Cucurbit Downy Mildew (*Pseudoperonospora cubensis*): Cucumber Resistance

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ABSTRACT

Pseudoperonospora cubensis (Bert. et Curt) Rost. is the causal agent of cucurbit downy mildew (CDM). It is the most damaging cucumber pathogen on the Eastern Shore of Virginia and eastern parts of the United States. *Pseudoperonospora cubensis* is an obligate oomycete pathogen, infecting crops within the Cucurbitaceae family. The disease is characterized by angular chlorotic lesions and a downy or felt-like appearance on the abaxial side of the leaf. Control of this pathogen includes use of resistant cucumber cultivars and costly fungicide programs. Continuous use has led to resistance to commonly used fungicides. This has become a major concern and in response, seed companies have developed cucumber cultivars which claim downy mildew resistance. This study evaluates different cucumber cultivars and assesses their level of resistance to CDM. The results indicate that an integrated management approach of reduced fungicide application and the use of resistant cultivars can suppress levels of CDM and yield a cucumber crop. Additionally, a molecular study was conducted, comparing the relative expression of genes encoding a basic PR-1 protein, a cytosolic ascorbate peroxidase protein and three resistance (R) gene proteins, in nineteen cultivars. All of the selected genes were analyzed using real-time PCR. The relative expression levels of the R-genes varied between cultivars. The basic PR-1 protein decreased expression in the majority of the cultivars, suggesting no involvement in the first twenty-four hours. Cytosolic ascorbate peroxidase relative expression levels suggest an increase in susceptible cultivars and a decrease in tolerant cultivars.

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CHAPTER 1: INTRODUCTION AND OBJECTIVES

Cucurbits are one of the most cultivated vegetables worldwide, second only to *Solanum lycopersicum* (tomato) (Pitrat, 1999). *Pseudoperonospora cubensis* (Berkeley & Curtis) Rostovtsev is the causal agent of cucurbit downy mildew. It is one of the most devastating diseases of cucurbit crops worldwide. It infects crops in the Cucurbitaceae or gourd family, including squash (*Curcurbita* ssp.), watermelon [*Citrullus lanatus* {Thumb}Matsum. & Nakai], melon (*Cucumis melo* L.), and cucumber (*Cucumis sativus* var. *sativus* L.) among others (Palti, 1975). The disease was first reported in 1868 by Berkeley and Curtis in Cuba, thus the species name "cubensis" (Berkeley and Curtis, 1868). Originally the pathogen was placed in the genus *Peronospora*, but in 1903 Rostovtsev suggested the name be changed to *Pseudoperonospora* (Waterhouse and Brothers, 1981). It is an obligate parasite, requiring live host tissue to thrive and reproduce (Palti, 1975). This pathogen belongs to kingdom Stramenopila; phylum Oomycota; subphylum Peronospororomycotina; class Peronosporomycetes (Oomycetes); order Peronosporales (downy mildews); family Peronosporaceae (Goker *et al.*, 2007; Voglmayr, 2008; Savory *et al.*, 2010).

Pseudoperonospora cubensis can be found worldwide, causing significant yield losses in the USA, Europe, and Asia (Thomas, 1996). It has been found in over 70 countries, across diverse environments (semi-arid to tropical) (Cohen, 1981). It can infect over 50 different species in 20 genera (Lebeda and Urban, 2007). *P. cubensis* and its cucurbit hosts cannot survive freezing temperatures; consequently it overwinters in warmer climates (Holmes *et al.*, 2004).

Morphology, Life Cycle & Mode of Infection

The morphology of *P. cubensis* varies widely dependent on the specific isolate, host crop and temperature (Iwata, 1942; Waterhouse and Brothers, 1981; Runge and Thines, 2010; Savory, 2010). *Pseudoperonospora cubensis* has true sporangia with a poroid apex, and a papilla at the distal end (Waterhouse and Brothers, 1981). The sporangiophores branch at acute angles with pointed tips which bear the sporangia. The spores are lemon-shaped, and are a light purple to gray color. The intercellular hyphae are clear and aseptate. The conidiophores branch irregularly, then become dichotomous. Zoospores are biflagellate, one flagellum is posterior and one anterior (Cohen, 1981).

The life cycle of *P. cubensis* starts when a sporangium lands on the adaxial leaf surface of a host plant. If conditions are conducive the sporangium will germinate and form biflagellate zoospores. Leaf wetness is required for the release and movement of zoospores (Lange *et al.*, 1989; Cohen, 1981). Zoospores will encyst in a leaf stoma and penetrate the surface via a germ tube. The mesophyll layer becomes colonized by the hyphae and clavate-branched haustoria. Sporulation occurs, and up to six sporangiophores emerge from a single stoma Emergence will not occur until there is sufficient moisture over the lesion. The sporangia are released to continue the disease cycle (Savory *et al.*, 2010). This pathogen reproduces predominantly asexually, but has been reported to reproduce sexually via the production of oospores, although oospore production is rare (Lebeda and Cohen, 2011; Palti and Cohen, 1980; Thomas,1996).

Symptoms vary depending on the host plant, but *P. cubensis* has some consistent identifying characteristics. It is a foliar pathogen that causes chlorotic lesions on the adaxial leaf surface. Primary lesions are between 3 -10 mm, as the disease progresses the lesions combine to

form larger lesions that can eventually cover the entire leaf (Lebeda and Cohen, 2010). The disease is characterized by a 'downy' or 'felt' appearance which is due to the sporangia found on the abaxial side of the leaf. The lesions can be angular and restricted by the veins of the leaf, particularly on *Cucumis sativus* (cucumber) (Savory *et al.*, 2010). However, *Citrullus lanatus* (watermelon) lesions are not bound by the veins (Thomas, 1996). Lesions on *Cucumis melo (*melon) tend to be less irregular and more circular than those found on other hosts (Cohen, 1981). As the infection worsens, chlorotic lesions become necrotic, particularly during hot and dry weather conditions (Oerke *et al.*, 2006). The incubation period from initial infection to visible symptoms varies dependent on field conditions and inoculum level, but generally ranges between 4 and 12 days (Cohen, 1977). Recent research has discovered that *P. cubensis* has 271 predicted effector proteins (Savory *et al.*, 2012). This is contradicts the original idea that the pathogen does not produce any toxins in the plant, except for the initial enzymes used for cell wall penetration (Lebeda *et al.*, 2001).

Epidemiology

The epidemiology of this pathogen depends greatly on environmental conditions. The sporangia life span does not exceed 48 hours and within this short time they must locate a susceptible host and germinate (Cohen and Rotem, 1971). The germination rate will decrease with warmer temperatures (above 30 ºC), with optimum temperature for germination being between 10-20 ºC (Lebeda and Cohen, 2011). Infection requires free water on the leaf surface for the zoospore to develop germ tubes. The minimum amount of time necessary for the sporangia to germinate and penetrate the leaf surface is two hours (Cohen, 1981). Morning dew aids penetration into the stomata and if dew is present for six hours on the leaf surface, additional free water is not needed. This process can be affected by light, because light can lead to

evaporation of dew which leads to shorter leaf wetness period (Cohen, 1971). First penetrations are observable about 5 hours after the original sporangium has landed on the leaf surface, if conducive conditions are present (Lebeda, 1990). Colonization of host tissue takes place in the leaf mesophyll layer (Lange *et al.*, 1989). Incubation period varies dependent on environmental conditions, the host the pathogen is infecting and the amount of initial inoculum. Optimum temperature for disease development ranges from 25 to 30 ºC during the day and 10-15 ºC at night (Palti and Cohen, 1980). If conditions are conductive for disease development, early symptoms can appear in as few as three days. The other factor that needs to be accounted for is the amount of initial inoculum. If there is a low concentration of inoculum (10 sporangia/cm^2 per leaf), it may take over a week to observe disease symptoms (Cohen and Eyal, 1977).

Sporangia and sporangiophores are affected by changes in relative humidity or temperature. If sporangia experience dry periods even for a short interval the integrity of the membrane is damaged and the spore is prevented from germinating (Ledeba and Cohen, 2011). Colonization of host tissue, symptom development and sporulation are greatly affected by temperature. While colonization benefits from low temperatures, symptom development will increase with higher temperatures and more light. Enhanced symptom development will lead to more chlorotic lesions. Hot and dry weather in the field increases the rate at which the lesions become necrotic, necrotic lesions terminate the survival of *P. cubensis,* thus ceasing sporulation (Cohen, 1981).

Dispersal and Transport

Generally, *Pseudoperonospora cubensis* is not capable of overwintering in geographical locations where there are killing frosts. Cucurbits are frost sensitive and without living host

tissue the pathogen will not survive (Thomas and Jourdain, 1992). In the United States, *P. cubensis* survives in the southern regions of Florida on both cultivated and wild host species of cucurbits (Cohen, 1981). The pathogen is dispersed through wind currents (anemochory) that carry the sporangia to new hosts. This method of dispersal can carry spores several hundred kilometers (Lebeda and Cohen, 2011). The sporangia of *P. cubensis* travel on wind currents from southern areas northward in the spring into the summer, surviving between movements on cultivated cucurbit crops. The progression of this disease was first tracked by Doran in 1932, from Florida to Georgia to the Carolinas. Similarly in 1943, Nusbaum and his collaborators traced the progression of the disease from Florida in April to Delaware in July, to Connecticut in the beginning of August and into Massachusetts in late August (Nusbaum, 1944). Environmental conditions have a major effect on the geographical spread of disease, they can increase the rate at which it is spread, increase the severity if conditions are favorable, or if they are non-conducive the disease will spread slower and not be as damaging. More recently the spread of disease has been linked to transplants grown in greenhouses were the conditions are warm enough for the pathogen to overwinter (http://cdm.ipmpipe.org/).

In 1998, North Carolina State University along with other researchers began a forecasting system for this pathogen (Holmes *et al*. 1998, 2004). This system has been updated and now involves many collaborators from many different states, and is called The Cucurbit Downy Mildew ipmPIPE (http://cdm.ipmpipe.org/) (Ojiambo *et al.*, 2011). The purpose of the program is to inform people when disease pressure will be greatest and when to apply fungicides for the most cost effective applications, by tracking the progression of the disease. To aid in the detection of the pathogen, universities set up sentinel plots throughout their respective states. Sentinel plots are planted in the spring and contain many different cucurbit hosts. A variety of

susceptible hosts are used to identify which pathotypes of the pathogen are in the area, as some hosts are susceptible only to certain pathotypes (Lebeda and Widrlechner, 2003). Once the disease is found, it is reported online through the IPMpipe system, indicating the location in which the disease was found, and on which host. Alerts are generated to inform the subscribers about the outbreaks (Ojiambo *et al.,* 2011).

Host Range and Preference

Pseudoperonospora cubensis has many different host species in the Cucurbitaceae family, infecting at least 50 species within 20 genera (Palti and Cohen, 1980). *Pseudoperonospora cubensis* isolates have been documented to show host preference, and specialization (Doran, 1932; Hughes and Van Haltern, 1952; Palti, 1974; Palti and Cohen, 1980). *Pseudoperonospora cubensis* isolates have shown variability in both virulence and pathogenicity depending on which type of cucurbit species it is infecting (Savory *et al.,* 2010).

Doran (1932), working in Massachusetts, inoculated a variety of cucurbit crops (cucumber, melon, squash, pumpkins and gourds) and found that *P. cubensis* was most severe on cucumber, and that disease was moderate on muskmelon, watermelon and gourd, and absent on pumpkin and squash (Doran, 1932). Hughes and Van (1952) continued this work testing cucumber, cantaloupe and watermelon, using two isolates, one collected from cucumber and one from watermelon. The cucumber isolate was able to infect all cucurbits examined, with disease progressing faster on cucumber than cantaloupe and watermelon. The watermelon isolate caused severe damage on the watermelon, but only moderate disease on cucumber and cantaloupe (Hughes &Van Haltern, 1952). Bains and Jhooty (1976) collected a *P. cubensis* isolate from muskmelon; then tested the isolate on both squash and ash gourd and found that it was not

pathogenic to either. Thomas *et al.* (1987) reported host range studies in USA, Israel and Japan with each country testing 26 cucurbit species using isolates from their own countries. The intensity of sporulation varied between hosts, with cucumber and cantaloupe being the most susceptible to the isolates tested with the reaction from the other cucurbit species being varied (Table 1.1) (Thomas *et al.*, 1987). More recent studies classified 13 physiological races (pathotypes) in the Czech Republic, France and Spain. These differences between the races were determined based on the virulence of isolates on different hosts (Lebeda and Gadasova, 2002; Lebeda and Widrlechner 2003).

Differences in infection severity on various hosts have been attributed to differing physiological races, and environmental and biotic conditions (Palti, 1974; Cohen *et al.*, 2003). There have been six physiological races identified in the USA, Israel, and Japan (Shetty *et al.*, 2003; Cohen *et al.*, 2003; Savory *et al.*, 2010). All six races have show pathogenicity on cucumber and muskmelon, but have displayed differences in pathogenicity on watermelon, squash, and pumpkin (Savory *et al.*, 2010). While these studies have shown the variability in the *P. cubensis* strains for virulence and pathogenicity, to date there are no data published on the genetics of the different strains.

Management and Fungicides

Successful management of *P. cubensis* involves utilizing resistant or tolerant cultivars, cultural practices, and fungicide applications. An aggressive fungicide program is often needed for prevention and protection of the crop to avoid yield losses when environmental conditions favor disease development (Savory *et al.*, 2010).

This pathogen, as with many oomycete pathogens, has been able to develop resistance to fungicides rather quickly. *Pseudoperonospora cubensis* is categorized by the Fungicide Resistance Action Committee (FRAC) as possessing a high risk of developing fungicide resistance (Russell, 2004). It has already developed resistance to phenylamides (FRAC code 4), such as metalaxyl, strobilurins (FRAC code 11), such as azoxystrobin, and carbamates (FRAC code 28), such as propamocarb (Urban and Lebeda, 2006). It was the first oomycete pathogen to develop resistance to metalaxyl (Reuveni *et al.*, 1980; Samoucha and Cohen, 1984; Cohen and Samoucha, 1984; Cohen and Reuveni, 1983). Because of the pathogen's ability to overcome fungicides, multiple fungicides with differing modes of action should be used when managing the disease. It is best to use preventative multisite inhibitors, mixed with systemic fungicides to reduce the risk of resistance (Urban and Lebeda, 2006). The two fungicides most commonly and recently used for control of *P. cubensis,* are cyazofamid (FRAC code 21, Ranman 3.33SC, FMC Corporation, King of Prussia, PA) and fluopicolide (FRAC code 43, Presidio 4SC, Valent U.S. A. Corporation, Walnut Creek, CA). Cyazofamid is a respiration inhibitor with strong lipophilic properties so it can adhere to the surface of the leaf (Mitabi, 2003). It is in the family of Quinone inside inhibitors (QiI), inhibiting the cytochrome bc (ubiquinone reductase) at the Qi site. Fluopicolide is a relatively new fungicide that is marketed by Valent, within the family of benzamides and has a novel mode of action. It has been determined that it works by delocalization of spectrin-like proteins, is systemic and has both preventative and curative actions (www.frac.info).

Host Resistance

One of the most important aspects of managing *P. cubensis* is the use of resistant cultivars. "Palmetto" was a cucumber cultivar released in 1948 and although it was not immune

to infection, it was considered highly resistant, producing fewer sporangia than susceptible cultivars (Barnes, 1948). The pathogen quickly overcame the "resistance" of Palmetto in 1951 (Epps and Barnes, 1952). A resistance gene was identified in cucumber (PI 197087), and was named *dm1* and determined to be a recessive gene (Epps and Barnes, 1954). The cultivar Poinsett was released in 1966, possessing resistance to strains of *P. cubensis*. This resistance was originally attributed to the single *dm1* gene, but more recent research has reported that there are multiple genes that encode for *P. cubensis* resistance (recessive alleles *dm1, dm-2, dm-3)* (Doruchowski and Lakowska-Ryk, 1992). While all resistant cultivars are still able to be infected by the pathogen, the *dm* resistance is characterized by a hypersensitive reaction and reduced sporulation. The recessive *dm* genes are still being used for resistant cultivars, though they have lost their original efficacy.

Cultural Practices

Although management of *P. cubensis* relies primarily on the use of resistant cultivars and fungicides, there are other types of management practices that may suppress the pathogen. The pathogen has to rely on wind currents to move up the east coast, thus cucurbits planted in the late spring, in areas where the pathogen does not overwinter, are at a lower risk of developing disease than those planted later in the growing season (cdm.ipmpipe.org). Thus, a common management technique is an earlier planting date. Another management technique is growing the cucurbits on polyethylene mulch, which was found to significantly reduce the amount of *P. cubensis* found on the cucumber plants, compared to those grown on bare ground; most likely due to reduced leaf wetness duration (Shtienberg *et al.*, 2009).

Cucumber

Cucumber is a valued crop in the U.S. and worldwide and is used both for fresh market slicing, and for pickling. The first wild cucumber was found in the foothills of the Himalayas in Nepal (Whitaker and Davis, 1962). It is believed that the common garden cucumber is of Asiatic origin and that cucumber has been cultivated for over 3,000 years in India and over 2,000 years in China (Colucci, 2008). The cucumber was brought to the Americas by Christopher Columbus in 1494 (Robinson and Decker-Walters, 1997).

The cucumber genome was sequenced by Huang *et al.* in 2009, revealing 61 probable resistance gene regions in the genome. Three quarters of these possible resistance genes are located in 11 clusters on the genome (Huang *et al.*, 2009). This is important information due to the fact that disease resistance to *Cladosporium cucumerinum* (cucumber scab) was found in a resistance gene cluster (Kang *et al.*, 2010). In contrast, the *Fusarium oxysporum* f. sp. *melonis* (fusarium wilt) resistance gene was found to be a single gene in the melon genome (Joobeur *et al.*, 2004). None of the putative R-genes in cucumber have been recognized for resistance to *P. cubensis.*

Molecular Aspects

Plants have developed several ways to defend against pathogen invaders. There is a general type of resistance called basal or innate immunity. This type can be activated by mechanical injury and insect feeding as well as infection by pathogens. There is also a more specific resistance called adaptive or R-gene mediated resistance. This type of resistance is activated when the pathogen secretes effector proteins into the plant. These secretions are called pathogen associated molecular patterns (PAMPS) (Bent and Mackey, 2007). If the plant has a

corresponding R-gene, it will recognize the pathogen-secreted effectors and a resistant reaction will ensue. If the plant is not resistant, thus susceptible, it will not recognize the pathogen and become infected. A gene-for-gene theory was originally proposed by H.H. Flor for the interaction between flax and flax rust (Flor, 1947. It states that as a plant and pathogen evolve together, a virulence gene develops in the pathogen and a corresponding resistance gene develops in the host plant.

All R genes contain nucleotide binding sites (NBS) and group into two classes depending on what their amino terminus is comprised of, a coiled coil or a Toll/Interleukin 1(TIR) domain. In several cases, the NBS domain binds the pathogen effectors directly and sends a signal to promote defense (Bent and Mackey, 2007). This signal can lead to production of pathogenesis-related proteins, called PR proteins. PR genes have been studied in other organisms, most notably in tomato (Van Loon *et al.*, 2006). There are multiple PR genes that have been studied; PR-8 was found in cucumber and is a chitinase gene, which works well against fungal pathogens, but not oomycetes which do not produce chitin

Objectives

When planting susceptible cucumber cultivars, growers need to make on average four fungicide applications throughout the season depending on disease pressure and weather. Fungicide applications are costly and time consuming and the use of resistant cultivars may be able to reduce these costs. Understanding the nature of the resistance is imperative to reducing costs without an accidental yield loss due to a misinterpretation of the resistant cultivars. *Pseudoperonospora cubensis* has shown the ability to develop resistances to fungicides; hence reducing the applications of fungicides would lessen the risk of the pathogen becoming resistant. Presently there is no public knowledge on genetic resistance to *P. cubensis* in cucumber, identifying a resistance gene, or pathogen related protein that defends the plant against the pathogen would benefit breeders.

The research objectives were:

1) Evaluate the inherent resistance and/or tolerance of commercially available cucumber cultivars and their utilization in reduced-fungicide application programs.

2) Determine expression levels of selected defense related proteins and possible R-genes during infection by *P. cubensis.*

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Table 1.1 Pathotype by *Pseudoperonospora cubensis* and host compatibility (Thomas *et al*., 1987)

		Pathotype				
Host	Common Name	1	2	3	4	5
Cucumis sativus	Cucumber	+	+	+	+	+
Cucumis melo var. *reticulatus*	Cantaloupe	+	+	+	+	+
Cucumis melo var. *conomon*	Oriental pickling melon.	-	+	+	+	+
Cucumis melo var. *acidulous*	Snap melon	-	-	+	+	+
Citrullus lanatus	Watermelon	-	-	-	+	+
Cucurbita spp.	Squash	-	-	-	-	+

+ Highly compatible host interaction
- Incompatible or very slightly compatible host-pathogen interaction.

CHAPTER 2: Effects of Integrating Cultivar Resistance and Reduced Fungicide Application for Control of Cucurbit Downy Mildew in Cucumber.

Abstract

Pseudoperonospora cubensis (Bert. et Curt) Rost. is the causal agent of cucurbit downy mildew (CDM). It is a damaging oomycete pathogen that can cause significant economic losses in cucumbers if not properly managed. Resistant cultivars are being developed and promoted to combat the pathogen. However, resistance levels vary between cultivars. Six field experiments were conducted in 2010, 2011, and 2012 to test the efficacy of cucumber cultivars against the pathogen in conjunction with reduced fungicide applications. For each year, two trials were conducted, one assessing slicer-type and one for pickling-type cucumber cultivars. Both cucumber cultivar and fungicide program had significant effects on disease incidence and severity AUDPC values and yield in all six trials. The slicer-type cultivars Tasty Green, Lider, Cobra and Dasher II yielded the highest across all fungicide programs, compared to remaining cultivars screened. The pickling cultivars, Expedition, Sassy and Supremo yielded significantly higher than the other cultivars. The results from this study indicate that an integrated management approach of reduced fungicide application and the use of resistant cultivars can suppress levels of CDM and optimize cucumber yield.

Keywords: *Pseudoperonospora cubensis*, Cucurbit Downy Mildew, *Cucumis sativus,* Cucumber, cyazofamid and fluopicolide.

Introduction

Production of both slicer and pickling type cucumber (*Cucumis sativus*) in the U.S. has decreased rapidly from 68,000 ha in 2004 to 48,500 ha in 2011. One potential cause of reduced production is the devastating pathogen *Pseudoperonospora cubensis* causal agent of cucurbit downy mildew (CDM), a destructive pathogen of many different cucurbit crops worldwide. It has a wide host range infecting crops in the Cucurbitaceae or gourd family, including cucumber (*Cucumis sativus* var. *sativus* L.), squash (*Curcurbita* ssp.), watermelon (*Citrullus lanatus)*, and melon (*Cucumis melo* L.), among others (Palti, 1975).

Pseudoperonospora cubensis is an obligate parasite that cannot overwinter in areas that experience freezing temperatures. In North America the pathogen commonly overwinters in Florida, Central America, and Mexico, then spreads north through the growing season infecting cucurbits if conditions are favorable. The disease is characterized by angular chlorotic lesions on the adaxial side of the leaf and a 'downy' or 'felt-like' appearance of sporulation on the abaxial side of the leaf (Thomas, 1996). It has the ability to infect host plants at any stage of plant growth, including the cotyledonary stage. This disease poses a great threat to cucumber and cucurbit production worldwide and an effective management approach is desperately needed to suppress economic losses and increase profitable cucumber production.

To aid in the tracking of CDM movement, sentinel plots containing susceptible cucurbit cultivars are established annually throughout the United States (www.cdm.IPMpipe.org). Alerts are sent via email when CDM is reported in the selected region to notify growers, researchers and industry, to take preventative measures. Because *P. cubensis* does not overwinter in most of the U.S., certain cultural control methods, such as crop rotation and debris destruction do not

impact disease occurrence the following year. Thus, CDM management traditionally requires heavy reliance on preventative fungicide applications complemented with the use of disease tolerant cultivars (Steve Rideout, personal communication, Oct. 2010). However, *P. cubensis* has a history of developing resistance to fungicides quickly. *P. cubensis* it is categorized by the Fungicide Resistance Action Committee (FRAC) as possessing a high risk of developing fungicide resistance (Russell, 2004). Strains of *P. cubensis* have been reported to be resistant to phenylamides (FRAC code 4), and strobilurins (FRAC code 11) (Urban and Lebeda, 2006). Virginia Cooperative Extension recommends the use of protective fungicide applications prior to disease onset, such as chlorothalonil (FRAC M5) or mancozeb (FRAC M3), and a diverse fungicide program using multiple modes of action once disease is present within the area or field. Over the past decade three recommended fungicides have been; propamocarb hydrochloride (FRAC code 28, Previcur Flex 6F 1.2pt, Bayer Corporation, Research Triangle Park, NC), fluopicolide (FRAC code 43, Presidio 4SC, Valent U.S. A. Corporation, Walnut Creek, CA) and cyazofamid (FRAC code 21, Ranman 3.33SC, FMC Corporation, King of Prussia, PA) (Wilson *et al.,* 2012). Satisfactory disease control, and minimizing yield lost, could require four or more fungicide applications per growing season, which can be costly especially if the fungicides have reduced efficacy due to resistance development or favorable disease conditions.

To aid in satisfactory CDM management an effective integrated pest management program is essential. Selection of cucumber cultivar is important because of the variation in the their ability to combat the pathogen. To date, complete resistance to *P. cubensis* has not been accomplished, as currently cultivars marketed as resistant can still be infected by the pathogen, though some can reduce pathogen sporulation (Holmes *et al.,* 2004).

As resistant cultivars are being developed, the durability of the resistance varies greatly among cultivars. Among those marketed as resistant, some have partial resistance, meaning that they reduce the ability of the pathogen to infect, colonize, or sporulate (Agrios, 2005). Others are tolerant of the disease, meaning that disease progress is not restricted but that growth and yield are less affected than in susceptible cultivars. It is not always known whether a particular variety has partial resistance, or tolerance, or a combination of the two, and in this thesis, the term tolerance will be used to cover both phenomena. Without continual screening of new cucumber cultivars against CDM it is impossible to know which cultivars possess substantial tolerance to the pathogen. Understanding CDM resistance in currently available cultivars, could prevent yield losses, and reduced fungicide inputs.

The objectives of this research are 1) to evaluate the claims of CDM resistance found in commercially available pickling and slicing cucumber cultivars, and 2) to utilize cultivar resistance to develop an effective IPM program to manage CDM. This was accomplished by evaluating each potentially resistant cultivar in conjunction with three different fungicide programs (0, 2 and 4 applications). The results of this research will provide an effective management strategy for *P. cubensis.*

Materials and Methods

Experimental Design

Two field trials, one for pickling and one for slicer type cucumbers were conducted in each of the years 2010, 2011, and 2012, examining disease resistance to CDM at Virginia Tech's Eastern Shore Agricultural Research and Extension Center (ESAREC) in Painter, on a Bojac sandy loam soil. Fertilizer was applied at the rate of 112 kg/ha, broadcast incorporated, which is equivalent to 224 kg/ha nitrogen after bed formation. Beds were fumigated with 112 kg/ha

methyl iodide:chloropicrin (Midas 50:50%) (Arysta Lifesciences, Cary, NC) in 2010, 2011 and fumigated with 112 kg/ha methyl bromide:chloropicrin (Tri-Con 50:50%) (Trical Inc, Hollister, CA) in 2012 and covered with reflective plastic mulch and equipped with trickle irrigation tubing. Individual cucumber cultivars were hand seeded into the beds on Sep 2, 2010; Aug 24, 2011; and Aug 22, 2012. Three seeds per hole (46 cm spacing) were planted with a row spacing of 1.83 meters. Insect and weed control was accomplished using Virginia Cooperative Extension recommended practices (Wilson *et al.,* 2012). For all trials, plots were single rows 4.57 m long with 3.05 m alleys between plots. Treatments were replicated four times and arranged in a split plot design, with cucumber cultivar as the main plots and fungicide program as the subplots. Fungicide regimes included: 1) non-treated control (NTC), 2) low input program (Lo) and 3) high input program (Hi). The Lo input regime included one application of fluopicolide (0.05 l/ha) and an application of cyazofamid (0.03l/ha) on an approximate 20-day interval. The Hi input regime included one application of fluopicolide followed by two applications of cyazofamid and a final application of fluopicolide on an approximate 10-day schedule. The adjuvant alkyl polyethoxyethanol sulfate (0.06 L/ha) (CoHere, Helena Chemical Company, Collierville, TN) was added to all applications of cyazofamid. Application dates for the fungicide programs can be found in Table 2.1**.** Due to an apparent loss of efficacy of fluopicolide displayed in 2011 trials, cyazofamid was applied first in 2012, followed by two applications of fluopicolide and a final application of cyazofamid, to prevent a complete loss of plants due to high disease pressure early in the season (Rideout *et al*., 2012). Fungicide applications were applied with a backpack CO_2-pressurized backpack sprayer that was calibrated to deliver 374 L/ha at 206 kPa through a boom consisting of three nozzles spaced 46 cm apart and outfitted with TeeJet 11003 twin flat fan tips (Spraying systems Co. Wheaton, IL). The two

outer nozzles were mounted on 23-cm drop tubes. Cultivars tested varied between years, due to seed availability and current CDM resistance claims. A known susceptible cucumber cultivar was included as a comparison in both the pickling and slicing trials each year (Table 2.2).

Disease Ratings and Yield Assessments

Individual plots were rated for both disease incidence (percentage of leaves infected) and severity (percent leaf area infected). The Area Under Disease Progress Curve (AUDPC) for both disease incidence and severity was determined using the formula:

$$A_k = \sum_{i=1}^{N_i-1} \frac{(y_i + y_{i+1})}{2}(t_{i+1} - t_i)$$

Were t was the time in days since the first rating, and y was the estimated amount of disease present (Shaner and Finney, 1977). Trials were harvested twice in 2010 (pickles Oct 27 and Nov 15; slicers Nov 5 and 15), three times in 2011 (Oct 12, 20, and Nov 2), and twice in 2012 (pickles Oct 8 and 22; slicers Oct 17 and Nov 5).

Data were analyzed using Agricultural Research Manager (Version 8.0, Gylling Data Management Inc., Brookings, SD). Analysis of variance (ANOVA) and mean comparison were completed for all data sets (Fisher's Protected LSD, $P \geq 0.05$). Data were not analyzed across years due to differences in cultivars examined. Data sets without a significant 'cultivar X fungicide program' interaction allowed for analysis across treatments. Thus the analysis was conducted for the cultivar means and the fungicide program means. When the interaction effect was significant, individual treatment combinations were compared for significance.

Results

Slicer-type Cucumber 2010 Trial

Disease pressure was moderate in the 2010 slicer trial. Both fungicide program and cultivar were found to have highly significant effects ($p \leq 0.01$) for cucumber yield, and disease incidence and severity AUDPC values (Table 2.3). The cultivar X fungicide interaction effect was not significant for either disease incidence or severity AUDPC values, so main effects were compared (Figure 2.1). Resistant cultivars produced lower disease incidence and severity AUDPC values than the known susceptible cultivar Straight Eight Elite (SEE). Dasher II and Acclaim exhibited lower disease incidence AUDPC values than the other 'resistant' cultivars, Munchmore, General Lee, Tasty Green, which were not different from each other. Slicemaster Select showed lower disease incidence AUDPC values than the susceptible SEE. The Hi input fungicide program reduced disease incidence and severity AUDPC values compared to both the Lo input and the non-treated control (Figure 2.2). The Lo input program also reduced disease compared to the non-treated control.

A significant cultivar X fungicide program interaction did not allow analysis across treatments for yield (Table 2.4)**.** In general, three cultivars displayed the highest yields regardless of fungicide program, Munchmore, Tasty Green and Dasher II. Poor germination of the cultivar Acclaim caused lower yields across all fungicide programs. In general, fungicide programs with more applications caused increased yield.

Slicer-type Cucumber 2011 Trial

Disease pressure in the 2011 trial was moderate. Both fungicide program and cultivar were found to have highly significant effects ($p \leq 0.01$) for cucumber yield and disease incidence and severity AUPDC values (Table 2.3). The 'cultivar X fungicide program' interaction effect was not significant for disease severity AUDPC values, so main effects and sub effects were compared. Cultivars means for disease severity AUDPC values indicated three levels of cultivar

resistance (Figure 2.3). The cultivars Cobra, Tasty Green and Lider had lower disease severity AUDPC values than the cultivars Marketmore, Stonewall, Olympian and Dasher II. All had lower disease severity AUDPC values than the susceptible SEE. The fungicide program means were significantly different; the Hi input program reduced disease severity AUDPC values compared to both the Lo input program and the non-treated control (Figure 2.4). The Lo input program also reduced disease compared to the non-treated control. .

A significant interaction between 'cultivar X fungicide program' did not allow for analyses across treatments for disease incidence AUDPC values and yield (Table 2.5). Three cultivars, Tasty Green, Lider and Cobra, were lower in disease incidence AUDPC values in the Hi fungicide input program and had lower disease incidence AUDPC values in the non-treated control than the other cultivars. Four cultivars, Marketmore 76, Stonewall, Olympian and Dasher II, had lower disease incidence AUDPC values than the susceptible cultivar SEE in the non-treated control, but were not different in the Lo input program. In general, three cultivars showed the highest yields regardless of fungicide program: Tasty Green, Cobra and Dasher II. Throughout all treatments, yield increased with increasing fungicide applications.

Slicer-type Cucumber 2012 Trial

Disease pressure was severe in this trial, with emerging cotyledons becoming infected. Both fungicide program and cultivar were found to have highly significant effects ($p \leq 0.01$) for cucumber yield, disease incidence AUDPC and severity AUPDC values (Table 2.3). A significant 'cultivar X fungicide program' interaction did not allow for analyses across treatments. In general, three cultivars, Tasty Green, Lider, and Cobra, had the lowest amount of disease for both incidence and severity AUDPC values, regardless of the fungicide program (Table 2.6). Garden Sweet Burpless had higher levels of both disease incidence and severity

AUDPC values than all other cultivars including the susceptible cultivar SEE regardless of fungicide program. Tasty Green yielded significantly greater in all fungicide programs than the remaining cultivars. Cultivars Thunder and Cobra yielded significantly greater than most of the other cultivars in the respective fungicide programs, and consistently higher than the susceptible SSE in all fungicide programs.

Pickling-type Cucumber 2010 Trial

Disease pressure was moderate in the 2010 pickle trial. Cultivar and fungicide program effects were found to be highly significant ($p \leq 0.01$) for disease incidence and severity AUPDC values (Table 2.3). Cultivar and fungicide program effects were not significant for cucumber yield due to a wet season causing a high variability in fruit production. A significant 'cultivar X fungicide program' interaction did not allow for analysis across treatments for disease severity AUPDC values. Cultivars Carolina and Cross Country had lower disease severity AUDPC values in the non-treated control than the susceptible cultivar SMR58 (Table 2.7). There were no other significant differences.

The 'cultivar X fungicide program' interaction effect was not significant for yield or disease incidence AUDPC values so analyses across treatments were conducted. The cultivar Carolina has lower disease AUDPC values than Cross County and the susceptible SMR58 (Figure 2.5). The fungicide program means show lower disease incidence AUDPC values with increasing fungicide applications (Figure 2.6). There were no differences among the cultivars means (Figure 2.7) or the fungicide program means (Figure 2.8) for cucumber yield.

Pickling-type Cucumber 2011 Trial

Disease pressure was moderate in the 2011 pickle trial. Both fungicide program and cultivar were found to have highly significant effects ($p \leq 0.01$) for cucumber yield, disease incidence and severity AUPDC values (Table 2.3). A significant 'cultivar X fungicide program' interaction did not allow for analyses across treatments for disease incidence and disease severity AUDPC values. In general, the cultivars Supremo and Sassy displayed lower disease incidence and severity AUDPC values than Carolina and susceptible cultivar SMR58, regardless of the fungicide program (Table 2.8). Carolina had less disease incidence and disease severity AUDPC values than the susceptible SMR58. In all treatments, increasing fungicide applications produced a decrease in disease incidence and disease severity AUDPC values.

The 'cultivar X fungicide program' interaction effect was not significant for yield, so analyses across treatments were conducted (Figure 2.9). Resistant cultivars Supremo, Sassy, and Carolina yielded more than the known susceptible cultivar SMR58, but did not yield different from each other. Plots receiving the Hi input fungicide program yielded more than the Lo fungicide program and the non-treated control (Figure 2.10). The Lo input fungicide program also yielded higher than the non-treated control.

Pickling-type Cucumber 2012 Trial

Disease pressure was high in the 2012 pickle trial. Both fungicide program and cultivar were found to have highly significant effects ($p \leq 0.01$) for cucumber yield, disease incidence and severity AUPDC values (Table 2.3). The 'cultivar X fungicide program' interaction effect was not significant for disease severity AUPDC values, so analysis across treatments was conducted. The cultivar means showed three levels of disease severity AUDPC values, cultivars Supremo, Sassy, and Eureka had less disease than the cultivar Expedition. The cultivar Calypso

had more disease than Sassy, but was not different from the other cultivars (Figure. 2.11). All had lower disease severity AUDPC values than the susceptible cultivar SMR58. The fungicide program means were significantly different, the Hi input program reduced disease severity AUDPC values compared to both the Lo input program and the non-treated control (Figure 2.12). The Lo input program reduced disease compared to the non-treated control.

A significant 'cultivar X fungicide program' interaction did not allow for analysis across treatments for disease incidence AUDPC values and cucumber yield (Table 2.9). Three cultivars, Sassy, Supremo and Eureka, had the lowest disease incidence AUDPC values regardless of fungicide program. Expedition and Calypso produced lower disease incidence AUDPC values than the susceptible cultivar SMR58 in the respective fungicide programs. In general two cultivars showed the highest yields regardless of fungicide program, Expedition and Sassy. Throughout all treatments yield increased with increasing fungicide applications.

Discussion

The availability of cucumber cultivars claiming *P. cubensis* resistance increases annually. Exact levels of disease tolerance have not been well documented making it difficult for growers to correctly select cucumber cultivars. This research confirms that no complete resistance has been developed in the cucumber cultivars evaluated, as all cultivars screened were infected and damaged by the pathogen. However, some cultivars displayed higher levels of tolerance that may be useful in an integrated disease management program.

The results of this study were effective in displaying the importance of cultivar selection for cucurbit downy mildew control. Cultivars with higher CDM tolerance can be successfully used in conjunction with a reduced fungicide program to effectively manage CDM. This study

further demonstrates that with selection of the correct cucumber cultivar and well timed fungicide applications, a high yield can be obtained using reduced fungicide inputs. However, when environmental conditions were favorable for disease development and high disease pressure was present (as seen in 2012) additional fungicide applications are required.

Fungicide selection is extremely important for successful CDM management. Incorporating effective fungicides with different modes of action is paramount. The study demonstrated a decrease in the efficacy of fluopicolide in the 2011 and 2012 trials against the pathogen. This pathogen has a high risk of developing resistance to fungicides and has been reported resistant to several other fungicides (Urban and Lebeda 2006). Fungicide efficacy and possible resistance development needs to be constantly monitored to ensure proper disease management.

In the three years of this study, disease was present and caused infection on all cultivars tested, through naturally occurring inoculum. This allowed for variation in disease pressure over the years, providing real field conditions for the cucumber cultivars and fungicide programs evaluated. The slicer-type trials displayed four cultivars with repeated successful seasons, Tasty Green, Dasher II, Lider and Cobra. Lider had lowest levels of disease in the group but also produced the lowest yields of the four. The pickle-type trials displayed less clarity in definite cultivar tolerance levels than the slicers. Cultivars Sassy and Supremo yielded well in both trials tested with low levels of disease present, but in 2012 the cultivar Expedition surpassed the yield of both Supremo and Sassy, even with increased disease values. In most cases, the validity of the disease assessment method was suggested by its correlation with yield.

Even with the opportunity to successfully manage CDM with the use of tolerant cultivars and reduced fungicide applications, it is important for future work to evaluate new fungicides to combat this disease due to the high risk of fungicide resistance. With the high variation in disease tolerance found in the commercially available cultivars, it is important that all new cultivars be carefully and properly screened for efficacy against *P. cubensis* over several seasons, to accurately determine proper fungicide application programs for individual cucumber cultivars. Additional experiments examining new cultivars with different reduced fungicide input programs are needed in the future.

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Table 2.1 Fungicide application dates by year applied to both the pickling cucumber cultivar trial and the slicing cucumber cultivar trial on the same date. Fungicides were applied on approximate 10 day intervals.

	Year		
Fungicide	**2010**	**2011**	**2012**
fluopicolide (Presidio 4SC, 0.05L/ha)	Sep 22 (Hi + Lo) Oct 25 (Hi only)	Sep 14 (Hi + Lo) Oct 11 (Hi only)	Sep 20 (Hi only) Oct 2 (Hi + Lo)
cyazofamid (Ranman 400SC, 0.03L/ha) + alkyl polyethoxyethanol sulfate (CoHere, 0.06L/ha)	Oct 4 (Hi only) Oct 14 (Hi + Lo)	Sep 22 (Hi only) Sep 30 (Hi + Lo)	Sep 11 (Hi + Lo) Oct 15 (Hi only)

Table 2.2. Cultivars screened for CDM resistance listed by year. Each year included one susceptible cultivar and multiple cultivars with varying levels of tolerance or 'resistance'.

	Year		
Cucumber Type	**2010**	**2011**	**2012**
Pickle			
'resistant' cultivars	Carolina Cross County	Carolina Supremo Sassy	Eureka Supremo Sassy
susceptible cultivar	SMR58	SMR58	SMR58
Slicer			
'resistant' cultivars	Tasty Green Acclaim Slicemaster Select General Lee Munchmore	Tasty Green Lider Cobra Marketmore 76 Olympian Stonewall	Tasty Green Lider Cobra Marketmore 76 Turbo Thunder Garden Sweet Burpless
susceptible cultivar	Straight Eight-Elite	Straight Eight-Elite	Straight Eight-Elite

Table 2.3. Significance *P*(F value) for the main effects of cultivar and fungicide program and the interactions among the main effects. By year and cucumber type for AUDPC values and total yield per plot.

	AUDPC Values[x]		Yield
Split-Plot Analysis	**Incidence[y]**	**Severity[y]**	**kg/plot**
Slicers			
2010			
Cultivar	0.0004*[z]	0.0001*	0.0001*
Fungicide Program	0.0001*	0.0001*	0.0001*
Cultivar x Fungicide	0.1579	0.0863	0.0271*
2011			
Cultivar	0.0001*	0.0001*	0.0001*
Fungicide Program	0.0001*	0.0001*	0.0001*
Cultivar x Fungicide	0.0234*	0.3324	0.0011*
2012			
Cultivar	0.0001*	0.0001*	0.0001*
Fungicide Program	0.0001*	0.0001*	0.0001*
Cultivar x Fungicide	0.0018*	0.0060*	0.0001*
Pickles			
2010			
Cultivar	0.0017*	0.0001*	0.6174
Fungicide Program	0.0001*	0.0001*	0.3122
Cultivar x Fungicide	0.4373	0.0002*	0.4777
2011			
Cultivar	0.0001*	0.0001*	0.0001*
Fungicide Program	0.0001*	0.0001*	0.0001*
Cultivar x Fungicide	0.0306*	0.0020*	0.1650
2012			
Cultivar	0.0001*	0.0001*	0.0001*
Fungicide Program	0.0001*	0.0001*	0.0001*
Cultivar x Fungicide	0.0052*	0.5405	0.0011*

[x]AUDPC= Area Under Disease Progress Curve.

[y] Incidence = percent leaves infected in each plot. Severity = percent leaf area infected in each plot.

[z]Significance at the 0.05 level is indicated by *.

Table 2.4. Total yield for 2010 slicer trial, separated by cultivar and fungicide program. Each treatment was analyzed due to a significant interaction effect.

Cultivar	Fungicide Program	Totals Yield kg/plot
Munchmore	Lo [x]	12.81 a[w]
Dasher II	Hi[y]	11.63 a
Munchmore	Hi	10.79 a
Tasty Green	Lo	10.07 ab
Tasty Green	Hi	8.29 bc
Munchmore	NTC[z]	8.10 bc
General Lee	Hi	7.98 bc
General Lee	Lo	7.67 bc
DasherII	Lo	7.63 bc
Tasty Green	NTC	6.96 c
Straight Eight Elite	Hi	5.35 cd
Slicemaster Select	Lo	4.66 cd
Slicemaster Select	Hi	4.58 cd
Dasher II	NTC	4.51 d
Straight Eight Elite	Lo	4.20 d
General lee	NTC	3.92 de
Slicemaster Select	NTC	1.85 de
Straight Eight Elite	NTC	1.08 e
Acclaim	Hi	0.18 e
Acclaim	Lo	0.36 e
Acclaim	NTC	0.00 e

[w]Means within each column with the same letter are not significantly different ($P \geq 0.05$, Fisher's Protected LSD).
[x] Lo fungicide input program 1 application of fluopicolide on 22 Sep and 1 application of cyazofamid on 14 Oct.
[y] Hi fungicide input program 2 applications of fluopicolide on 22 Sep and 4 Oct and 2 applications of cyazofamid on 14 and 25 Oct
[z] Non-treated control.

Table 2.5. AUDPC values for disease incidence and total yield for 2011 slicer trial, separated by cultivar and fungicide program. Each treatment was analyzed due to a significant interaction effect.

		AUDPC Values[v]	Yield
Cultivar	**Fungicide Program**	**Incidence**	**kg/plot**
Straight 8 Elite	NTC[z]	1454.4 a[w]	0.50 i
Marketmore 76	NTC	1346.9 b	0.05 i
Straight 8 Elite	Lo[x]	1338.8 b	4.40 fg
Stonewall	NTC	1317.5 bc	1.13 hi
Olympian	NTC	1282.5 bc	0.36 i
Dasher II	NTC	1243.1 c	2.68 ghi
Lider	NTC	1121.1 d	0.23 i
Marketmore 76	Lo	1079.4 de	5.22 efg
Olympian	Lo	1065.6 de	4.08 gh
Cobra	NTC	1058.0 def	2.72 ghi
Straight 8 Elite	Hi[y]	1048.1 def	12.11 c
Dasher II	Lo	1012.5 ef	10.25 d
Stonewall	Lo	988.8 ef	7.57 def
Tasty Green	NTC	967.5 fg	12.88 c
Cobra	Lo	860.3 h	11.70 c
Marketmore 76	Hi	862.5 h	8.35 de
Lider	Lo	860.3 h	4.85 fg
Olympian	Hi	835.0 hi	10.43 cd
Dasher II	Hi	828.8 hi	16.56 b
Stonewall	Hi	791.9 hi	13.61 bc
Tasty Green	Lo	768.1 ij	20.68 a
Tasty Green	Hi	682.5 jk	19.14 ab
Lider	Hi	643.9 k	8.21 de
Cobra	Hi	620.0 k	18.33 ab

[v] AUDPC= Area Under Disease Progress Curve values derived from disease assessments on 5 , 22 Oct and 1 Nov. Incidence = percent leaves infected in each plot

[w] Means within each column with the same letter are not significantly different ($P \geq 0.05$, Fisher's Protected LSD).

[x] Lo fungicide input program 1 application of fluopicolide on 14 Sep and 1 application of cyazofamid on 30 Sep.

[y] Hi fungicide input program 2 applications of fluopicolide on 14 Sep and 14 Oct and 2 applications of cyazofamid on 22 and 30 Sep.

[z] Non-treated control.

Table 2.6. AUDPC values for disease incidence and severity for 2012 slicer trial, separated by cultivar and fungicide program. Each treatment was analyzed due to a significant interaction effect found in all variables.

		AUDPC Values[v]		Yield
Cultivar	**Fungicide Program**	**Incidence[v]**	**Severity[v]**	**kg/plot**
Garden Sweet Burpless	NTC[z]	2726.25 a[w]	1426.25 a	0.14 k
Straight 8 Elite	NTC	2535.0 b	1296.88 b	0.90 kl
Marketmore 76	NTC	2394.88 c	1152.25 c	2.31 ijk
Thunder	NTC	2261.88 d	1130.63 c	4.15 ghij
Turbo	NTC	2250.0 de	1107.88 cd	0.65 jk
Garden Sweet Burpless	Lo[x]	2170.63 de	1029.38 d	5.41 fghi
Garden Sweet Burpless	Hi[y]	2147.50 de	1004.38 de	7.74 efg
Straight 8 Elite	Lo	2049.25 f	944.63 de	3.80 ghijk
Lider	NTC	1889.38 g	921.50 ef	1.11 jk
Turbo	Lo	1837.75 g	847.00 f	3.68 hijk
Tasty Green	NTC	1811.50 g	802.00 fg	17.84 c
Marketmore 76	Lo	1795.00 g	837.13 f	6.62 fgh
Straight 8 Elite	Hi	1789.63 g	759.38 fg	7.07 fgh
Cobra	NTC	1680.00 h	759.88 fg	4.15ghij
Thunder	Lo	1666.13 h	731.88 fg	14.04 d
Turbo	Hi	1621.00 hi	757.13 fg	8.36 ef
Marketmore 76	Hi	1555.00 i	665.50 gh	10.79 de
Thunder	Hi	1498.75 ij	661.50 gh	19.40 c
Lider	Lo	1416.63 j	612.50 gh	5.16 fghi
Lider	Hi	1382.75 k	597.38 h	8.30 ef
Tasty Green	Lo	1371.25 k	570.00 hi	32.21 b
Cobra	Lo	1236.00 l	506.50 i	12.50 d
Tasty Green	Hi	1204.38 l	494.88 i	36.14 a
Cobra	Hi	1003.63 m	423.50 i	18.95 c

[v] AUDPC= Area Under Disease Progress Curve values were derived from disease assessments on 9, 15 and 25 Sep and 4 and 16 Oct. Incidence = percent leaves infected in each plot. Severity = percent leaf area infected in each plot.

[w] Means within each column with the same letter are not significantly different ($P \geq 0.05$, Fisher's Protected LSD).

[x] Lo fungicide input program 1 application of fluopicolide on 2 Oct and 1 application of cyazofamid on 11 Sep.

[y] Hi fungicide input program 2 applications of fluopicolide on 20 Sep and 2 Oct and 2 applications of cyazofamid on 11 and 15 Sep.

[z] Non-treated control.

Table 2.7. AUDPC values for disease severity 2010 pickle trial, separated by cultivar and fungicide program. Each treatment was analyzed due to a significant interaction effect between cultivars X fungicide program in AUDPC values for disease severity.

		AUDPC Values[v]
Cultivar	**Fungicide Program**	**Severity[v]**
SMR58	NTC[z]	303.75 a[w]
Carolina	NTC	195.00 b
Cross Country	NTC	191.25 b
SMR58	Lo[x]	54.00 c
Carolina	Lo	30.38 c
Cross Country	Lo	26.44 c
SMR58	Hi[y]	18.38 c
Carolina	Hi	14.63 c
Cross Country	Hi	12.56 c

[v]AUDPC= Area Under Disease Progress Curve. Severity = percent leaf area infected in each plot.
[w]Means within each column with the same letter are not significantly different ($P \geq 0.05$, Fisher's Protected LSD).
[x] Lo fungicide input program 1 application of fluopicolide on 22 Sep and 1 application of cyazofamid on 14 Oct.
[y] Hi fungicide input program 2 applications of fluopicolide on 22 Sep and 4 Oct and 2 applications of cyazofamid on 14 and 25 Oct
[z] Non-treated control.

Table 2.8. AUDPC values for disease incidence and disease severity for 2011 pickle trial, separated by cultivar and fungicide program. Each treatment was analyzed due to a significant interaction effect for AUDPC values for disease incidence and severity.

		AUDPC Values[v]	
Cultivar	**Fungicide Program**	**Incidence[v]**	**Severity[v]**
SMR58	NTC[z]	1637.5 a[w]	827.5 a
SMR58	Lo[x]	1392.5 b	720.0 b
Sassy	NTC	1138.3 c	552.5 c
SMR58	Hi[y]	1135.8 c	525.8 cd
Carolina	NTC	1058.5 cd	471.3 d
Carolina	Lo	1036.0 cd	469.5 de
Supremo	NTC	1025.2 cd	507.3 cd
Sassy	Lo	963.3 d	395.8 ef
Carolina	Hi	822.7 d	337.8 fg
Supremo	Lo	793.3 e	303.0 g
Supremo	Hi	779.2 e	316.0 g
Sassy	Hi	752.5 e	301.8 g

[v] AUDPC= Area Under Disease Progress Curve values derived from disease assessments on 5 , 22 Oct and 1 Nov. Incidence = percent leaves infected in each plot Severity = percent leaf area infected in each plot.
[w] Means within each column with the same letter are not significantly different ($P \geq 0.05$, Fisher's Protected LSD).
[x] Lo fungicide input program 1 application of fluopicolide on 14 Sep and 1 application of cyazofamid on 30 Sep.
[y] Hi fungicide input program 2 applications of fluopicolide on 14 Sep and 14 Oct and 2 applications of cyazofamid on 22 and 30 Sep.
[z] Non-treated control.

Table 2.9. AUDPC values for disease incidence and total yield for 2012 pickle trial, separated by cultivar and fungicide program. Each treatment was analyzed due to a significant interaction effect.

		AUDPC Values[v]	Yield
Cultivar	**Fungicide Program**	**Incidence[v]**	**kg/plot**
SMR58	NTC[z]	2696.25 a [w]	0.02 f
SMR58	Lo[x]	2405.64 b	0.73 f
SMR58	Hi[y]	2300.63 b	1.15 f
Expedition	NTC	2289.38 b	3.08 ef
Calypso	NTC	2131.88 c	1.87 f
Eureka	NTC	2111.25 c	2.11 ef
Sassy	NTC	2089.13 c	2.09 ef
Supremo	NTC	1899.38 d	4.83 e
Expedition	Lo	1723.13 e	9.67 bcd
Calypso	Lo	1649.23 ef	8.84 cd
Calypso	Hi	1625.63 ef	9.14 bcd
Eureka	Lo	1610.25 ef	6.91 de
Supremo	Lo	1586.63 ef	7.72 cd
Expedition	Hi	1559.25 f	13.64 a
Sassy	Lo	1441.88 fg	9.91 bc
Supremo	Hi	1421.25 fg	9.23 bcd
Eureka	Hi	1402.50 g	8.67 cd
Sassy	Hi	1325.63 g	11.68 ab

[v] AUDPC= Area Under Disease Progress Curve values were derived from disease assessments on 9, 15 and 25 Sep and 4 and 16 Oct. Incidence = percent leaves infected in each plot. Severity = percent leaf area infected in each plot.
[w] Means within each column with the same letter are not significantly different ($P \geq 0.05$, Fisher's Protected LSD).
[x] Lo fungicide input program 1 application of fluopicolide on 2 Oct and 1 application of cyazofamid on 11 Sep.
[y] Hi fungicide input program 2 applications of fluopicolide on 20 Sep and 2 Oct and 2 applications of cyazofamid on 11 and 15 Sep.
[z] Non-treated control.

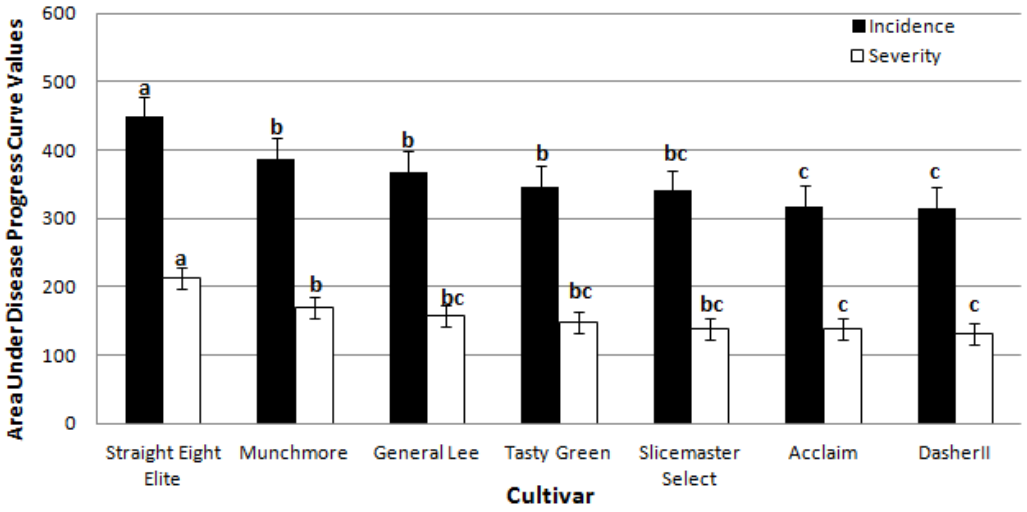

Figure 2.1 2010 slicer-type cucumber trial cultivar means for AUDPC values for disease incidence and severity. Trial was conducted in Painter, VA at Virginia Tech's Eastern Shore Agricultural Research and Extension Center. For each variable, bars with the same letter are not significantly different according to Fisher's LSD ($P \leq 0.05$).

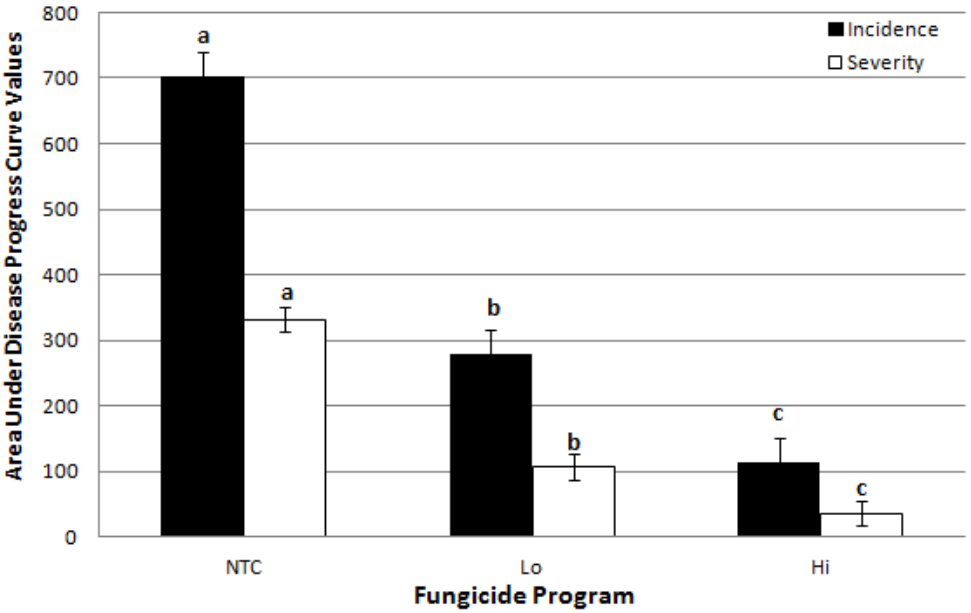

Figure 2.2 2010 slicer-type cucumber trial fungicide program means. Trial was conducted in Painter, VA at Virginia Tech's Eastern Shore Agricultural Research and Extension Center. For each variable, bars with the same letter are not significantly different according to Fisher's LSD ($P \leq 0.05$).

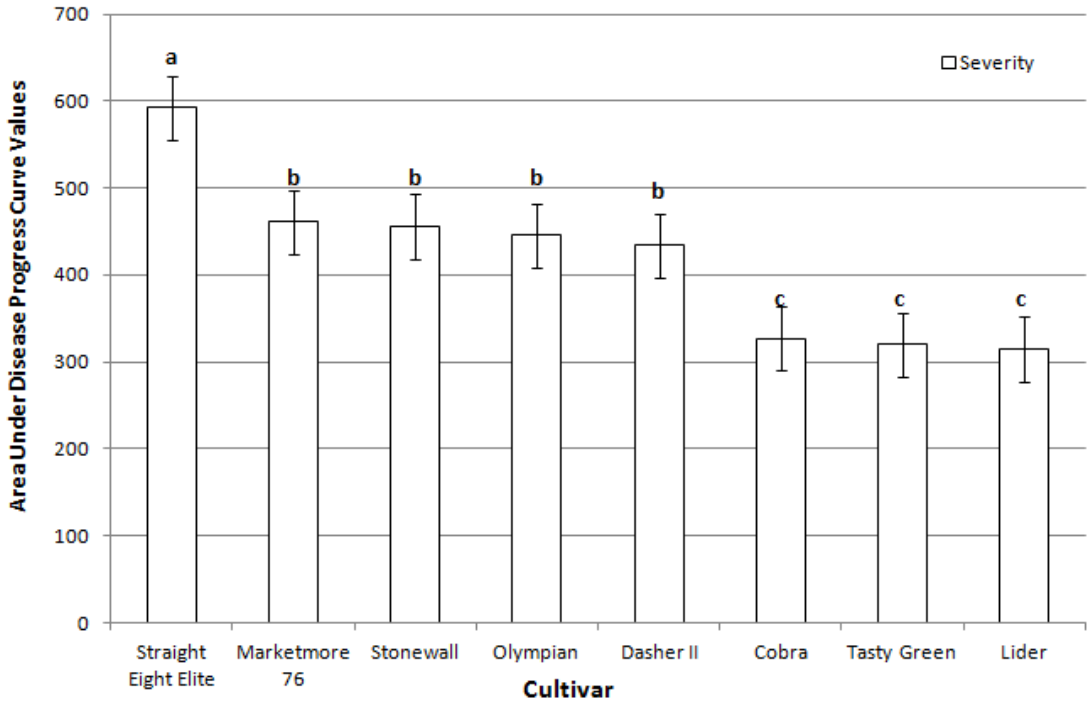

Figure 2.3. 2011 slicer-type cucumber trial cultivar means for AUDPC values for disease severity. Trial was conducted in Painter, VA at Virginia Tech's Eastern Shore Agricultural Research and Extension Center. Bars with the same letter are not significantly different according to Fisher's LSD ($P \leq 0.05$).

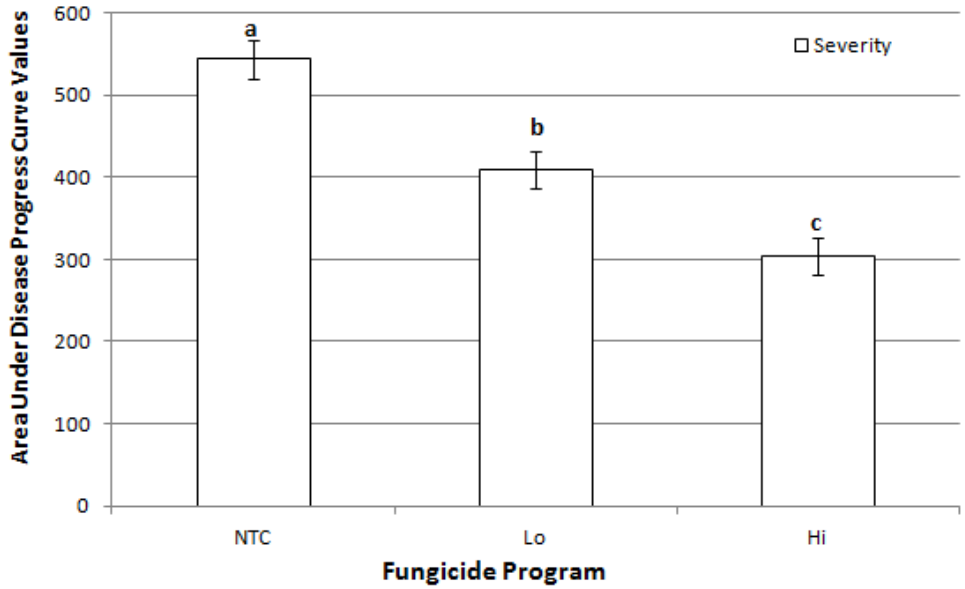

Figure 2.4. 2011 slicer-type cucumber trial fungicide program means. Trial was conducted in Painter, VA at Virginia Tech's Eastern Shore Agricultural Research and Extension Center. Bars with the same letter are not significantly different according to Fisher's LSD ($P \leq 0.05$).

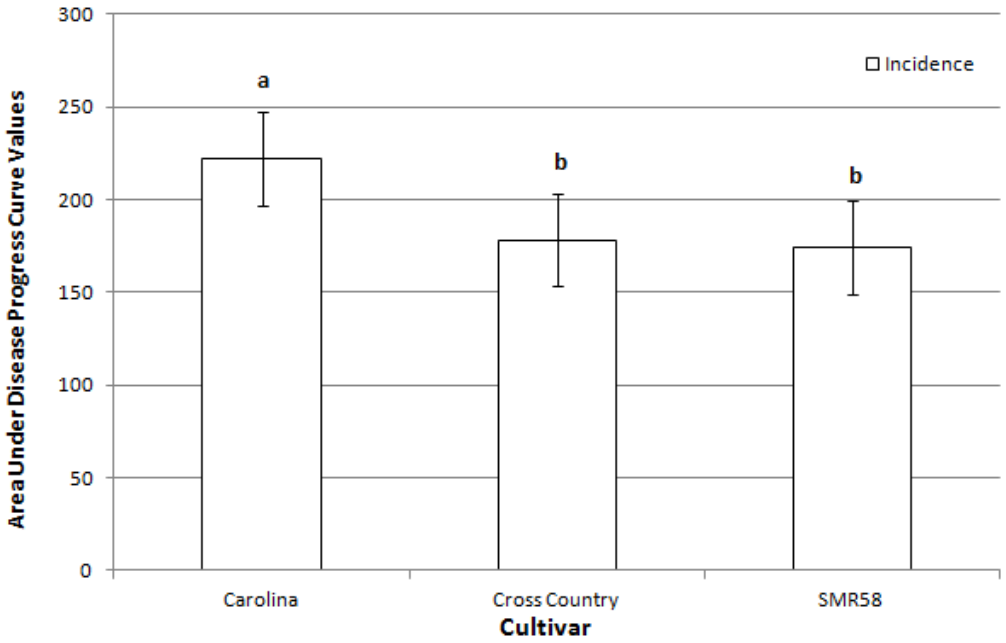

Figure 2.5. 2010 pickle-type cucumber trial cultivar means for disease incidence AUDPC values. Trial was conducted in Painter, VA at Virginia Tech's Eastern Shore Agricultural Research and Extension Center. Bars with the same letter are not significantly different according to Fisher's LSD ($P \leq 0.05$).

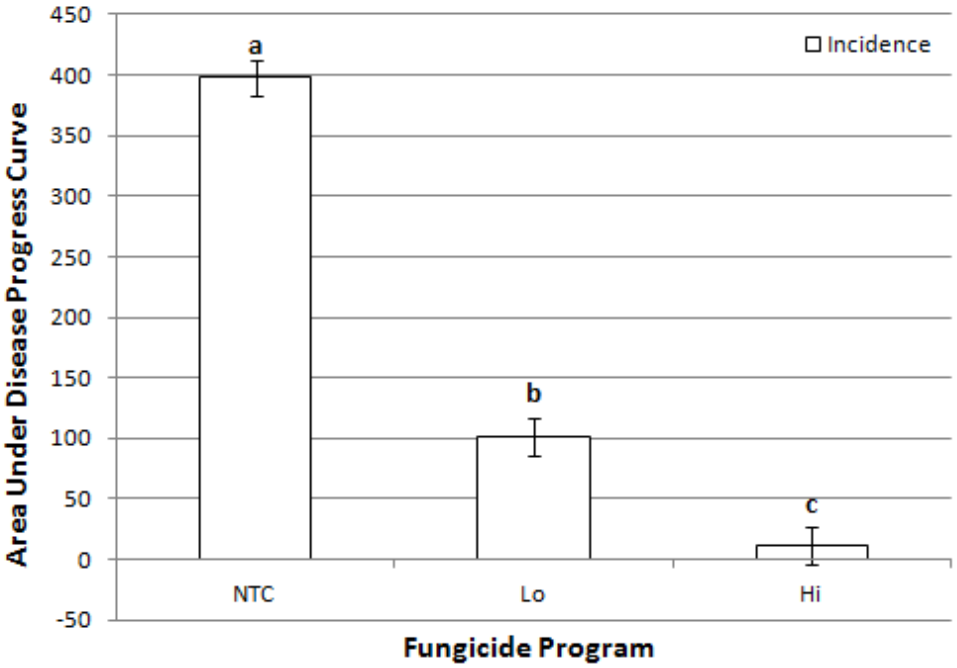

Figure 2.6. 2010 pickle-type trial fungicide means for disease incidence AUDPC values. Trial was conducted in Painter, VA at Virginia Tech's Eastern Shore Agricultural Research and Extension Center. Bars with the same letter are not significantly different according to Fisher's LSD ($P \leq 0.05$).

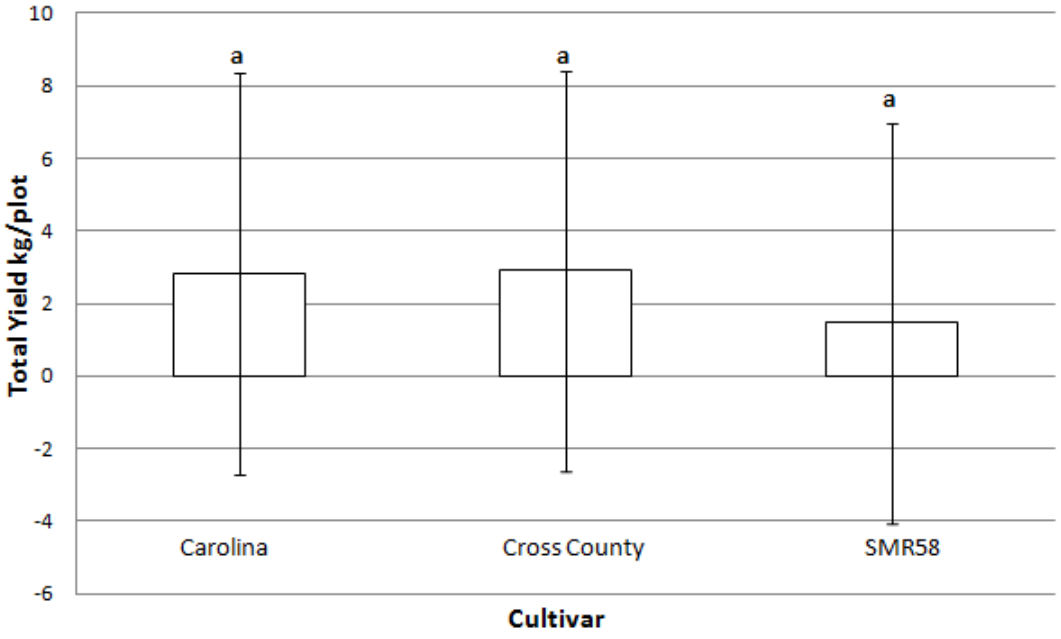

Figure 2.7. 2010 pickle-type cucumber trial cultivar means for total yield per plot .Trial was conducted in Painter, VA at Virginia Tech's Eastern Shore Agricultural Research and Extension Center. Bars with the same letter are not significantly different according to Fisher's LSD ($P \leq 0.05$).

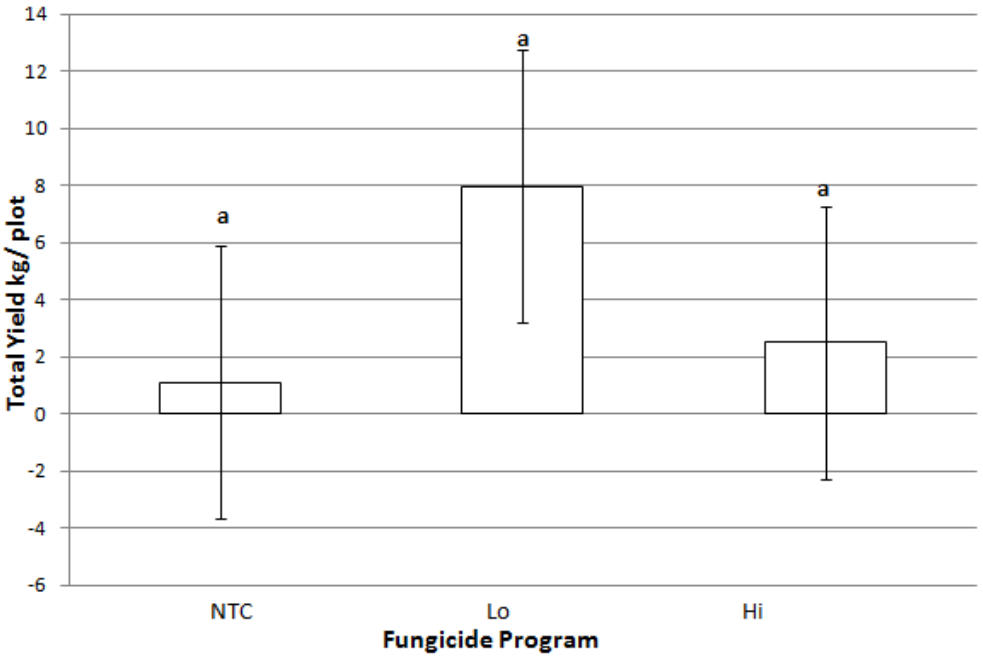

Figure 2.8. 2010 pickle-type cucumber trial fungicide means for total yield per plot. Trial was conducted in Painter, VA at Virginia Tech's Eastern Shore Agricultural Research and Extension Center. Bars with the same letter are not significantly different according to Fisher's LSD ($P \leq 0.05$).

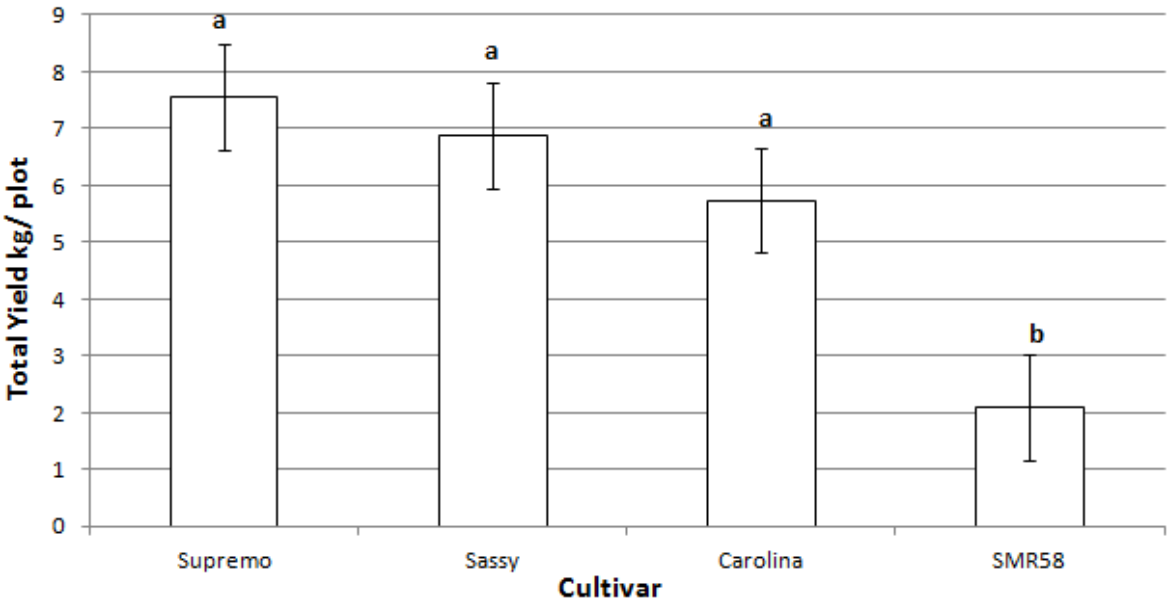

Figure 2.9. 2011 pickle-type cucumber trial cultivar means for total yield per plot. Trial was conducted in Painter, VA at Virginia Tech's Eastern Shore Agricultural Research and Extension Center. Bars with the same letter are not significantly different according to Fisher's LSD ($P \leq 0.05$).

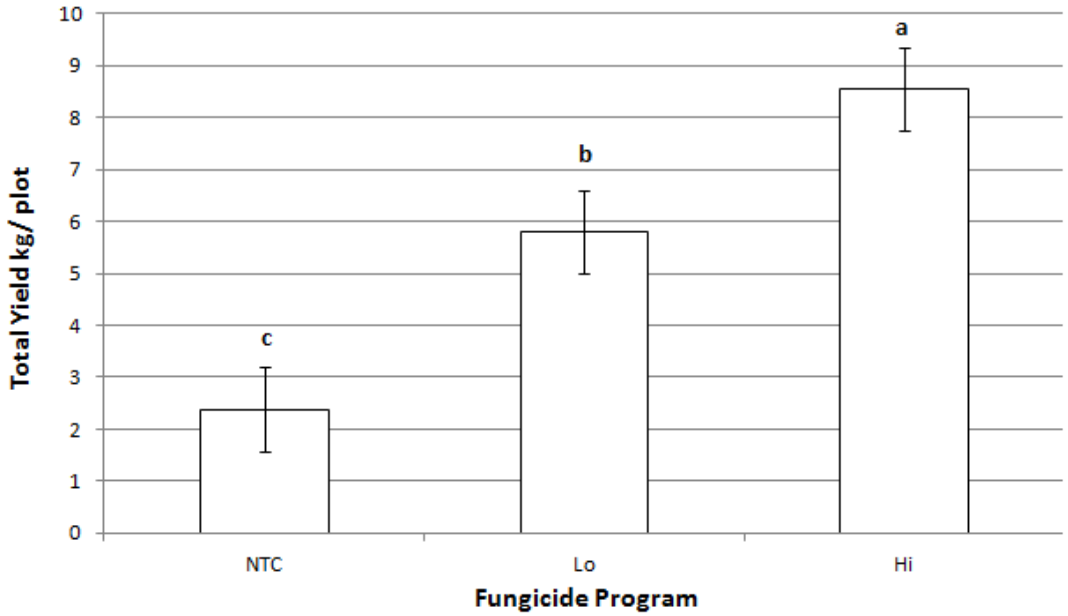

Figure 2.10. 2011 pickle-type cucumber trial fungicide means for total yield per plot. Trial was conducted in Painter, VA at Virginia Tech's Eastern Shore Agricultural Research and Extension Center. Bars with the same letter are not significantly different according to Fisher's LSD ($P \leq 0.05$).

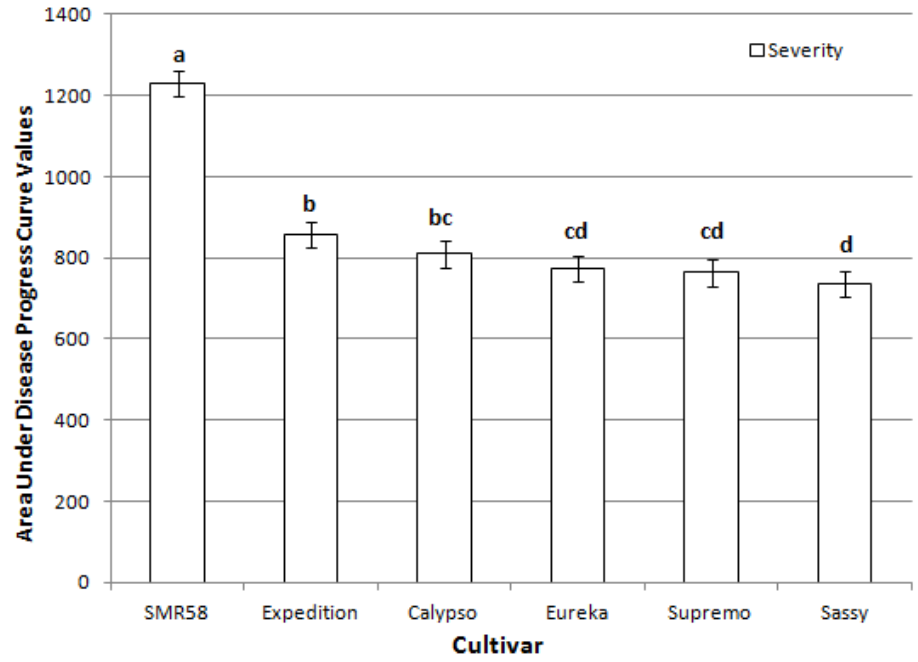

Figure 2.11. 2012 pickle-type cucumber trial cultivar means for AUDPC values for disease severity. Trial was conducted in Painter, VA at Virginia Tech's Eastern Shore Agricultural Research and Extension Center. Bars with the same letter are not significantly different according to Fisher's LSD ($P \leq 0.05$).

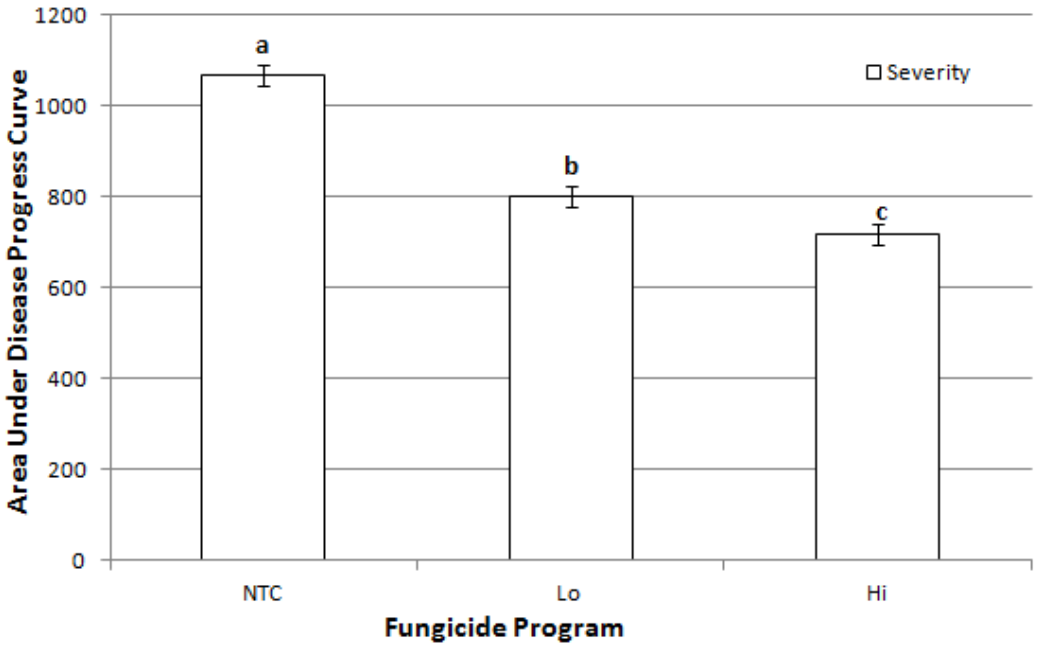

Figure 2.12. 2012 pickle-type cucumber trial fungicide means for disease severity AUDPC values. Trial was conducted in Painter, VA at Virginia Tech's Eastern Shore Agricultural Research and Extension Center. Bars with the same letter are not significantly different according to Fisher's LSD ($P \leq 0.05$).

CHAPTER 3: Variance in defense related gene expression in nineteen different cucumber (*Cucumis sativus*) cultivars to *Pseudoperonospora cubensis.*

Abstract

No complete resistance has been found in cucumber (*Cucumis sativus)* against the pathogen *Pseudoperonospora cubensis* and little is known about the molecular events involved in differing levels of cultivar tolerance. This study investigates the relative expression of genes encoding a basic PR-1 protein, a cytosolic ascorbate peroxidase protein and three resistance (R) gene proteins containing nucleotide binding sites, all of which may possibly be involved in defense response to *P. cubensis* in nineteen cucumber cultivars with various levels of disease tolerance. Relative expression levels for each R-gene varied between cultivar with no correlation between disease susceptibility and tolerance, suggesting no involvement between the genes of interest and defense against the pathogen. The basic PR-1 protein displayed very little significance in the expression levels. The majority of the cultivars had decreased expression indicting that defense against the pathogen does not require this gene. The pattern of the cytosolic ascorbate peroxidase suggests an increase in relative expression in susceptible cultivars and a decrease in the tolerant cultivars, suggesting a relationship between the pathogen recognition and cytosolic ascorbate peroxidase production.

Keywords: *Pseudoperonospora cubensis*, Cucurbit Downy Mildew, *Cucumis sativus,* Cucumber, PR-1, cytosolic ascorbate peroxidase, CSA06757, CSA06757, CSA06724.

Introduction

Pathogen and Host Importance

Pseudoperonospora cubensis (Bert. et Curt) Rost. is the casual agent of cucurbit downy mildew (CDM) which causes devastating economic losses worldwide. It is an oomycete obligate biotrophic pathogen with the ability to proliferate quickly, defoliating entire fields seemingly overnight. While *P. cubensis* can infect many different types of cucurbit crops, it is the most damaging foliar disease of cucumber (*Cucumis sativus)* (Savory *et al.,* 2011). The pathogen overcame the cucumber's genetic resistance in 2004 and presently there are no completely resistant cultivars commercially available (Savory *et al.,* 2011). Cucumber is a highly valuable crop worldwide; in the United States alone, it is estimated at about $360 million per annum (Anonymous, 2011). There are many different cucumber cultivars available on the market and many claim resistance to *P. cubensis,* but complete resistance has not been found. The cucumbers cultivars in this study have differing abilities to combat the pathogen. Some of the cultivars display partial resistance to the pathogen, reducing sporulation, while others display various levels of disease tolerance, allowing severe disease levels and but producing high yields. Some cultivars allow high disease development with reduced yields, but are not as affected as the susceptible cultivars. Due to the variability found, the term tolerance will be used here.

Nineteen different cucumber cultivars were selected for use in this study, including both pickling and slicer-type cucumbers. The cultivars are separated for this study based on ranking of their disease tolerance as either mildly tolerant or a slightly higher, moderate tolerance. Two known susceptible cultivars were included for comparison, a slicing-type Straight Eight Elite and a pickling-type SMR58. Nine of the cultivars used were ranked mildly tolerant, two pickling-types; Diva and Cross Country, and seven slicing-types; Stonewall, General Lee,

DasherII/Poinsett76, Munchmore, Slicemaster-Select, Marketmore 76, and Poinsett 76. Seven cultivars tested had moderate levels of tolerance including two pickling-types; Supremo and Sassy, and five slicing-types, Olympian, Dasher II Tasty Green, Lider, and Cobra. All of the cultivars tested were susceptible in that they could be infected by the pathogen, and the pathogen was able to sporulate on each cultivar (Cooper, 2012 unpublished data)

Disease Resistance- Searching for Answers

The cucumber genome was sequenced in 2009 by Huang *et al.* The sequence revealed 61 genes that encoded for nucleotide binding sites (NBS), which are a canonical domain found in plant resistance (R) genes (Dangl and Jones, 2001). These NBS are associated with detecting the pathogen inside the cell and eliciting a resistant reaction. Three quarters of the NBS genes found are located in 11 clusters in the genome (Huang *et al.,* 2009). For this study, three of the NBS genes were chosen, CSA06757, CSA06758, CSA06724; they are all found in a cluster on the second chromosome of *C. sativus.* A cluster of R-genes was chosen because resistance to the cucumber pathogen *Cladosporium cucumerinum* (cucumber scab) was found in a cluster of R-genes (Kang *et al.,* 2010). An additional reason for choosing these specific NBS genes is based upon the type of NBS, Toll-Interleukin-1 receptor type Nucleotide-binding sites with a leucine rich repeat (TIR-NB-LRR). This type of receptor has been associated with ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) in the model plant *Arabidopsis thaliana* (Wiermer *et al.,* 2005; Falk *et al.,* 1999) The EDS1 pathway has been thoroughly studied in *A. thaliana;* it is believed to be activated by TIR-NB-LRR-type resistance genes (upstream), and once activated, it can elicit a resistance response via pathogen related proteins (PR) or reactive oxygen species (ROS) (downstream) (Wiermer *et al.,* 2005; Falks *et al.,* 1999; Rogers and Ausubel, 1997). A related oomycete pathogen of *A. thaliana, Hyaloperonospora arabidopsidis,* was found to

produce effector proteins that are able to bind to the TIR-NB-LRR class of R-genes in *A. thaliana* thus activating EDS1 (Aarts *et al.*, 1998).

This study investigates the relative expression of two downstream proteins thought to be activated by the EDS1 pathway, a gene encoding basic PR-1 protein, and a cytosolic ascorbate peroxidase protein. Pathogenesis related proteins (PR) are inducible plant disease related proteins. They have been found to combat many different types of pathogens, and when induced can reduce disease severity (Van Loon *et al.*, 2006). Cytosolic ascorbate peroxidase is produced when the plant undergoes oxidative stress, which can be caused by ROS such as H_2O_2 or $–O_2$ (Davletova *et al.*, 2005). When EDS1 is activated it can cause an increase in production of ROS, which can cause a hypersensitive response in the cell. A high quantity of peroxisomes was found in *Cucumis melo* (wild melon) when infected with *P. cubensis,* causing a hypersensitive response and resistance to *P. cubensis* (Eckardt, 2004). Cytosolic ascorbate peroxidases are produced to scavenge the damaging ROS, thus preventing the hypersensitive response and cell death (Rizhsky *et al.*, 2004; Davletova *et al.*, 2005).

The hypothesis of this study is that the TIR-NB-LRR R-genes (CSA06757, CSA6758, CSA06724) will detect the pathogen, and signal through EDS1, which will in turn activate PR-1 and ROS production to combat the pathogen. The increase in ROS production will cause oxidative stress and an increase of cytosolic ascorbate peroxidase production. Increased relative expression for the R-genes and the PR-1 protein is expected in the moderately tolerant cultivars and reduced expression for the cytosolic ascorbate peroxidase as hypersensitive response would lead to reduced sporulation of the pathogen and less disease.

Clarifying the molecular pathway that the tolerant cultivars are using to combat this pathogen could theoretically help pave the way for developing a cultivar with higher tolerance, as well as better elucidate the interaction between the plant and pathogen.

Materials and Methods

Plant Material

Individual cucumber cultivar seeds were sown directly into 128 well Styrofoam flats filled with Premier Pro-Mix BX (sphagnum peat moss 85 %, perlite and vermiculite). Replicate 1 was planted on July 22, 2011, replicates 2 and 3 were planted June 25, 2012. Following germination (3-5 days after planting), the seedlings were fertilized with liquid fertilizer containing nitrogen, phosphorus and potassium (15-30-15). Plants were grown under natural light conditions in a greenhouse in Painter, VA. Plants were kept in the greenhouse until all cultivars developed into the second leaf stage (16-20 days). Due to variation in growing time for each cultivar, some cultivars reached the third leaf stage before inoculation.

Inoculation and Tissue Collection

Infected cucumber leaves were collected from cucumber plants in cucurbit downy mildew sentinel plots located in Newark, DE and Painter, VA for replicate 1 and replicates 2 and 3, respectively. The leaves were washed with sterile distilled water and a sporangia suspension of 2,000 spores/ml was made. The spore concentration was determined using a hemocytometer. Cucumber plants were placed in plastic bags and inoculated with the spore suspension and kept at 18.5 ^{o}C for 24 hours in the dark. Bags were sealed to maintain high humidity during the infection period. A control set of cultivars was sprayed with sterile distilled water and kept at the same conditions. Inoculations for replicate 1 differ from replicates 2 and 3, as the first was conducted at University of Delaware in Newark, DE and was kept in a dark, cold room during

the infection period. Replicates 2 and 3 were conducted at Virginia Tech's Eastern Shore Agricultural Research and Extension Center (ESAREC) and were kept in a growth chamber at the same conditions following inoculations. At 24 hours following inoculation, the second leaf from each plant was removed, wrapped in tin foil and flash frozen in liquid nitrogen immediately following collection and stored at -80 ^{o}C for RNA extraction.

RNA Extraction and cDNA Synthesis

Two different protocols were used for total RNA extraction for replicates 1 versus 2 and 3. Leaves for replicate 1 were extracted by grinding the frozen tissue by hand and using triazol reagent to extract the RNA (Appendix A). Replicates 2 and 3 were extracted using FastRNA® Pro Green Kit (MP biomedical, Solon, OH) using the FastPrep 24 instrument for grinding the sample (Appendix A). Approximately 200 mg of fresh leaf tissue was used in accordance with the protocols. RNA concentration and purity were determined by spectrophotometry (Nano-drop). The Nano-drop revealed that some RNA samples were contaminated with cellular mucopolysaccharides. Contaminated samples were further purified using either an RNeasy Mini Kit (Qiagen, Germantown, MD), or the additional purification steps provided by the protocol (Appendix A). The RNA integrity was confirmed by gel electrophoresis. A 1.5% agarose gel was used to check for integrity of the RNA product prior to cDNA synthesis. Synthesis of cDNA was accomplished using cDNA Omniscript RT kit (Qiagen, Germantown, MD). PCR was performed on the cDNA product with EF1 and actin primers to ensure the synthesis was successful prior to qRT-PCR (Table 3.1).

Primer Design

Primer pairs for real time qRT-PCR were designed using the Primer Quest program accessed through integrated DNA technologies (www.idtdna.com). Resulting primers can be found in Table 3.1. To determine whether the primers worked, each was tested on DNA that was previously extracted from each cultivar using DNA fast spin kit (MP biomedical, Solon, OH). Primers that amplified the genes of interest in all nineteen cultivars were used for real-time qRT-PCR.

Quantification of Specific Gene Expression

Real-time quantitative PCR reactions were conducted in an Eppendorf Mastercycler® (Eppendorf, Hamburg, Germany). Each reaction was done in triplicate using 5'SYBR green kit at 20 µl per reaction in 96-well eppendorf plates (Eppendorf, Hamburg, Germany). Reaction parameters were set at 95 ^{o}C for 2 minutes, followed by 40 cycles of 95 ^{o}C for 15 seconds, 58 ^{o}C for 20 seconds, and 72 ^{o}C for 25 seconds; this was followed by a melting curve to check for contaminating fragments. A water control lacking a cDNA template was included for each primer set used. Elongation-factor 1 (EF1) gene and alpha-tubulin (A-TUB) were used as housekeeping genes, as they are generally constitutively expressed, to normalize the expression, and a control plant (non-inoculated) was used to compare.

Data analysis

Relative expression values (REVs) were calculated using the $2^{-\Delta\Delta CT}$ method for each gene. Three different biological replicates were conducted and each biological replicate had three technical replicates. The Ct values for each reaction was calculated and used in the $2^{-\Delta\Delta CT}$ equation (Livak and Schmittgen, 2001). Effects were considered significant if the standard error

bars did not intersect with the control relative expression level of 1. Thus each bar in the figures represents the average REVs for all three replicates for each cultivar ± standard error. Standard error was calculated using a total of 9 data points from the qRT-PCR reactions.

Results

Calculating the $2^{-\Delta\Delta CT}$ value using the geometric mean of both housekeeping genes (EF1 and A-TUB) caused large variation in the error bars. Consequently the $2^{-\Delta\Delta CT}$ value was recalculated using only the housekeeping gene EF1 to reduce the standard error. Outliers, which were most likely due to human errors in pipetting, were removed from each data set before the analysis was conducted (Table 3.2- 3.6).

R-Gene Relative Expression

The cluster of R genes tested did not express as a unit in all cultivars, which suggests that they operate individually. Compared with the non-inoculated control, inoculated plants of the susceptible cultivars Straight Eight Elite and SMR58 had significantly reduced expression of the R genes tested except for CSA06757 which was not significantly reduced in Straight Eight Elite (Figure 3.1).

The mildly tolerant cultivars varied greatly in R gene expression. CSA06758 relative expression value was significantly increased in cultivars; Marketmore 76, Slicemaster Select, Carolina, and Diva and was significantly reduced in the cultivars; Stonewall, DasherII/Poinsett76, General Lee and Poinsett 76 (Figure 3.1). CSA06724 had high relative expression in six cultivars, namely Slicemaster Select, Diva, Cross Country, Marketmore 76, and decreased in cultivars; General Lee, and Dasher II/Poinsett 76 (Figure 3.1). CSA06757 had increased relative expression in five of the mildly tolerant cultivars; Marketmore 76, Stonewall,

Slicemaster Select, Carolina, and Diva, and had decreased relative expression in the three cultivars, Dasher II/Poinsett 76 and General Lee (Figure 3.1).

The moderately tolerant cultivars also varied in the relative expression of their R genes. CSA06724 had significant expression in only three cultivars; it was increased in cultivars Sassy and Olympian and decreased in Dasher II. The remaining four cultivars did not have significant changes in gene expression (Figure 3.1). CSA06757 had increased expression in two cultivars, Cobra and Sassy, and decreased expression in two cultivars, Olympian and Supremo (Figure 3.1). The last R gene CSA06758 had increased relative expression in the cultivars Lider and Sassy and decreased expression levels in the cultivar Olympian (Figure 3.1).

There was no relationship between R gene relative expression values and disease tolerance levels, except for the reduced expression in the two susceptible cultivars.

Cytosolic Ascorbate Peroxidase Expression

Cytosolic ascorbate peroxidase produced the most interesting results in expression levels. Susceptible cultivars displayed an increase in relative expression levels. The increase for SMR58 was highly significant, the increase for SEE was not (Figure 3.2).

Mildly tolerant cultivars Dasher II/Poinsett 76, Munchmore, Slicemaster Select had an increase in cytosolic ascorbate peroxidase expression. Marketmore 76, Carolina, and Cross Country had decreased expression. The remaining three cultivars did not have a significant change in expression level during infection (Figure 3.2).

The moderately tolerant cultivar Supremo was the only cultivar in this group that had a increase in relative expression values. All of the remaining six cultivars had a decrease in relative expression value (Figure 3.2).

PR-1 Relative Expression

Ten of the nineteen cultivars had decreased relative expression levels, three of them had increased expression and the remaining six did not have significant changes in their expression levels. These results suggest that PR-1 is not activated during the first 24 hours of *P. cubensis* infection. The two susceptible cultivars both had significantly reduced expression of the PR-1 gene (Figure 3.3).

The mildly tolerant cultivars with decreased expression levels were Marketmore 76, Stonewall, DasherII/Poinsett76, General Lee, and Cross Country. The only cultivar with mild tolerance and increased PR-1 expression was Poinsett 76.

The moderate tolerant cultivars Olympian and Cobra were the only cultivars with increased relative expression of the PR-1 gene, cultivars Lider and Supremo had no significant change in expression levels. The three remaining cultivars, Tasty Green, Dasher II and Sassy, all had decreased expression levels (Figure 3.3).

Discussion

While expression profiling of cucumber during *P. cubensis* infection has been conducted, it was completed in the cucumber cultivar 'Valspik' (Adhikari *et al.*, 2012). This study is the first to compare relative expression differences of multiple genes in multiple cultivars. Examining the molecular differences between cultivars with various levels of tolerance gives insight into the possible plant pathogen interactions. Uncovering the pathway the cucumber uses to battle *P. cubensis* could lead to the development of a cultivar highly resistant to the pathogen. The results of this study provide fundamental information on the molecular basis of resistance; our data both supports one section of the hypothesis, and disproves others.

Unraveling the Role of R-Genes

With the large number of putative R-genes revealed in the sequencing of the cucumber genome, there is much research need to discover the role that each gene plays in disease resistance (Huang *et al.,* 2009). This study looks at only a small set (3) of the 61 NBS genes revealed in the sequence. The genes tested are located on the second chromosome in a cluster. A previous study in cucumber found a cluster of R-genes working together causing a resistance reaction to *Cladosporium cucumerinum* (cucumber scab) (Kang *et al.,* 2010). Our results disprove the hypothesis that the selected gene cluster expresses as a group. Gene expression differed individually regardless of the tolerance levels in different cultivars, suggesting that they may not be playing a vital role in disease resistance to *P. cubensis.*

The Unknown PR-1 Protein

The exact role of PR-1 in disease resistance is still unknown; it has been studied in other systems (*Arabidopsis thaliana, Nicotiana tabacum*) and found to play a role in signaling in a resistance reaction (Van Loon *et al.,* 2006). It has been recognized to play a role with both bacterial and Oomycete pathogens (Van Loon, 1997, Rogers and Ausubel, 1997). The data from my study did not display an interaction between *P. cubensis* and the gene encoding for the basic PR-1 like protein. The majority of cultivars tested displayed reduced or non-significant change in relative expression levels. While the protein has displayed an active presence in other pathways, these data demonstrate that it is unlikely the gene plays a role in the infection of cucumber by *P. cubensis*. With the large number of pathogenesis-related proteins already identified, there are many other opportunities, to examine the expression levels of each PR gene during infection.

Cytosolic Ascorbate Peroxidase - Tackling H_20_2

Though the PR-1 protein is not likely being used against *P. cubensis*, in contrast cytosolic ascorbate peroxidase did display some interesting results. If the EDS1 pathway is being activated by an unknown R-gene, it is possible that ROS are being produced to cause a hypersensitive response and inhibiting pathogen development within the plant. This hypothesis is supported by the significant differences in relative expression levels discovered in this study and the high levels of peroxidase that were reported to be produced during cucumber infection with *P. cubensis* in a recent study by Adhikari *et al.* (2012). The susceptible cultivars had highly significant expression of cytosolic ascorbate peroxidase gene, thus the ROS species would be removed by the cytosolic ascorbate peroxidase and there would be no hypersensitive response (Rizhsky *et al.*, 2004). Cultivars with higher levels of tolerance had significantly down regulated relative expression levels of the gene encoding for the cytosolic ascorbate peroxidase, suggesting a larger amount of ROS present in the cell. This high level of ROS could result in a hypersensitive response, causing partial resistance to the pathogen, by inhibiting cell colonization and thus stopping sporulation and further spread of the pathogen.

Future Work

Genes encoding for pathogen effectors have been uncovered in *P. cubensis.* Over 270 candidate effector proteins have been identified with RXLR motifs (Savory *et al.*, 2012). H.H. Flor's gene for gene hypothesis states that there are corresponding genes in susceptible hosts for these putative effector proteins (Flor, 1947). While this study did produce some interesting results of gene expression in cucumber, there is still much to be done in searching for virulence gene interactions. There are still many questions that need to be answered about the cucumber

and *P. cubensis* interaction: Which R-genes interact with the pathogens effectors? Does the pathogen have the ability to suppress the plant's defenses? And what is the cost of resistance, a large amount of programmed cell death along with a reduction in yield? With so many questions unanswered, there is still much work to be done, uncovering the pathway to develop stronger resistance in cucumber to *P. cubensis.*

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Table 3.1. Primers used in the study.

Gene Name	Author	Accession #	Primer name	Sequence	Product size
Csa006724	Huang *et al.* 2009	ACHR00000000	006724For	ACAAACTGGTTGGTTTGGAGGAGC	106bp
			006724Rev	ACCAGCTAAGTTGGCAGCAGTAGT	
Csa006757	Huang *et al.* 2009	ACHR00000000	006757For	AACCGGGTCGAAATGCATGACTTG	155bp
			006757Rev	ATGACTTTCACAGCCCTTGCTTCC	
Csa006758	Huang *et al.* 2009	ACHR0000000	006758 For	GCATGGAATTGGAGGTATGGGCAA	132bp
			006758 Rev	CGAACAAGTCCCTCGTGTTGCTTT	
Cytosolic Ascorbate Peroxidase 37020723	Deepak *e al.* 2008	Gi 166985	Peroxidase 1 For	TTACCAGTTGGCTGGTGTTGTTGC	178bp
			Peroxidase 1 Rev	AATGTCCTGGTCCGAAAGACCCAT	
PR-1	Cools,H.J. Ishii,H.	ALL84768.1	PR-1 For	TCAAGACTTCGTCGGTGTCCACAA	213bp
			PR-1 Rev	TCCACCCACAACTGAACTGCATCT	
Elongation factor 1-alpha	Hongjian *et al.* 2010	EF446145.1	EF1 For	ACTGTGCTGTCCTCATTATTG	98bp
			EF1 Rev	AGGGTGAAAGCAAGAAGAGC	
Actin	Hongjian *et al.* 2010	ab010922	ACT For	TTCTGGTGATGGTGTGAGTC	149bp
			ACT Rev	GGCAGTGGTGGTGAACATG	
Alpha-Tubulin	Hongjian *et al.* 2010	aj715498	TUA For	ACGCTGTTGGTGGTGGTAC	106bp
			TUA Rev	GAGAGGGGTAAACAGTGAATC	

Table 3.2 Individual Relative Expression Values of TIR-NB-LRR type resistance gene CSA06758. Bolded data points were considered outliners in the data set and were removed prior to mean analysis. Each of the triplicate biological replicates had three individual technical replicates**.**

	Biological Replicate 1			**Biological Replicate 2**			**Biological Replicate 3**		
Cultivar	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep
Marketmore	7.835	4.438	**11.959**	1.050	1.110	1.231	1.064	1.292	0.946
Olympian	1.125	0.470	0.993	0.835	0.973	0.742	0.889	0.702	0.732
Lider	0.131	1.717	1.064	0.758	0.807	0.801	1.087	0.908	1.057
Supremo	0.624	**11.158**	1.717	1.206	1.189	1.173	0.674	0.559	0.732
Cobra	0.993	2.000	4.228	0.812	0.933	0.927	0.986	0.901	0.281
Sassy	1.376	3.387	4.724	1.206	1.329	1.223	2.694	2.868	3.249
Stonewall	0.035	0.075	0.240	0.901	0.973	1.157	0.717	0.933	0.758
DasherII/Poinsett	0.507	0.812	1.141	0.940	1.173	1.102	0.590	0.651	0.486
General Lee	0.460	0.117	0.901	1.079	1.214	0.979	0.078	0.045	0.066
Tasty Green	9.383	5.134	4.959	1.117	1.173	1.007	0.655	0.595	0.633
Dasher II	4.028	1.591	1.495	1.117	1.094	1.157	0.779	0.642	0.551
Cross County	4.857	3.655	2.395	0.859	0.927	1.007	0.486	0.426	0.664
Carolina	1.376	1.778	0.865	1.558	1.526	1.454	0.454	0.620	0.642
Diva	**18.507**	1.257	0.376	1.110	1.474	1.395	0.432	0.473	0.620
Straight Eight Elite	0.358	0.043	0.435	0.678	0.758	0.702	1.000	0.953	1.064
Munchmore	0.167	0.107	3.706	0.660	0.525	0.590	0.914	0.629	0.835
Poinsett 76	0.126	0.111	0.078	1.110	1.117	1.079	0.914	0.732	0.895
Slicemaster Select	2.621	2.532	4.141	1.357	1.444	1.505	0.796	0.807	0.877
SMR58	0.020	0.041	0.047	0.186	0.143	0.151	1.275	1.094	1.275

Table 3.3 Individual Relative Expression Values of TIR-NB-LRR type resistance gene CSA06757. Bolded data points were considered outliners in the data set and were removed prior to mean analysis. Each of the triplicate biological replicates had three individual technical replicates.

	Biological Replicate 1			**Biological Replicate 2**			**Biological Replicate 3**		
Cultivar	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep
Marketmore	9.781	7.062	5.134	1.079	0.841	0.841	0.940	0.927	1.133
Olympian	1.464	0.993	0.503	0.270	1.231	0.085	0.216	0.301	0.257
Lider	0.172	0.138	0.136	9.918	**94.353**	18.636	1.028	0.927	0.901
Supremo	0.532	0.308	0.204	1.223	1.064	1.141	0.574	0.603	0.763
Cobra	0.293	0.237	0.180	3.531	5.242	4.563	0.986	0.853	1.094
Sassy	1.110	0.920	0.712	3.095	3.340	3.074	3.031	3.117	3.204
Stonewall	2.129	3.458	1.613	2.848	2.751	2.329	0.712	0.727	0.801
DasherII/Poinsett	0.920	0.877	0.853	0.551	0.637	0.620	0.432	0.448	0.432
General Lee	0.742	0.503	0.209	0.933	0.737	0.901	0.304	0.356	0.330
Tasty Green	0.363	0.219	0.210	7.464	7.516	6.498	1.197	1.028	1.110
Dasher II	2.514	4.141	10.411	0.013	0.008	0.570	0.570	0.566	0.518
Cross County	0.590	4.438	1.042	0.457	0.371	0.392	0.480	0.624	0.807
Carolina	0.015	0.019	0.005	9.918	12.996	12.042	0.202	0.158	0.167
Diva	1.892	1.149	1.444	9.580	8.634	10.556	2.266	1.602	1.828
Straight Eight Elite	2.085	3.095	1.197	0.056	0.037	0.064	0.807	0.727	0.633
Munchmore	0.224	0.824	0.451	2.713	1.376	1.892	0.507	0.497	0.470
Poinsett 76	0.316	0.457	0.637	0.420	0.979	0.986	0.933	1.125	1.050
Slicemaster Select	7.621	18.252	4.790	0.629	0.467	0.566	0.717	0.624	0.693
SMR58	0.001	0.006	0.003	0.166	0.122	0.092	1.189	0.742	1.021

Table 3.4 Individual Relative Expression Values of TIR-NB-LRR type resistance gene CSA06724. Bolded data points were considered outliners in the data set and were removed prior to mean analysis. Each of the triplicate biological replicates had three individual technical replicates.

	Biological Replicate 1			**Biological Replicate 2**			**Biological Replicate 3**		
Cultivar	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep
Marketmore	1.729	3.837	2.713	0.599	0.590	0.693	1.165	0.993	1.021
Olympian	2.657	1.320	0.582	4.228	1.376	2.676	0.678	0.586	0.616
Lider	1.133	1.376	1.165	1.257	2.567	0.206	1.050	0.877	0.824
Supremo	0.824	0.507	0.774	1.079	1.014	1.102	0.883	0.986	0.933
Cobra	3.630	2.585	1.079	0.859	0.812	1.338	0.790	0.895	0.829
Sassy	2.099	2.014	0.096	1.110	1.223	1.231	3.706	4.028	3.531
Stonewall	0.233	0.233	0.233	1.338	1.257	1.338	0.966	0.883	1.057
DasherII/Poinsett	0.518	0.518	0.518	1.057	0.973	1.117	0.707	0.727	0.371
General Lee	0.262	0.356	0.106	0.768	0.829	0.908	0.109	0.125	0.111
Tasty Green	0.470	0.480	0.179	1.765	1.815	2.071	1.444	1.548	1.366
Dasher II	1.266	1.945	0.196	0.914	1.223	1.028	0.702	0.678	0.865
Cross County	5.979	2.144	7.160	0.801	0.841	0.824	0.390	0.532	0.457
Carolina	0.035	0.497	0.451	1.404	1.986	1.454	0.253	0.212	0.232
Diva	0.702	0.611	0.490	1.636	1.357	1.357	1.647	0.257	3.605
Straight Eight	0.044	0.061	0.051	0.669	0.727	0.646	0.841	0.865	1.173
Elite Munchmore	0.004	0.004	0.004	1.516	1.110	1.414	1.157	1.072	1.050
Poinsett 76	0.883	0.444	0.578	1.102	1.125	1.266	1.338	1.157	1.102
Slicemaster	**92.411**	29.041	**51.984**	0.493	0.483	0.438	0.818	0.871	1.035
Select SMR58	0.012	0.017	0.016	0.096	0.104	0.098	1.320	1.110	1.223

Table 3.5 Individual Relative Expression Values of the gene encoding for a basic PR-1 protein Bolded data points were considered outliners in the data set and were removed prior to mean analysis. Each of the triplicate biological replicates had three individual technical replicates.

	Biological Replicate 1			**Biological Replicate 2**			**Biological Replicate 3**		
Cultivar	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep
Marketmore	0.266	0.023	0.025	0.500	0.532	0.493	0.574	0.620	0.732
Olympian	9.580	14.621	12.042	0.732	0.707	0.702	0.140	0.129	0.140
Lider	0.073	2.042	2.313	0.669	0.812	0.651	0.432	0.363	0.371
Supremo	0.406	0.908	4.257	1.240	1.000	1.057	0.532	0.511	0.543
Cobra	13.361	**18.896**	5.938	0.908	0.763	1.021	0.940	0.966	0.883
Sassy	1.050	0.141	0.035	0.062	0.054	0.062	0.120	0.110	0.173
Stonewall	1.223	0.507	0.297	0.200	0.182	0.215	0.035	0.025	0.033
DasherII/Poinsett	0.224	0.376	0.232	1.659	1.625	2.042	0.438	0.376	0.356
General Lee	0.551	1.117	0.031	1.505	1.495	1.495	0.003	0.002	0.002
Tasty Green	0.342	0.444	0.835	0.304	0.261	0.253	0.247	0.215	0.279
Dasher II	0.027	0.019	0.025	1.197	1.320	1.248	0.017	0.014	0.014
Cross County	1.079	0.753	0.669	1.110	1.094	0.946	0.222	0.117	0.152
Carolina	4.287	3.010	4.317	0.105	0.090	0.088	0.018	0.017	0.017
Diva	4.287	4.595	6.190	0.310	0.328	0.390	0.063	0.076	0.051
Straight Eight	0.118	0.295	0.243	0.847	0.732	0.889	0.036	0.060	0.058
Elite Munchmore	0.245	4.532	1.057	1.206	0.966	0.973	0.529	0.480	0.454
Poinsett 76	15.780	11.392	5.098	1.102	1.042	1.094	0.202	0.247	0.240
Slicemaster	2.990	1.214	0.927	1.404	1.591	1.485	0.046	0.043	0.040
Select SMR58	0.012	0.017	0.016	0.096	0.104	0.098	1.320	1.110	1.223

Table 3.6 Individual Relative Expression Values of the gene encoding for cytosolic ascorbate peroxidase. Bolded data points were considered outliners in the data set and were removed prior to mean analysis. Each of the triplicate biological replicates had three individual technical replicates.

	Biological Replicate 1			**Biological Replicate 2**			**Biological Replicate 3**		
Cultivar	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep	Tech Rep
Marketmore	0.104	0.100	0.176	0.293	0.463	0.435	1.181	1.064	1.014
Olympian	0.790	0.966	0.953	0.170	0.330	0.470	0.005	0.007	0.004
Lider	4.377	2.969	3.272	0.009	0.017	0.022	0.015	0.011	0.005
Supremo	0.063	0.063	0.063	6.681	7.160	5.098	0.035	0.042	0.037
Cobra	3.434	0.467	1.087	0.908	0.853	0.993	0.266	0.001	0.081
Sassy	1.028	0.590	0.518	0.004	0.005	0.006	0.002	0.002	0.002
Stonewall	9.126	9.126	9.126	0.008	0.014	0.013	0.003	0.005	0.002
DasherII/Poinsett	0.027	0.027	0.027	12.817	14.221	12.381	0.467	0.493	0.390
General Lee	14.420	51.625	69.551	2.928	3.434	2.809	0.003	0.003	0.002
Tasty Green	0.529	0.717	0.829	0.005	0.005	0.005	0.016	0.011	0.005
Dasher II	0.075	0.384	0.064	0.129	0.346	0.426	0.006	0.005	0.005
Cross County	0.268	0.232	0.310	0.697	0.642	0.415	0.518	0.532	0.732
Carolina	12.042	2.056	4.469	0.006	0.005	0.005	0.002	0.002	0.001
Diva	6.681	6.063	8.056	0.012	0.018	0.005	0.323	0.029	0.036
Straight Eight	0.117	0.193	0.151	2.809	2.657	2.694	0.000	0.000	0.000
Elite Munchmore	0.001	0.001	0.002	2.868	2.445	2.129	6.453	0.779	0.540
Poinsett 76	12.467	9.190	7.311	1.454	2.395	1.414	0.000	0.000	0.000
Slicemaster	2.789	1.803	6.635	11.632	7.013	7.781	0.026	0.015	0.022
Select SMR58	0.871	0.742	0.697	**190.019**	**170.0718**	**298.1718**	2.189	2.189	2.189

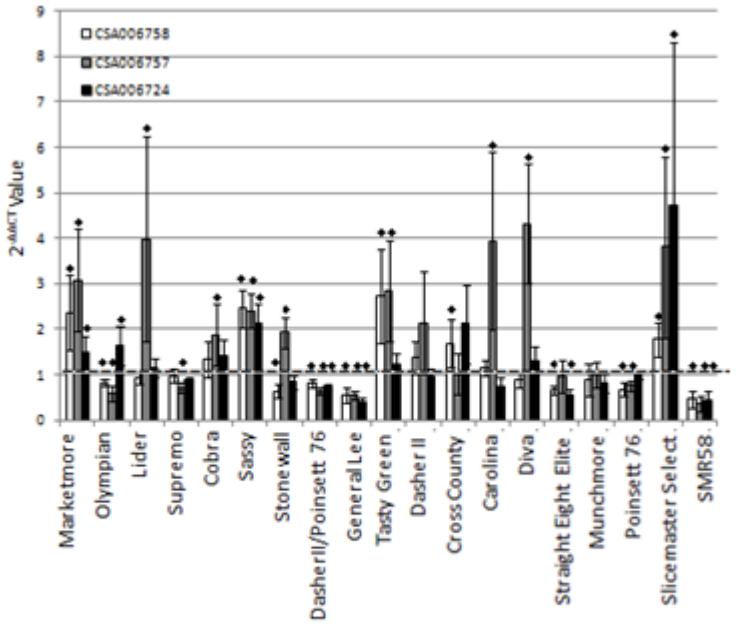

Figure 3.1 Relative expression values for the putative r-genes in cucumber (CSA006758, CSA06757, CSA006724). Values were calculated using the $2^{-\Delta\Delta CT}$ equation. Significant is relative to the dotted line and is indicated with a *.

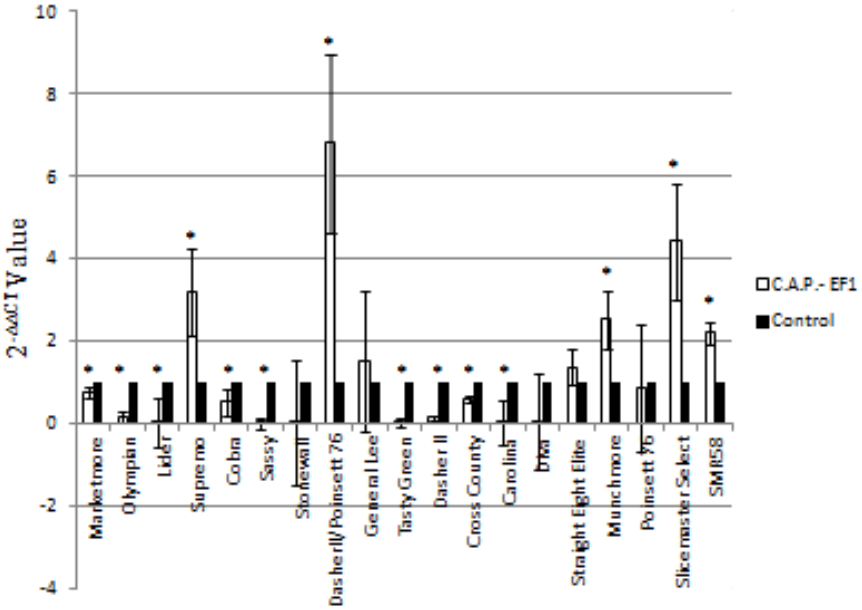

Figure 3.2 Relative expression values for the gene encoding for cytosolic ascorbate peroxidase (C.A.P.). Values were calculated using the $2^{-\Delta\Delta CT}$ equation. Significant is relative to the control bar and is indicated with a *.

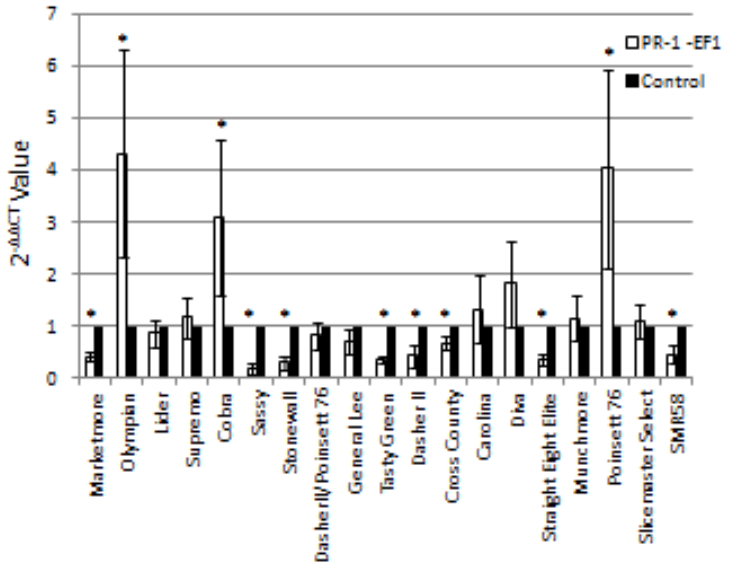

Figure 3.3 Relative expression values for the gene encoding for a basic PR-1 protein. Values were calculated using the $2^{-\Delta\Delta CT}$ equation. Significant is relative to the control bar and is indicated with a *.

APPENDIX A

FastRNA® Pro Green Kit

1. For each 100-300mg sample to be processed, add 1 ml RNApro® Solution to a green-cap containing Lysing Matrix D.
2. Add 100-300 mg plant tissue sample to the tube containing RNApro® Solution and Lysing Matrix D
3. Securely close the cap to prevent leakage in the next step. (Do not overfill the tube)
4. Process the sample in the tube in the FastPrep or FastPrep 24 instrument for 40 seconds at a setting of 6.0. (keep samples on ice to prevent sample degradation)
5. Remove the sample tube and centrifuge at minimum of 12,000 x g for 5 minutes at 4ºC or room temperature.
6. Transfer the upper phase to a new microcentrifuge tube. Avoid transferring the debris pellet and lysing matrix.
7. Incubate the transferred sample 5 minutes at room temperature to increase RNA yield.
8. Add 300ul of chloroform. Vortex for 10 seconds
9. Incubate 5 minutes at room temperature to permit nucleoprotein dissociation and increase RNA purity.
10. Centrifuge the tubes at a minimum of 12,000 x g for 5 minutes at 4ºC.
11. Transfer the upper phase to a new microcentrifuge tube without disturbing the interphase.
12. Add 500ul of cold absolute ethanol to the sample; invert 5x to mix and store at -20 ºC for 30 minutes
13. Centrifuge at a minimum of 12,000 x g for 15 minutes at 4ºC and remove the supernatant. The RNA will appear as a white pellet in the tube.
14. Wash the pellet with 500 ul of cold 75% ethanol.

15. Remove the ethanol, air dry 5 minutes at room temperature, and resuspend the RNA in 100 ul of DEPC-H_20 for short-term storage.
16. Incubate 5 minutes at room temperature to facilitate RNA resuspension
17. Nano-drop sample

Additional Purification Steps

18. Add 400ul of H_20 to RNA solution.
19. Add 300 ul of choloroform:isoamyl alcohol (12:1) Vortex for 10 seconds
20. Incubate 5 minutes at room temperature to permit nucleoprotein dissociation and increase RNA purity.
21. Centrifuge the tubes at a minimum of 12,000 x g for 5 minutes at 4ºC.
22. Transfer the upper phase to a new microcentrifuge tube without disturbing the interphase.
23. Add 500ul of cold absolute ethanol to the sample; invert 5x to mix and store at -20 ºC for 30 minutes
24. Centrifuge at a minimum of 12,000 x g for 15 minutes at 4ºC and remove the supernatant. The RNA will appear as a white pellet in the tube.
25. Wash the pellet with 500ul of cold 75% ethanol.
26. Remove the ethanol, air dry 5 minutes at room temperature, and resuspend the RNA in 100 ul of DEPC-H_20 for short-term storage.
27. Incubate 5 minutes at room temperature to facilitate RNA resuspension
28. Nano-drop sample

Trizol Reagent RNA extraction Protocol

1) Clean bench, pipettors, mortars and pestles with Rnase away + 70% ETOH
2) Freeze mortars and pestles in liquid nitrogen

3) Place a small tissue sample in mortar and pour on liquid nitrogen
4) Once the nitrogen evaporates, grind the tissue into a powder
5) Use a metal spatula (frozen) to remove the tissue and place in a micro centrifuge tube
6) Add 1ml of tri reagent to the tube
7) Invert the tube to separate proteins
8) Leave at room temperature for 10 minutes
9) Add 200 ul of chloroform and vortex
10) Centrifuge for 15 minutes at 4 C at 12,000 x g
11) Transfer supernatant add 500 ul of isopropanol
12) Centrifuge for 10 minutes at 4 C at 12,000 x g
13) Decant supernatant and wash pellet with 75% ETOH (vortex)
14) Centrifuge for 5 minutes at 4 C at 12,000 x g
15) Decant supernatant and air dry pellet
16) Add 35-50 ul of sterile water
17) Nano-drop sample