid nitrogen and stored at -80°C until used.

Protein Extraction Using Ammonium Sulfate: Dogwood leaves were placed in a pre-chilled mortar, polyvinylpyrrolidone (PVP) (see appendix) added to each sample, and the mixture was then ground with the pestle into a fine powder. Extraction buffer (see appendix) was added (3X the sample) and the sample was immediately re-ground. The sample was allowed to thaw and centrifuged twice at 14,000 rpm (23.5 g) (see appendix). Following centrifugation the supernatant was removed and placed in an Oak Ridge tube (Nalgene, Rochester, NY). The supernatant was saturated to 40% followed by 60% ammonium sulfate on ice for 1-2 hours and then centrifuged for 30-40 minutes at 14,000 rpm (23.5 g). The supernatant was removed and the pellet was redissolved in water.

The solution was dialyzed against water overnight in molecular porous membrane tubing, of 3,500 kDa (Spectrum Medical Industries, Inc., Laguna Hills, CA). Protein

concentration was determined by the Bradford method (Bradford, 1976) at 595nm with a Shimadzu UV VIS-160A Recording Spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Samples were then stored at -20°C until use.

Protein Extraction Without Ammonium Sulfate: Protein to be used for electrophoresis were extracted as explained above with the exception of ammonium sulfate was not added. Samples (100 μl) were stored at -80°C until use.

Chitinase Assay: Chitinase activity was assayed using a modification of the Sigma protocol (See Appendix). The protein sample (0.8-0.9 mg/ml) (see appendix) was dissolved in 2 ml of a 1.25% w/v chitin suspension in a test tube and incubated at 45°C for 3 hours on a rotary shaker. The sample was boiled for 5 minutes and placed on ice to allow cooling to room temperature. One unit of β-N-acetylglucosaminidase (Sigma, St. Louis, MO.) was added to the solution and the sample incubated for 30 minutes at 45°C on a rotary shaker. Samples were then centrifuged at 2500 rpm (10.6 g) for 10 minutes. Supernatant was removed and centrifuged at 14,000 rpm for 10 minutes. One milliliter of sample was removed and placed in a test tube which contained 2 ml ddH20 and 1.5 ml of color reaction solution (see appendix). The solution was boiled for 10-15 min. and placed on ice to allow it to cool to room

temperature. Chitinase content was determined using a Shimadzu UV-VIS Recording Spectrophotometer at 540nm.

Electrophoresis: Protein samples were electrophoresed according to Pan et al. (1991) with some modifications. Samples were electrophoresed in either 10% or 15% native polyacrylamide resolving gel at 30mA constant current for 2-8 hours at 8°C using a Bio-Rad Protein II unit (Bio-Rad Laboratories, Hercules, CA). Gels were overlayed with a 7.5% polyacrylamide gel containing 0.04% glycol chitin following electrophoresis and incubated for 4-12 h at 45°C under moist conditions. Each overlay gel was incubated with 0.01% (w/v) Calcofluor white M2R (fluorescent brightener 28) in 0.5M Tris-HCl (PH 8.9) for 5 minutes and then placed at 15°C overnight. Protein bands were visualized with a UV transilluminator and photographed with Polaroid MP-4 unit or a ISO-1000 Digital Imaging System (Alpha Innotech Corporation, San Leandro, CA).

After electrophoresis the chitinase band was removed and electrophoresed in a 10% acrylamide gel at 200 V for 6 hours with the addition of Kaleidoscope Prestained Protein

Standard (Bio-Rad Laboratories, Hercules, CA) to determine the molecular weight.

Iso-electric focusing: Protein samples that were precipitated without the use of ammonium sulfate were dialyzed against water overnight at 4°C to prepare for iso-electric focusing. Protein samples were focused in a pre-cast, IEF phastgel ampholyte pH 3-9 range using a Phastsystem (Pharmacia Biotech, Piscataway, NJ).

Samples were focused at 2000 V, 2.5mA, 3.5 W, 15°C for a total of 410 VH (Phastsystem file # 100). Following electrophoresis, each pre-cast gel was overlayed with glycol chitin and incubated as described earlier.

Preliminary Test of Chitinase With Discula destructiva: One isolate of GA-1 on PDA was used for this experiment. Under sterile conditions, chitinase (Sigma) was applied to 4, 1/8" squares of Whatman filter paper at a rate of 1 μl, 2 μl, 5 μl and 10 μl. The chitinase was then placed around the actively growing mycellium, of the plate, of GA-1. *Discula* plates were incubated for 4 days at 16 hours light, 8 hours dark and 17°C. Visual inspection of the plate occurred every 24 hours for 3 days.

Chapter IV

Results

Colorimetric Chitinase Assay: Proteins precipitated with ammonium sulfate was assayed for chitinase using a modification of Sigma Quality Control Test Procedure for assaying chitinase (Sigma, St. Louis, MO). Activity of chitinase was measured after 12 hr, 1, 2, 4, 6, 8, 10, 12 days following inoculation with *Discula destructiva* GA-1 as described earlier.

Chitinase was detectable in *Cornus mas* after 4, 6, 8, 10, and 12 days following inoculation with *Discula*. The following chitinase levels were recorded (Figure 1): 4 days - 0.146 units/ml protein; 6 days - 0.102 units/ml protein; 8 days - 0.06 units/ml protein; 10 days - 0.140 units/ml protein; 12 days 0.102 units/ml protein. Chitinase was detected in *Cornus florida* (Figure 1) only on day 8 at a rate of 0.012 units/ml protein sample. Non-inoculated *Cornus mas* demonstrated chitinase activity (Figure 1) at day 4 - 0.203 units/ml protein; and day 12 - 0.224 units/ml protein. There was no detectable chitinase activity in non-inoculated *Cornus florida*. (Units Definition: One unit will liberate 1.0 mg of N-acetyl-D-glucosamine from chitin per hour at pH 6.0 at 40° C in a two-step reaction with β-N-acetylglucasaminidase.)

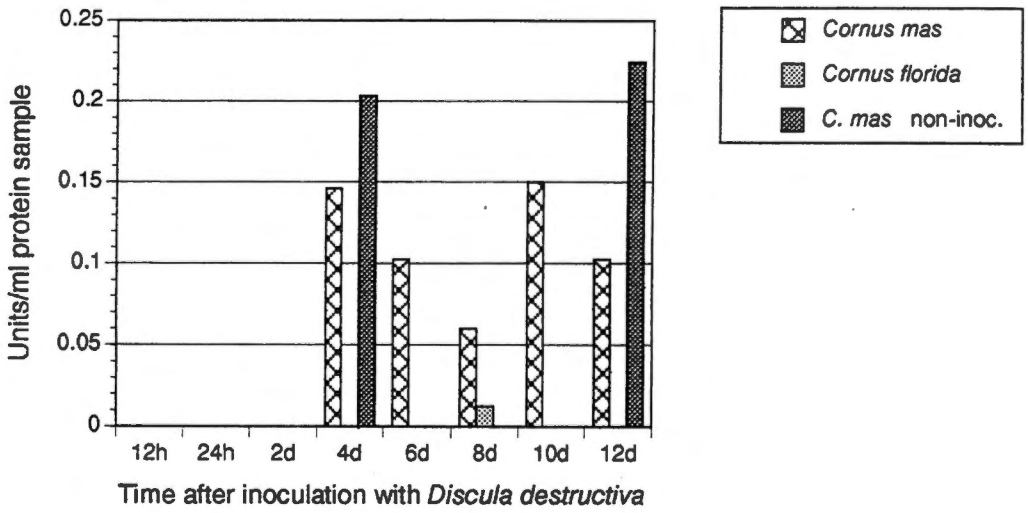


Figure 1. Detection of chitinase activity in ammonium sulfate precipitated proteins at days 4, 6, 8, 10, and 12 after inoculation in *Cornus mas*. Chitinase activity in *C. florida* was only detectable at day 8. Chitinase activity was also detected at days 4 and 12 in non-inoculated *C. mas*.

Chitinase Assay Using Overlay Polyacrylamide Gel: Proteins extracted without ammonium sulfate precipitation were electrophoresed in either a 10% or 15% polyacrylamide gel and overlayed with 7.5% glycol chitin in polyacrylamide overlay. Glycol chitin is the substrate for chitinase (Koga and Kramer, 1983) and when chitinase diffuses from the native polyacrylamide gel it will digest the glycol chitin. The fluorescent brightener 28 will then bind to the undigested glycol chitin by affinity (Shen et al., 1991; Maeda and Ishida, 1967). When the chitinase digests the glycol chitin in the overlay gel the fluorescent brightener 28 produces nonfluorescent dark bands against a fluorescent background (Shen et al., 1991) on a UV transilluminator at a wavelength of 312 nm (Shen et al., 1991).

These studies have shown that both *Cornus mas* and *Cornus florida* protein preparations contain chitinase throughout the experimental test period following inoculation with GA-1 in 10% polyacrylamide gel. Using 7.5% glycol chitin overlays, *Cornus mas* preparations demonstrated only one band with chitinase activity per protein sample (Figure 2). *Cornus florida* also contains one band per lane also identifying chitinase activity (Figure 3).

Protein separation using a 15% polyacrylamide gel and a 7.5% glycol chitin overlay, *Cornus mas* contains three

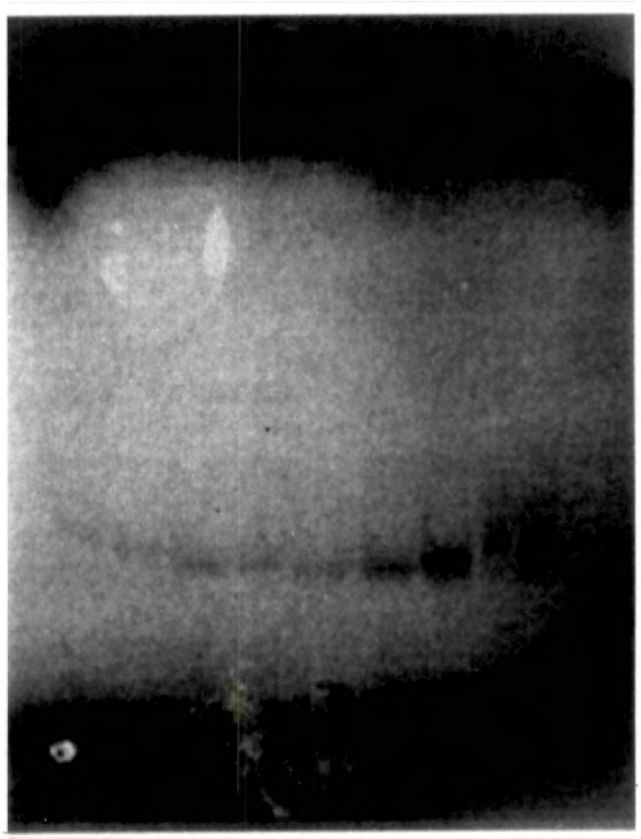


Figure 2. 7.5% glycol chitin polyacrylamide gel of *Cornus mas*. Lane 1:12 hours; Lane 2:24 hours; Lane 3:2 days; Lane 4:4 days; Lane 5:6 days; Lane 6:8 days; Lane 7:10 days; Lane 8:12 days after inoculation with *Discula* isolate GA-1.

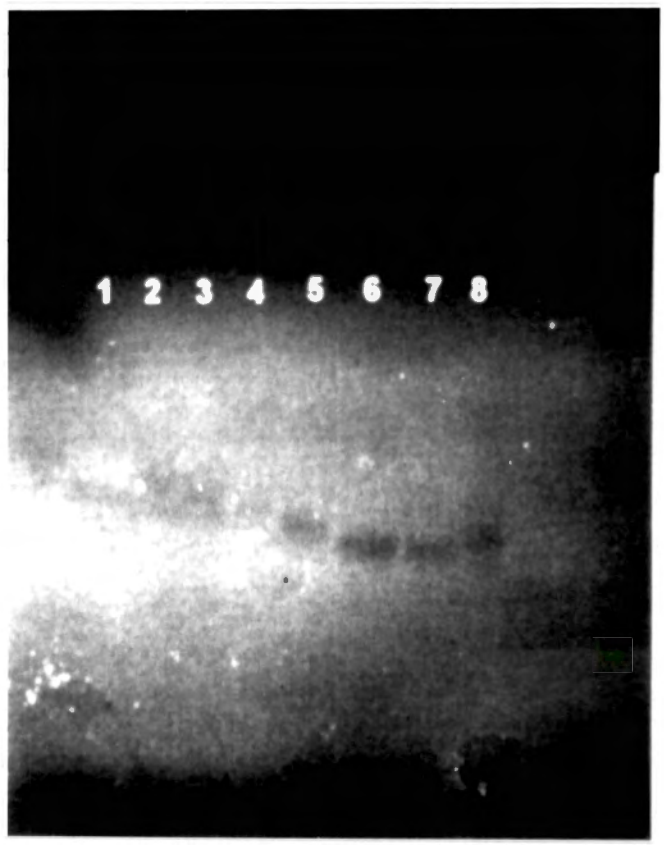


Figure 3. 7.5% glycol chitin polyacrylamide overlay gel of *Cornus florida*. Lane 1:12 hours; Lane 2:24 hours; Lane 3:2 days; Lane 4:4 days; Lane 5:6 days; Lane 6:8 days; Lane 7:10 days; Lane 8:12 days after inoculation with *Discula* isolate GA-1.

bands per lane indicating the existence of three chitinase isozymes. (Figure 4) In contrast, *Cornus florida* protein preparations showed only two isozyme band (Figure 4). This overlay gel indicates that a 15% polyacrylamide gel is able to separate more isozyme patterns than in the 10% gel (figure 4).

Evidence was provided that chitinase in the protein sample is reacting with the glycol chitin in the overlay gel. Protein samples of *Cornus mas* and *Cornus florida* were boiled to denature the protein and were then loaded onto a 10% polyacrylamide gel with corresponding unboiled samples (Figures 5 and 6). This overlay provides good evidence that chitinase is reacting with the glycol chitin in the overlay gels. None of the boiled samples reacted with the overlay gel as seen in both figures.

The molecular weight of the two isozyme forms of chitinase is estimated at 65 +/- 5 kDa and 21 kDa in *Cornus mas* and 21 kDa in *Cornus florida* following SDS-PAGE system (not shown).

The Phastsystem identified three pI bands per lane on the glycol chitin overlay gel in *Cornus mas*. These chitinase bands for *Cornus mas* were estimated to have a pI of 5.6, 6.8 and 8.9 (Figure 7). The Phastsystem estimated the pI of *Cornus florida* at 5.6 and 6.8 (Figure 7).

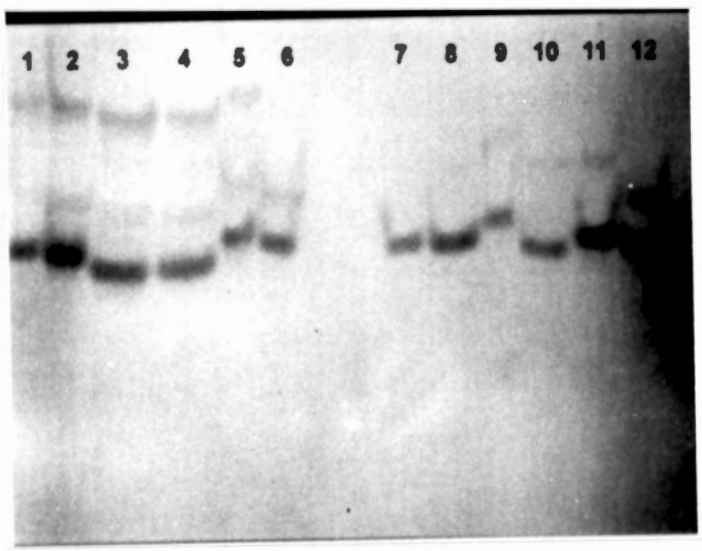


Figure 4. 7.5% glycol chitin overlay gel of a 15% polyacrylamide gel with *Cornus mas* and *C. florida*. Lane 1-6 are *Cornus mas* and lanes 7-12 and *Cornus florida*. Lane 1:24 hours, Lane 2:2 days, Lane 3:4 days, Lane 4:6 days, Lane 5:8 days, Lane 6:10 days, Lane 7:24 hours, Lane 8:2 days, Lane 9:4 days, Lane 10:6 days, Lane 11:8 days, Lane 12:10 days after inoculation with *Discula* isolate GA-1.

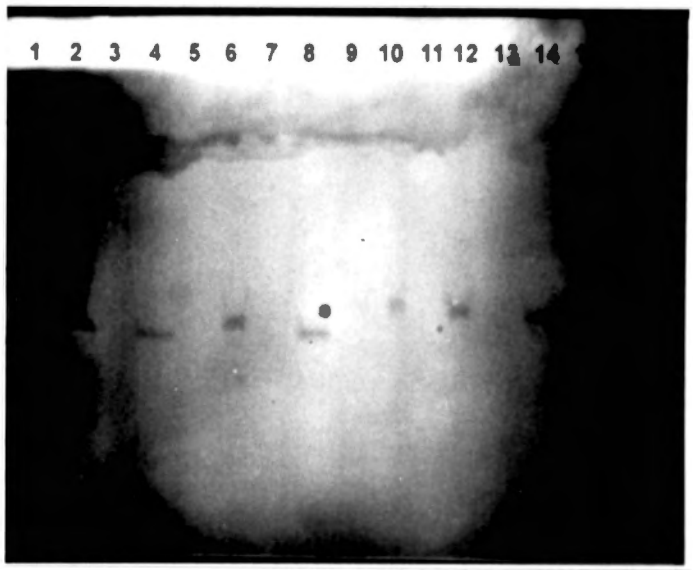


Figure 5. 7.5% glycol chitin overlay gel of 10% polyacrylamide gel with boiled and non-boiled samples of *Cornus mas*. Lane 2 chitinase control, Lane 4:1 day, 6:2 days, Lane 8:4 days, Lane 10:6 days, Lane 12:8 days, Lane 14 10 days. Odd lanes are boiled protein samples corresponding to the non-boiled protein samples.

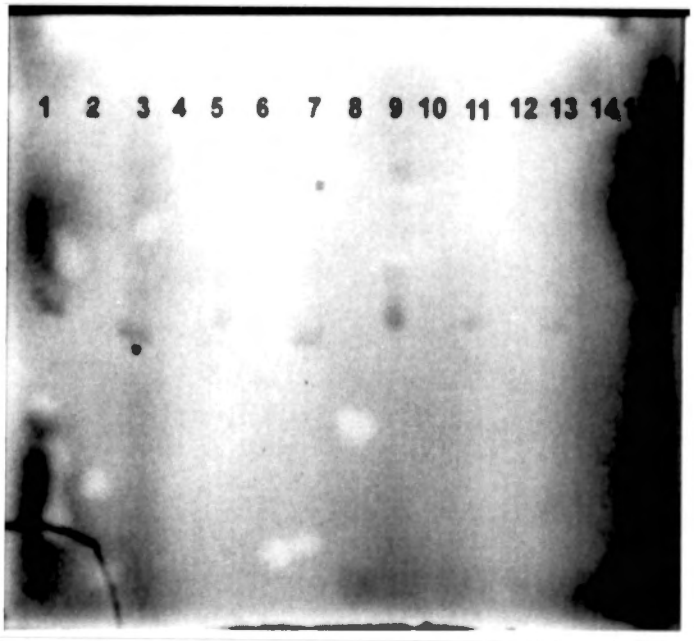


Figure 6. 7.5% glycol chitin overlay gel of a 10% polyacrylamide gel of *Cornus mas*=CM and *Cornus florida*=CF boiled and non-boiled protein samples. Lanes 1:CM 12 days, Lane 3:CF 24 hours, Lane 5:CF 2 days, Lane 7:CF 4 days, Lane 8:CF 6 days, Lane 11:CF 8 days, Lane 13:CF 10 days, Lane 15:CF 12 days. Even lanes are corresponding boiled samples.

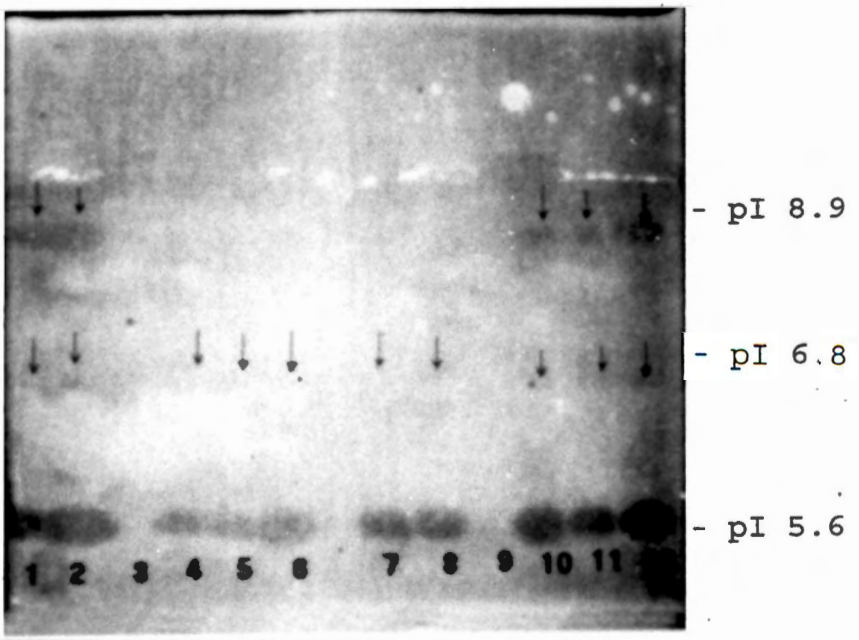


Figure 7. Phastsystem glycol chitin overlay gel containing *Cornus mas*=CM and *Cornus florida*=CF. Lane 1:CM 24 hours, Lane 2:CM 4 days, Lane 3:Protein Standard, Lane 4:CF 24 hours, Lane 5:CF 4 days, Lane 6:CF 6 days, Lane 7:CF 8 days, Lane 8:CF 10 days, Lane 9:Protein Standard, Lane 10:CM 4 days, Lane 11:CM 6 days, Lane 12:CM 8 days.

Preliminary Test of Chitinase With *Discula destructiva*:

There was no reaction of chitinase with *Discula* cultures. The inoculum continued to grow over and under the Whatman paper containing the chitinase.

Chapter V

Discussion

The primary goals of this study were: 1.) to determine whether *Cornus mas* and *Cornus florida* express pathogenesis-related proteins known as chitinases and 2.) to characterize chitinase in *Cornus mas* and *Cornus florida*. There are many similarities and differences between the resistant and susceptible *Cornus* species as explained below.

Chitinases were shown to be present in both *Cornus mas* and *Cornus florida*. Chitinase activity was detectable first at day 4 following inoculation of *Cornus mas* with *Discula destructiva*. It's activity increased thereafter and leveled off. In contrast, negligible chitinase activity was observed in *Cornus florida* only at days 8 and 12 following inoculation, a species susceptible to dogwood anthracnose.

Non-inoculated *Cornus mas* resulted in a significant amount of detectable chitinase activity . This result appears to indicate that chitinase is also induced at an elevated level as high or higher than inoculated *Cornus mas*. Chitinase activity was not detected in non-inoculated *Cornus florida*. The reason for this result could be: 1.) chitinase activity had not been induced to the level where activity could be detected 2.) chitinase activity is induced at a much slower rate compared to *Cornus mas*.

3.) Wounding also has the capability of inducing the production of one or more chitinases causing a systemic response (Graham and Sticklen, 1993). C h i t i n a s e induced by wounding depends on what plant part has been wounded. For example, an insect continuously feeding on the host tissue causes a long induction of chitinase. A minor abrasion causes a short but quick response from the plant tissue (Graham and Sticklen, 1993).

When *Cornus mas* and *Cornus florida* proteins were electrophoresed in a 10% polyacrylamide gel and overlayed with glycol chitin-containing gels, chitinase activity was present for all protein preparations obtained after inoculation. Under those electrophoretic conditions, chitinase appeared to be present as one band form. In contrast, 15% polyacrylamide gels revealed a three band pattern in *Cornus mas*. This same gel revealed only a two band pattern in *Cornus florida*. This indicated that a 15% gel is able to separate more isozyme forms in *Cornus mas* and *Cornus florida*. This is possible because the protein samples separate as they migrate through the running gel according to their individual molecular weights and charges. The 15% gel contained a smaller pore size compared to a 10% gel, thereby allowing a better separation of the two isozyme forms in *Cornus mas* (Chrambach and Rodbard, 1971). The 10% gel allowed for the migration of

both bands at the same point, probably because it contains a larger pore size.

The molecular weight of chitinases in *Cornus mas* were estimated at 65 +/- 5 kDa and 21 kDa as determined by SDS-PAGE. The iso-electric points (pI) for the detected species were estimated at 5.6, 6.8, and 8.9. The molecular weight of chitinase in *Cornus florida* was estimated at 21 kDa. The iso-electric point (pI) for the detected species in *Cornus florida* were estimated at 5.6 and 6.8.

The difference in results between the colorimetric and the gel overlay assays may be a result of the methods used to precipitate the proteins. When the proteins are precipitated with ammonium sulfate, it appeared that much of the chitinase activity was lost at this step. This result has been reported in the study of sweet orange callus (Wolfgang et al., 1994). That study revealed total activity of chitinase decreased approximately by half and the yield also decreased by nearly half from 100% to 58% when protein was precipitated with ammonium sulfate. Alternatively, ammonium sulfate may not precipitate the 21 kDa chitinase, therefore the colorimetric assay only detects the 65 kDa chitinase present early in *Cornus mas*.

The detection of chitinase activity over all days tested in the 10% and 15% polyacrylamide gels can result from the fact that overlay assay was not quantitative and

may be more sensitive compared to the colorimetric assay. Also, the chitinase was concentrated in the polyacrylamide gel bands.

The preliminary test of *Discula destructiva* with the chitinase appeared to have no visual reaction. This could be due to: 1.) chitinase is unstable under the same incubation conditions as *Discula*, 2.) that in order to destroy the hyphal strand, chitinase needs to be continuously produced or manufactured over time, and 3.) the presence of glucanases may be required to disable growing hyphal tips. The second statement is most likely true, because after the resistant *Cornus mas* was inoculated, chitinase was produced continuously over time.

All of the above information identifies similarities and differences in chitinase activity. It appears that chitinase activity is an inducible protein with different isozymes being produced. *Cornus mas* has an isoenzyme present which does not appear in *Cornus florida*. This third isozyme could possibly be the link to disease resistance in *Cornus mas*, rather than in *Cornus florida*. The literature has provided evidence that susceptible species produce chitinase, but it is a matter of how quick and how much is produced to provide resistance. This could possibly explain what is occurring in the susceptible *Cornus florida*. There is chitinase activity, but there is a significant difference in how much is produced and how

rapidly. The use of a 15% polyacrylamide gel identified a chitinase isozyme induced in *Cornus mas* that was not detected in *Cornus florida*. This isozyme may have been present in *Cornus florida* but its activity was not expressed as rapidly as in *Cornus mas*.

In conclusion, chitinase was expressed in both *Cornus mas* and *Cornus florida*. The activity of chitinase increased over several days in *Cornus mas* and was only detectable at day 8 in *Cornus florida* using the Sigma Chitinase Assay. The chitinase was detectable in all days following inoculation in *Cornus mas* and *Cornus florida* after electrophoresis. Chitinase has been suggested to have antifungal properties in other species. These results identify chitinase to be present in *Cornus mas* and *Cornus florida*. In order to examine whether the protein present is produced in different amounts over time between the two dogwood species, chitinase activity needs to be quantified.

What type of chitinase is being induced in *Cornus mas* and *Cornus florida*? As mentioned earlier there could be four possible chitinases. Class 1, accounting for most of the chitinolytic activity (Graham and Sticklen, 1993). Class 2 accounts for the first line of defense (Mauch and Stahelm, 1989). Classes 3 and 4 are also suggested to have an antifungal role (Graham and Sticklen, 1993).

Discula destructiva cannot positively be identified as a pathogen which induces chitinases. However, the literature supports the fact that the probability is great. Other host-pathogen relationships have recognized the ability of pathogens to induce chitinases, such as in a study of pepper stems (Kim and Byung, 1994). This study found that several chitinases were induced by *Phytophthora capsici* infection. Early studies revealed that chitinases were undetectable in non-inoculated pepper stems (Kim and Young, 1994). This study provides evidence that chitinases are induced by pathogen infection.

The chitinase expressed in this study cannot be identified as being induced by *Discula destructiva*, because there are many other substances that could possible induce this enzyme. Although, two of the chitinases were common to both *Cornus* species, one was specific to *Cornus mas*, and may represent an inducible chitinase.

Chitinase can be induced by ethylene, elicitors such as β-1,3-glucans and chitin, organic molecules, salt, growth regulators and certain environmental conditions (Graham and Sticklen, 1993). Ethylene was produced at high rates within the first 5-7 hours following exposure to a fungal pathogen and then dropped as in the study of pea pods Graham and Sticklen, 1993; Mauch et al., 1984). A

study of parsley infected with a fungal pathogen showed an increase in ethylene in 6 hours (Chappel et al., 1984).

Since chitinase has been identified in both *Cornus* species, the next experimental procedure should be to quantify the production of chitinase in resistant versus susceptible species and clones. If the quantity of chitinase is different, then to identify what classes of chitinase are being produced.

If chitinase is proven to be induced by the pathogen, then the production of a transgenic *Cornus* that would express chitinase at elevated levels could be sought. This type of research could be achieved by attaching a promoter region from a resistant *Cornus* species onto a chitinase gene and transforming susceptible *Cornus* species with this construct to allow for rapid induction. The above research could contribute greatly to the understanding of defense responses in plant cells and increase our understanding of the molecular biology of *Cornus* species.

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APPENDIX

Section 1: Buffers and Solutions for Electrophoresis and Overlay Gels

Protein Extraction Buffer (1 L) (Santamour et al.,1986)

0.121 g Tris base

0.098 g maleic acid

1.112 g potassium metabsulphite

1.212 g cysteine

1% (10 ml) Tween 20

Combine all chemicals together except for Tween 20 and ph to 7.0 with 5 M NaOH. Combine Tween 20 with solution and bring to 1 L. Store at 4°C.

30% Acrylamide/BIS Solution (200 ml)

60 g Acrylamide

1.6 g BIS

Add chemicals to 140 ml ddH20 and QS to 200 ml. Store in a dark bottle at 4°C.

Resolving Buffer (PH 8.8, 250 ml)

45.4 g Tris-base

Add chemical to 170 ml ddH20, ph to 8.8 with HCl. Store at 4°C.

Electrode Buffer (PH 8.3, 3 L)

9.08 g Tris-base

43.2 g glycine

Add chemicals to 2500 ml and ph to 8.3 with 5 M NaOH.

1.5% APS (10 ml)

1.5 g APS

Store at -20°C

1 M Sodium Acetate Buffer (PH 5.0, 1 L)

82.03 g Sodium Acetate

Add chemical to 500 ml ddH20 and ph to 5.0 with glacial acetic acid.

0.1 M Sodium Acetate Buffer (PH 5.0, 1 L)

100 ml 1 M Sodium Acetate ph 5.0

Dilute in 900 ml ddH20

0.01% (w/v) Fluorescent Brightener 28 (200 ml)

0.02 g fluorescent brightener 28

Dissolve in 200 ml 0.5 M Tris-HCl

0.5 M Tris-HCL (PH 8.9, 1 L)

60.55 g Tris base

Dissolve in 500 ml ddH20 and PH to 8.9 with 4 M HCl.

3.0 M Tris-HCL (PH 8.8, 250 ml)

90.08 g Tris-base

Dissolve in 200 ml ddH20 and PH to 8.8 with 4 M HCL

1% Glycol Chitin (Trudel and Asselin, 1989)

Glycol chitin prepared and kindly provided by Dr. Kimberly Gwinn of Entomology and Plant Pathology of The University of Tennessee, Knoxville

10% Resolving Gel (2 Gels 1.5 mm thick)

40 ml 30% Acrylamide/BIS

29.6 ml Resolving Buffer ph 8.8

48 ml water

9 g sucrose

800 µl APS

120 µl TEMED

Combine all solutions except for TEMED and APS, degas for 15 minutes. Add TEMED and APS. Load solution in gel rig with a 60 cc syringe.

15% Resolving Gel (2 Gels 1.5 mm thick) (Shen et al., 1991)

30 ml 30% Acrylamide/BIS

7.5 ml 3.0 M Tris-HCl PH 8.8

3 ml 1.5% APS

19.5 ml ddH20

15 µl TEMED

Combine all solutions except for TEMED and degas for 15 minutes. Add TEMED. Load gels with 60 CC syringe.

Chitin Overlay Gel (2 Gels 1.5 mm thick) (Pan et al., 1991)

25 ml 30% Acrylamide/BIS

74 ml Sodium Acetate ph 5.0

400 µl 1% glycol chitin

500 µl APS

50 µl TEMED

Combine solutions, load gels with a 60 cc syringe

Section 2: **Protein Extraction with and without Ammonium Sulfate Precipitation**

Protein Extraction without Ammonium Sulfate Precipitation (Santamour et al., 1986)

1. Grind 2-3 g plant tissue, to a fine powder, in pre-chilled mortar and pestle with the addition of .5 g PVP.
2. Add 6-9 ml of extraction buffer, mix and allow mixture to thaw at room temperature for app. 30 minutes.
3. Aliquot 1 ml samples into 1.5 ml microcentrofuge tubes and spin samples at 14,000 rpm for 15 minutes.
4. Place the supernatant in clean microcentrifuge tubes and centrifuge again as in step #3.
5. Aliquot 100 μl samples in 0.5 ml microcentrifuge tubes and store at -80°C until use.

Protein Extraction with Ammonium Sulfate Precipitation

1. Step 1 same as above
2. Place sample in 50 ml Oak Ridge tubes and spin for 15 min at 14,000 rpm at 4°C.
3. Remove supernatant and dissolve 40% ammonium sulfate and allow solution to sit on ice for 2 hours.
4. Place solution in 30 ml Oak Ridge tubes and spin for 30 minutes at 4°C.
5. Remove supernatant and dissolve in 60% ammonium sulfate and allow to sit on ice for 1 hour.
6. Place supernatant in 30 ml Oak Ridge tubes and spin for 40 minutes.

7. Discard supernatant and dissolve pellet in 0.5 ml ddH20.

8. Dialyze sample overnight at 4°C against ddH20.

Section 3: **Solutions for *Discula destructiva* isolate GA-1 Growth**

PDV8 Preparation (2 L) (Dhingra and Sinclair, 1985)

12 oz. V8 juice

3 g Calcium Carbonate

24 g potato dextrose

36 g bactoagar

1. Place 35-40 ml of V8 juice in 50 ml centrifuge tubes and spin at 2400 rpm for 15 minutes.
2. Discard pellet and pour liquid in a 1 liter beaker and stir in calcium carbonate for 30 minutes.
3. Allow calcium carbonate to settle, pour off liquid and raise to 2 liters with ddH20.
4. Add potato dextrose in solution and add bactoagar
5. Autoclave solution for 20 minutes

PDA Preparation (200 ml)

6 g potato dextrose

4.25 g bactoagar

1. Combine solutions in ddH20 and autoclave for 20 minutes

Section 4: Sigma Protocol and Solutions

Buffers and Solutions

200 mM Potassium Phosphate Buffer, PH 6.0 with 2 mM Calcium Chloride (100 ml)

2.7 g Potassium Phosphate, Monobasic

294 mg Calcium Chloride

Dissolve chemicals in 80 ml of ddH20. PH with 1 N KOH and QS to 100 ml.

1.25% (w/v) Chitin Suspension (25 ml)

Dissolve 25 ml of the above solution with 1.25 g chitin. Stir solution for app. 15 minutes.

5.3 M Sodium Potassium Tartrate Solution (8.0 ml)

12.0 g Sodium Potassium Tartrate, Tetrahydrate

8.0 ml 2 M NaOH

Dissolve sodium potassium tartrate in 2 M NaOH. Heat solution in a boiling water bath, but do not boil solution.

96 mM 3,5-Dinitrosalicylic Acid Solution (20 ml)

438 mg 3,5-Dinitrosalicylic Acid

Dissolve chemical solution in 20 ml ddH2O. Heat solution in boiling water bath, but do not boil.

Color Reaction Solution (40 ml) (Clr Rgt Soln)

8.0 ml 5.3 M Sodium Potassium Tartrate Solution

20 ml 96 mM 3,5-Dinitrosalicyclic Acid Solution

While stirring, add the first solution, to the second solution. Dilute solution to 40 ml with ddH20. Store in a dark bottle.

Beta-N-Acetylglucosamindase (NAGase)

Dissolve 20 µl of NAGase in 980 µl 200 mM Potassium Phosphate Buffer with 2 mM Calcium Chloride

0.1% (w/v) N-Acetyl-D-Glucosamine (NAG)

Dissolve 0.1 g in 10 ml ddH2O.

Sigma Protocol

1. Dissolve 2 ml chitin solution with 0.5 ml protein extraction in a test tube.
2. Incubate solution on a rotary shaker for 3 hours at 40 C.
3. Place sample in boiling water bath for 5 minutes and cool to room temperature by placing test tube on ice.
4. Add 10 µl of NAGase. Incubate for 30 minutes at 40 C on a rotary shaker.
5. Centrifuge solutions at 2500 rpm and keep the supernatant.
6. Place 1.0 ml supernatant to a test tube containing 2.0 ml ddH2O and 1.50 ml color reaction.
7. Place test in boiling water bath for 15 minutes.
8. Cool to room temperature by placing in ice for app. 15 minutes.
9. Record chitinase at 540 nm.

Standard Curve for Sigma Protocol

Pipette the following reagents (in milliters) into cuvettes and record at 540 nm for each and analyze data with Statistical Analysis System (SAS). Plot the 540 nm of the standards versus milligrams of NAG.

	NAG	Chitin Buffer	Clr Rgt Soln
Std 1	0.1	2.9	1.5
Std 2	0.2	2.8	1.5
Std 3	0.3	2.7	1.5
Std 4	0.4	2.6	1.5
Std 5	0.5	2.5	1.5
Std 6	0.0	3.0	1.5

VITA

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