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ABSTRACT OF DISSERTATION

MERARI FELICIANO-RIVERA

THE GRADUATE SCHOOL

UNIVERSITY OF KENTUCKY

2011

EFFICACY OF ORGANICALLY CERTIFIABLE MATERIALS AND NATURAL COMPOUNDS AGAINST FOLIAR HEMIBIOTROPHIC AND NECROTROPHIC FUNGI IN CANTALOUPE AND TOMATO

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy
in the College of Agriculture
at the University of Kentucky

By

Merari Feliciano-Rivera

Lexington, Kentucky

2011

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ABSTRACT OF DISSERTATION

EFFICACY OF ORGANICALLY CERTIFIABLE MATERIALS AND NATURAL COMPOUNDS AGAINST FOLIAR HEMIBIOTROPHIC AND NECROTROPHIC FUNGI IN CANTALOUPE AND TOMATO

Kentucky reported a solid 13.1% growth in certified organic land from 1997 to 2002. The relative lack of research on disease management practices in Kentucky consistent with organic regulations is an issue that needs to be addressed to provide more reliable information to local farmers. Thus, the first objective of this research was to investigate the potential disease control obtained with natural, organically certifiable spray materials against *Colletotrichum orbiculare in vitro* and *in vivo*. The second objective was to test certifiable spray materials in combinations to identify synergistic interactions. The third objective was to evaluate Organic Material Review Institute (OMRI)-certified materials for managing Septoria leaf spot and early blight in tomato under field conditions. The fourth objective was to evaluate chitosan-based products against *C. orbiculare in vitro* and *in vivo*.

Essential oils, Trilogy®, and Actinovate®, failed to suppress *C. orbiculare in vitro* as well as cucurbit anthracnose. Bicarbonate salts, Regalia®SC, Sonata®, copper based-products, lime sulfur and water-soluble chitosan showed high antifungal activity *in vitro*. Bicarbonate salts, Sonata®, Serenade Max®, Soil Gard 12G®, copper based-products and lime sulfur reduced anthracnose disease severity *in vivo*.

In the s
ynergism experiments only a limited number of mixtures showed synergistic interactions, but even in those cases, the effect was not consistent between experiments. The main response obtained was antagonism.

In field experiments the most effective fungicides for managing Septoria leaf spot and early blight of tomato were copper-based fungicides. None of the biological-based products (Sonata® and Serenade Max®)), plant-based extracts (Trilogy® and Regalia® SC), chitosan, ammonium bicarbonate nor horticultural lime sulfur provided a significant reduction in disease severity.

For the fourth objective, water-soluble chitosan with a molecular weight between 3 to 10 kDa (80 and 85% deacetylated) showed the highest antifungal activity among all chitosan-based products evaluated *in vitro*. Also, combining the *in vitro* and *in vivo*

results suggest that the antifungal activity of chitosan-based products is molecular weight- and concentration-dependent. These results provide a significant advance in the evaluation of the efficacy of OMRI-certified materials and natural materials to help organic farmers in Kentucky and the USA to manage diseases.

KEYWORDS: *Alternaria solani*, *Septoria lycopersici*, OMRI-certified materials, chitosan, synergism

Merari Feliciano Rivera

Student Signature

5.31.2011

Date

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DISSERTATION

Merari Feliciano-Rivera

The Graduate School

University of Kentucky

2011

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DISSERTATION

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"A thankful heart is not only the greatest virtue, but the parent of all other virtues."
Cicero

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CHAPTER 1

LITERATURE REVIEW

Plant disease is an ongoing problem that leads to losses of yield and quality in crops worldwide. In a demanding market for high-quality vegetables, growers must manage and produce crops that meet the requirements of the market and which are profitable. The costs of meeting all of the market requirements in an organic production system in humid climates can be very expensive in the presence of severe disease epidemics, which may result in financial losses. Practices such as crop rotation, the use of resistant cultivars, pesticide applications, weed management, soil fertility management and sanitation are employed by farmers to manage and prevent economic losses by plant diseases. However, under high disease pressure these practices, used individually, can fail; thus, an integrated pest management plan is the best way to reduce disease severity and prevents yield losses. It remains an elusive challenge to find a single control measure which provides consistent and absolute control of pathogens and the diseases they cause.

One of the most extensively used disease-management practices in the United States and around the world is chemical control. In the USA, approximately 1,200 million pounds of pesticides are used annually and 6% of this is comprised of fungicides (EPA, 2002). Since the first fungicide (sulfur) was discovered 2,000 years ago, significant advances have been made developing new chemical fungicides with high antifungal activity and low application rates. With the widespread use of systemic fungicides since the 1970s, new fungal strains resistant to these fungicides have appeared (Agrios, 2005).

Therefore, the development of resistance to fungicides is a problem more and more frequently reported in highly chemical-dependant agricultural production systems.

Large-scale producers are often highly dependent on pesticides in order to prevent yield losses that reduce incomes. However, for many organic growers the highly intensive use of fungicides is not an attractive option. Many organic-certified pesticides approved by the Organic Material Review Institute (OMRI) are available for organic growers, but the lack of information in the efficacy of these pesticides is one of the main issues that must be addressed.

ORGANIC PRODUCTION SYSTEMS

Organic farming is the oldest form of agriculture on Earth. Current conventional production systems are highly dependent on the application of chemicals for disease control, whereas organic productions systems typically use them as a last alternative.

Organic agriculture is the production of food following regulations established in the Organic Foods Production Act (OFPA) of 1990 by the National Organic Program Board (USDA, 2011). The prohibition of most conventional pesticides, fertilizers made with synthetic ingredients or sewage sludge, and transgenic crops (=”genetically modified” crops or GMO’s) is included in those regulations. Also in the USA is defined by the production of food in an ecological management system that promotes biodiversity, biological cycles and soil biological activities. An organic production system promotes the increase and maintenance of soil fertility through practices such as crop rotation, composting, green manures and cover crops. The goal is to create a safe environment where the plant, microbial biodiversity and the community can coexist in a natural

environment where all the components in the system can have mutual benefits, without the use of synthetic materials or genetically modified plants (GMO's).

In recent years, there has been a notably increased interest in preserving the environment, as well as public concern about pesticide residues in food, all of which has spurred interest in organic products and organic production systems. Organic prices often range from 20 to 400 percent above conventional prices, depending on the season, country and the availability of the crop (Delate, 2003). However that hasn't limited the rapid and steady growth of the organic industry around the world, including in the U.S. Worldwide, the market for organic products is estimated to be $28 billion and the USA has the highest growth, where organic sales reached $12.2 billion in 2005 (Zehnder et al., 2007). Retail sales of organic foods increased from $3.6 billion in 1997 to $21.1 billion in 2008 (Dimitri and Oberholtzer, 2009). Organic vegetable production is one of the fastest growing organic agriculture sectors in USA. Certified organic land for vegetable production increased from 48,227 acres in 1997 to 98,525 in 2005 (Dimitri and Oberholtzer, 2009). In Kentucky a 13.1% increase in certified organic acreage during the period of 1997 to 2002 has been reported and in 2003 approximately 8,700 acres were certified as organic (Baird et al., 2005).

Disease control in organic crop production systems is one of the greatest challenges that farmers face, especially in humid climates. Regulations by the National Organic Program (NOP) established and regulated the inputs (including pesticides) used to manage diseases. Among the products allowed for use in an organic production system to manage disease are: essential oils, plant extracts, biological agents, soil extracts, bicarbonate salts, copper and sulfur. Organic farming systems employ practices like crop

rotation, sanitation, resistant varieties, reduced or no-tillage, organic amendments, biocontrol agents, use of cover crops, and proper soil fertility to manage diseases (Delate, 2003; van Bruggen, 1995). Sanitation is one of the most important practices employed in organic production systems to prevent diseases, because it helps to eradicate initial inoculum of many pathogens.

According to Delate (2003), the basis of insect, disease and nematode management in organic farming system is the reliance on the inherent equilibrium in nature by the use of natural enemies, natural products or commercial preparations, and cultural practices that do not affect natural enemies (Delate, 2003). However, under high disease pressure and favorable conditions for the development of a disease epidemic, the inherent equilibrium in nature is not enough and specific control measures, such as the use of certified organic products, is required to prevent significant yield losses. Thus, the Organic Materials Review Institute (OMRI) provides a complete list of materials allowed in organic production systems.

Materials allowed in organic production systems

OMRI-certified materials

OMRI, the national nonprofit organization that determines which input products are allowed for use in organic production systems, had approved over 2000 products by 2010, providing many product options for certified organic farmers (OMRI, 2010). OMRI-certified materials include pesticides, fertilizers, adjuvants and products for livestock production. Some of the most commonly certified materials used in the USA to manage diseases in organic agriculture include: botanical oils like sesame, rosemary and

tea tree; botanical extracts such giant knotweed and garlic; chemicals like hydrogen peroxide, potassium bicarbonate and mineral oil; biocontrol agents; and plant defense inducers (McGrath 2003; Wszelaki and Miller, 2005). Copper-based fungicides like Kocide®, Bordeaux mixture and Champion® WP are listed as synthetics and must be used following the restrictions established by the NOP. With the rapid growth of organic agriculture and the increased number of OMRI-certified products allowed for use, the need for more research to evaluate the efficacy of these products is pressing.

Essential oils

Essential oils are complex volatile compounds obtained from leaves, flowers, bark and roots (Delespaul et al., 2000; Herath and Abeywickrama, 2006). Essential oils have been used as natural controls against insects as well as plant pathogens (Herath and Abeywickrama, 2006; Soylu et al., 2006; Moretti et al., 2002). The possibility of using essential oils as fungicides is very attractive because volatile compounds can evaporate without leaving residues on crops, are thought to be safe to the environment and sensitive ecosystems, and are of extremely low toxicity to mammals (Yeop et al., 2005; Isman, 2000). They can affect insects and fungi by contact and as fumigants (Isman, 2000). Moreover, certain plant essential oils have a broad spectrum of control against insects, fungi, nematodes and bacteria (Isman, 2000).

A wide variety of terpene hydrocarbons and their oxygenated isoprenoid compounds are present in essential oils and have been shown to have antimicrobial activity against fungi and bacteria (Soylu et al., 2006; Müller-Riebau et al., 1995; Prabuseenivasan et al., 2006). Essential oils and their constituents have been effective against numerous plant pathogenic fungi, including *Alternaria alternata*, *Botrytis cinerea*, *Phytophthora*

infestans, Phytophthora capsici, Fusarium verticillioides, Rhizoctonia solani, and *Sclerotium sclerotiorum* (Fandohan et al., 2004; Feng and Zheng, 2005; Müller-Riebau et al., 1995; Soliman and Badeaa, 2002; Soylu et al., 2006; Wilson et al., 1997). Essential oils extracted from rosemary, thyme, oregano, lavender, sage, basil, cinnamon and marigold have showed antifungal activity against foliar and postharvest pathogens (Fandohan et al., 2004; Soliman and Badeaa, 2002; Daferera et al., 2000).

Essential oils exhibit different levels of antifungal activity depending on the type of oil, its concentration, and the species of phytopathogenic fungus evaluated (Wilson et al., 1997; Soliman and Badeaa, 2002). Also, essential oils can differentially affect different stages of the fungus, mycelial growth and spore germination (Fandohan et al., 2004; Feng and Zheng, 2005). The antifungal effects provided by essentials oils can vary by the oil concentration and the sensitivity of the pathogen to the antifungal chemical compounds present in the essential oils. As an example of differential sensitivity of pathogens, *in vitro* experiments using basil oil at 500 µg/ml provided 30% inhibition of mycelial growth of *Aspergillus flavus*, 71% of *Aspergillus parasiticus* and 58% of *Fusarium moniliforme* (Soliman and Badeaa, 2002). I*n vitro* studies using the same essential oil at 250 µg/ml showed no inhibition of mycelial growth of *Botrytis cinerea*, *Penicillium digitatum* and, *Geotrichum citri-aurantii* (Bouchra et al., 2003). Concentration also plays an important role in the antifungal activity of essential oils. Evaluation of essential oils against *P. infestans* showed that fennel oil was toxic at a low concentration (6.4 µg/ml); however, rosemary oil inhibited mycelial growth only at a higher concentration (51.2 µg/ml) (Soylu et al., 2006). Thus, the antifungal activity of the essential oils depends on the pathosystem under investigation, the chemical composition of the essential oils and

the concentration tested. Furthermore, the growing conditions, the season in which the aromatic plants were collected, the dehydration procedure, storage conditions and, the method used to extract the oil all can influence the chemical composition of the essential oil, and therefore, its antimicrobial activity (Hawthorne et al., 1993; Kokkini et al., 1997; Tarantilis and Polissiou, 1997; Russo et al., 1998).

At present, essential oil-based pesticides like Cinnamite™ (aphidicide/miticide/fungicide) and Valero™ (miticide/fungicide) with cinnamon oil as the active ingredient have been registered for use in grapes, berry crops, citrus and nuts (Isman, 2000). Other OMRI-certified products with essential oils as active ingredients include AZA-Direct® (azadirachtin), Neem Oil RTU (neem oil), NeemGard® (neem oil), Neemix® 4.5 (azadirachtin), SPORAN® EC (rosemary, clove and thyme oils), SPORATEC® (rosemary, clove and thyme oils), Triact® 70 EC (clarified hydrophobic neem oil) and Trilogy® (clarified hydrophobic neem oil) (Zitter, 2010). Among these, Trilogy® is the most commonly used in organic production systems (Wszelaki and Miller, 2005).

Bicarbonate salts

The US Environmental Protection Agency stated that bicarbonates were exempt from residue tolerances on all agricultural commodities, and the USDA classified various carbonates and bicarbonates as approved ingredients on organic labeled products (Smilanick et al., 1999). Bicarbonates have been tested against fungal plant pathogens and have shown inhibitory effects *in vitro* and *in vivo* (Palmer et al., 1997; Homa et al., 1981; Horst et al., 1992; Ziv and Zitter, 1992). Sodium bicarbonate, potassium bicarbonate and ammonium bicarbonate are among of the most common bicarbonate salts

tested *in vitro* and *in vivo* against plants pathogens (Palmer et al., 1997; Homa et al., 1981; Horst et al., 1992; Ziv and Zitter, 1992). Fungal foliar pathogens of cucurbits (*Alternaria cucumerina, Colletotrichum orbiculare, Didymella bryoniae,* and *Ulocladium cucurbitae*) were inhibited *in vitro* and caused less disease on greenhouse-grown plants treated with sodium bicarbonate, potassium bicarbonate and ammonium bicarbonate salts (Ziv and Zitter, 1992). Fungal pathogens differ in their sensitivity to bicarbonates: a significant reduction in colony growth of *Botrytis cinerea* was obtained on media amended with sodium bicarbonate, potassium bicarbonate and ammonium bicarbonate salts (Palmer et al., 1997), whereas no inhibition of spore germination and mycelial growth of *Helminthosporium solani* was observed using sodium bicarbonate (Hervieux et al., 2002). As with the essential oils, the antifungal activity of bicarbonate salts depends on several factors such as the kind of salt tested, its concentration and the sensitivity of the target organism.

OMRI-certified products containing potassium bicarbonate and labeled for crop pest, weed, and disease control include: Bi-Carb Old Fashioned Fungicide, Kaligreen® Potassium Bicarbonate Soluble Powder, MilStop® Broad Spectrum Foliar Fungicide and PHC® MilStop Plus® (OMRI 2011). These products had showed to be effective against biotrophic pathogen like powdery mildews which obtain nutrients only from living cells. Significant reductions in powdery mildew severity have been obtained with MilStop® fungicide (Moyer and Peres, 2008). However, tomato plants treated with Kaligreen® showed no reduction in disease severity caused by the necrotrophic pathogens (which obtain nutrients from dead organic matter) *Septoria lycopersici* and

Alternaria solani compared with the water-treated control plants (Wszelaki and Miller, 2005).

CUCURBIT ANTHRACNOSE AND MANAGEMENT IN ORGANIC PRODUCTION SYSTEMS

Cucurbit anthracnose is an economically important disease that affects watermelon, melon, cucumber and other members of the genera *Cucumis*, *Citrullus*, *Cucurbita*, *Lagenaria*, and *Luffa* (Zitter et al., 1996; Prusky et al., 2000). Losses of 30% or more can occur in susceptible crops where control practices are not followed (Yeop et al., 2005). In the U.S., the disease is most severe in states in the midwestern and eastern regions, causing yield losses of up to 60% (Monroe et al., 1997; Thompson and Jenkins, 1985).

Colletotrichum orbiculare (Berk. & Mont.) Arx (syn. *C. lagenarium* (Pass) Ellis & Halst.) is the causal agent of cucurbit anthracnose (Figure 1.1) (Zitter et al., 1996). Given that this pathogen can affect all above-ground parts (leaves, stems and fruits) (Monroe et al., 1997; Anonymous, 1996), effective control measures should be employed; otherwise this disease can be a serious threat for organic growers as well as conventional growers.

C. orbiculare is a filamentous fungus characterized by having two growth stages in the host: a brief biotrophic phase and a destructive necrotrophic phase (Prusky et al., 2000). For the pathogen to be able to infect, a series of morphological changes must take place. Once the spore lands on the leaf surface via water splash, a period of leaf wetness is necessary for spore germination and successful infection (Leben and Daft, 1968; Zitter et al., 1996). After the spore germinates, a melanized appressorium is formed which is essential for penetration of the epidermal cells by the penetration peg. Following initial

penetration a spherical infection vesicle is formed and one or more primary hyphae grow out from the vesicle to colonize the host cell intercellularly (Prusky et al., 2000; Baley et al., 1996). Noticeable symptoms appear 96 h after the initial infection and are characterized by circular light brown or reddish lesions where the spores are produced (Zitter et al., 1996; Kwack et al., 2005).

The appressorium has been the focus of several research efforts because of its importance in the infection process (Sakaguchi et al., 2010; Kimura et al., 2001; Tsuji et al., 2003) and the possibility of using this essential event in the infection process as a target to develop control measures. The *CMK1* MAPK gene has been shown to be required for conidia germination, appressorium formation, and invasive growth (Takano et al., 2000). Conventional fungicides in the groups MBI-R (Melanin Biosynthesis Inhibitor-Reductase) and MBI-D (Melanin Biosynthesis Inhibitor- Dehydratase) prevent melanin biosynthesis by affecting reductase and dehydratase in the melanin biosynthesis pathway (FRAC, 2009). Experiments with melanin biosynthesis inhibitors and melanin-deficient mutants of *C. orbiculare* have shown that melanin is essential for successful penetration into the host (Kubo et al., 1985). However, fungicides under these two groups are not allowed for use in an organic production system as well as conventional production systems.

Resistant cultivars, fungicide applications, certified pathogen-free seed, crop rotation for at least one year and sanitation are the control practices recommended to manage anthracnose disease in cucurbit (Zitter et al., 1996; Thompson and Jenkins, 1985). Use of protectant and eradicant fungicides is the most intensive management approach for this disease (Pernezny et al., 2006). Fungicide programs for managing

fungal foliar diseases in muskmelons and melon in Kentucky have recommended the used of chlorothalonil, mancozeb, copper and azoxystrobin beginning one week after transplant (Rowell et al., 2010-2011). Nevertheless, organic growers cannot apply these materials to their crops except for the copper-based products, which must be applied following the restrictions established by the OMRI. Several OMRI-certified products are labeled for anthracnose. However, reliable data on the efficacy of these products in field trials are limited, which complicates the decisions that organic growers have to make in order to integrate them as part of the plant disease management plan.

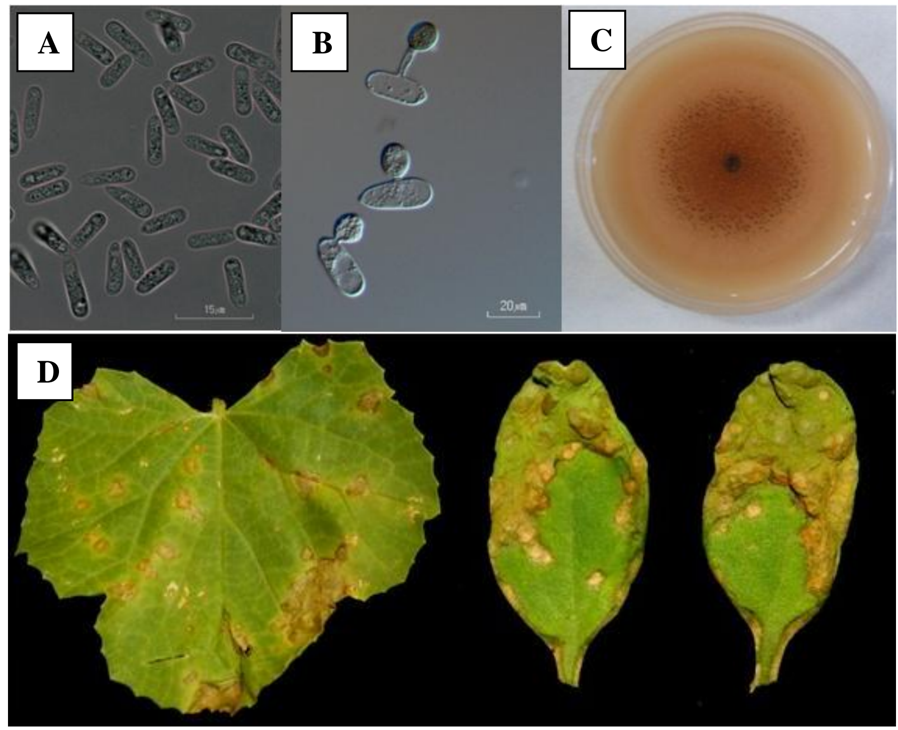

Figure 1.1. ***Colletotrichum orbiculare.*** A) Conidia, B) appressorium, C) *C. orbiculare* in green bean agar media and D) symptoms caused by the fungus in cantaloupe leaves and cotyledons five days post-inoculation.

EARLY BLIGHT AND SEPTORIA LEAF SPOT IN TOMATO

Alternaria solani

Diseases caused by members of the genus *Alternaria* are among the most common diseases affecting a wide variety of plants including annual plants, vegetables, ornamentals and trees (Agrios, 2005). Among fungal genera, *Alternaria* ranks 10th among fungal genera in terms of total number of plant hosts (Farr et. al., 1989). Due to the number of plants affected by the species in this genus, it is considered one of the most economically important in term of losses (Agrios, 2005).

Alternaria solani (Ell. & Mart.) L. R. Jones & Grout, is a necrotrophic fungus responsible for causing early blight in tomato (*Lycopersicon esculentum* Mill) and potato (*Solanum tuberosum* L.). Early blight is the most common fungal disease of tomato in Kentucky (Coolong et al., 2009). This ascomycete is one of the most important and extensively studied members of the genus *Alternaria* worldwide. The pathogen can affect all aerial parts of the plant at all stages of development; however it is frequently first observed affecting older or senescing tissue (Jones, et al., 1991). The disease is favored by mild temperatures (24-29˚C), high relative humidity and rainy weather (Chaerani and Voorrips, 2006; Jones, et al., 1991). Under high disease pressure in the absence of management, the disease induces early defoliation of tomato plants which may trigger suppression of yields and reduction in the number and size of fruits (Jones, et al., 1991). Lack of fruit set has been correlated with high disease severity caused by *A. solani* (Horsfall and Heuberger, 1942). Control measures include regular application of

fungicides, use of resistant or tolerant cultivars, crop rotations of 3-5 years, sanitation, pathogen-free seed and, weed management (Jones, et al., 1991; Madden et al., 1978).

Septoria lycopersici

Septoria leaf spot, caused by *Septoria lycopersici* Speg., is an important foliar disease of tomato in the U.S. and worldwide (Jones et al., 1991). Disease development is favored by temperatures between 20-25˚C, high relative humidity and rain events (Jones et al., 1991; Parker et al., 1995). The disease affects leaves, stems, petioles and calices. Despite the fact that the pathogen does not attack the fruits, in seasons with high disease pressure and favorable environmental conditions, the disease can destroy the foliage resulting in yield loss, furit sunscald, and/or improper maturation of fruits (Jones et al., 1991). Septoria leaf spot usually is first observed in the lower canopy and eventually spreads to the upper canopy after the first fruit sets (Coolong et al., 2009; Jones et al., 1991). Circular lesions with dark borders and tan-brown centers are typical of this disease (Coolong et al., 2009). In a conventional production system, the disease is mainly controlled with fungicides applied on a regular basis (Parker et al., 1997). Use of pathogen-free seed, resistant cultivars, sanitation, and crop rotation are practices recommended to manage Septoria leaf spot (Coolong et al., 2009; Jones et al., 1991).

Management of early blight and septoria leaf spot in organically produced tomato

Tomato (*Lycopersicon esculentum* Mill.) is one of the most important vegetables cultivated worldwide. China and U.S. are the highest tomato producing countries in the world (FAOSTAT, 2009). In 2009, 14141850 tons of tomatos were produced in USA

(FAOSTAT, 2009). Worldwide, pests and diseases are the primary constraints for conventional as well as organic farmers.

Conventional and organic farming differ mainly in tillage methods, crop rotations, fertilizer applications, and pest control methods (Reganold et al., 1987). The production of tomatoes is a very labor-intensive process, with each staked acre of tomatoes requiring approximately 350 hours of work (Konsler and Shoemaker, 1980). Despite the intensive work and good management practices, one of the greatest challenges to organic tomato growers in humid climates is disease management. Diseases of organically grown tomatoes in humid regions are called the "Achilles heel" (Diver et al., 1999). The management of necrotrophic fungi in conventionally grown tomatoes is by the use of protectant fungicides like mancozeb and chlorothalonil or systemic fungicides in the strobilurin class (Rowell at al., 2010-2011; Zitter et al., 2005). In contrast, management of diseases in organically grown tomatoes is done by a combination of organic soil management practices, integrated pest management practices, natural remedies, and limited fungicide use (Diver et al., 1999). The use of tolerant or resistant cultivars is a management practice recommended to conventional farmers as well as organic farmers, but high levels of resistance to early blight in cultivated tomatoes is rare (Chaerani and Voorrips, 2006). Tomato varieties in the Mountain series which are tolerant to early blight but not to septoria leaf spot, are recommended to organic growers to manage the former (Diver et al., 1999).

Several products have been registered and OMRI-approved for use on organically grown tomatoes in U.S. However, data on efficacy of these products are limited and on occasion inconsistent. In the majority of trials testing the efficacy of organic certified

materials, copper-based products like Kocide® 2000, Kocide® 3000 and Champion WP have provided the best results in terms of disease control but not in terms of yield. For example, in an evaluation of OMRI-certified materials such as Plantshield, Mycostop, Trilogy®, CaCO3, SW-3 Seaweed, Humega and Champion WP (active ingredient (a.i.) copper hydroxide) to manage foliar disease in tomatoes in New York, Champion WP was the only product that significantly reduced disease severity (Seaman et al., 2004), although in that trial disease control did not result in yield improvement. Similarly, in Ohio a significant reduction in disease severity was obtained with Champion WP but no improvement in yield was observed (Wszelaki et al., 2003). In contrast, in a study in Iowa, copper fungicide not only controlled disease but also provided for yields 60% more than the other treatments (Joslin and Taber, 2003). Despite the fact that copper-based products have shown themselves to be very effective for controlling foliar disease in numerous trials, they also have failed in others trials. As an example, in field trials, plants treated with Champion WP showed no differences in disease control compared with control plants (McGrath, 2007). Copper-based fungicides are routinely used for disease control in organic tomato production in the Eastern of United States; however, it can be toxic to earthworms and nitrogen-fixing soil microbes (blue-green algae) and can build up in the soil to phytotoxic levels to the plants (Diver et al., 1999). Thus, interest in the use of biological-based products for disease control in organic production has increased in recent years. Products containing *Bacillus* spp. *Streptomyces lydicus*, *Coniothyrium* and *Trichoderma harzianum* as active ingredients have been registered and approved to manage disease in organically-grown tomatoes. However, the limited amount of data on

efficacy of these products makes the selection of these materials to be use by organic farmers difficult.

Products like Sonata® (ai. *Bacillus pumilis*) and Serenade Max® (ai. *Bacillus subtilis*) have been evaluated alone and in combination with other OMRI-certified materials, commonly resulting in no disease control and/or inconsistent results. Also, improvement of yields by these biological-based products has been very inconsistent. For example, plants treated with combinations of Sonata® with Kocide® 2000 showed more damage from early blight than the water control or either fungicide used alone (Wszelaki and Miller, 2005). Curiously, plants treated with the Sonata/Kocide combination yielded the most. These results illustrate that there is not necessarily a correlation between disease control and yield improvement. In another trial, no effect on yields and Septoria leaf spot severity was obtained by the application of compost tea and Sonata® compared with the untreated control (McGrath and Moyer, 2003), illustrating that yield improvements obtained with OMRI-approved products may be inconsistent.

Combinations of Serenade Max® with Champion WP provided a reduction in disease severity caused by *A. solani* and *S. lycopersici*, compared with the water control (Wszelaki and Miller, 2005). In contrast to this result, a combination of Serenade Max® with Champion WP and Biotune showed no effect on severity of early blight (Zitter and Drennan, 2005). No improvement in yield was observed in these two trials by combinations of Serenade Max® and Champion WP. These showed inconsistent results in terms of disease control and also no correlation between disease control and yield improvement.

All of these results demonstrate that the efficacy of OMRI approved materials against foliar pathogens has been inconsistent in published work. With the rapid growth of the U.S. organic industry more data in the efficacy of these OMRI-certified materials is required to provide more tools and more options for organic growers.

Another material that has gained interest among organic farmers for foliar disease control in vegetables like tomatoes is compost tea. Compost tea is an unheated, on-farm infusion made from compost and used to promote plant growth as well as to control foliar and root diseases (Ingram and Millner, 2007). Reduction in Septoria leaf spot but not early blight has been observed in treatments using compost as a soil amendment (Baysal et al., 2009). However, foliar application of compost tea did not reduce severity of Septoria leaf spot as well as copper fungicide (Joslin and Taber, 2003). These results suggest that the efficacy of compost tea preparations may depend on the mode of application and other factors such as base materials used for the compost, age of compost and additives included. More research is needed to fully understand the effect of these compost tea preparations on disease control under field conditions. Despite the increasing interest in the use of compost tea to manage disease in organic production systems, data on the efficacy of the compost tea are limited. Moreover, new concern about the levels of human pathogens likes *Escherichia coli* and *Salmonella* in compost teas preparations led to the formation of a national Task Force called the NOSB (National Organic Standard Board) Task Force on Compost Teas, with the responsibility to make recommendations on concerns over these and other human pathogens in compost tea preparations (USDA-GOV, 2004).

CHITOSAN

Chitosan is a polysaccharide obtained mainly from the exoskeleton of crustaceans and derived from a low-acetyl form of chitin, composed mostly of glucosamine and *N*-acetylglucosamine (Uthairatanakij et al., 2007). It is also found in the cell walls of Zygomycetes, green algae, yeast and insects (Stössel and Leuba, 1984; Fang et al., 2008). The production of chitosan from chitin takes place by boiling chitin in a concentrated alkali (40-45% sodium hydroxide) for 1-3 hours at 120˚C (Raafat and Sahl, 2009). During this process the *N*-deacetylation of chitin is not complete, thus chitosan is consider a partially deacetylated form of chitin (Figure 1.2) (Raafat and Sahl, 2009).

Chitosan is a weak base and is insoluble in water and organic solvents (Qin et al., 2006). Most commercially available chitosans are dissolved in acetic- or lactic- acids. The solubility of chitosan decreases as the pH rises above 6.0-6.5 (Varum et al., 1994). Recently, water-soluble chitosan has become more available worldwide but the amount of research using this chitosan against fungi is limited. Against bacteria, it has been established that acid-soluble chitosan is more effective than water-soluble chitosan (Qin et al., 2006).

The antimicrobial activity of acid-soluble chitosan has been demonstrated against bacteria, fungi, viruses and insects (Muñoz et al., 2009; Stössel and Leuba, 1984; El Ghaouth et al., 1992; El Ghaouth et al., 1994; Iriti and Faoro, 2008). In addition, improvement in plants growth and development has been observed in plant treated with chitosan (Jayaraj et al., 2009), although field evaluations have been limited. In addition to its reported antimicrobial activity and improvement of plant growth, induction of resistance to certain pathogens has been observed in plants treated with chitosan (Jayaraj

et al., 2009; Lizama-Uc et al., 2007). These studies indicate that chitosan can act in multiple ways against plant pathogens.

Factors such as molecular weight, degree of polymerization, degree of deacetylation, pH and temperatures can affect the antimicrobial activity of chitosan (Rhoades and Rastall, 2003). In general, the antimicrobial activity of chitosan can vary with the type of chitosan (e.g. water soluble vs. acid soluble), the target organisms and the environment and conditions under which it is tested.

Antifungal activity of chitosan

Chitosan can affect spore germination and mycelial growth; in addition, it can induce morphological and structural changes in cells of several fungi (El-Ghaouth, 2000; Bell et al., 1998; Vesentini et al., 2007). Antifungal activity of chitosan has been demonstrated against powdery mildews, soilborne fungi, foliar pathogens, and postharvest fungi (Muñoz et al., 2009; Stössel and Leuba, 1984; El Ghaouth et al., 1992; El Ghaouth et al., 1994). The specific mode of action of chitosan against fungi is not well-understood. However, it has been suggested that its polycationic properties as well as the length of the polymer are involved in the antifungal activity (Hirano and Nagao, 1989). Experiments performed using chitosan against oomycetes and deuteromycetes showed that the antifungal activity of chitosan depended on the degree of deacetylation, the particle diameter of the polymer and the pH (Stössel and Leuba, 1984). A 32% reduction in the radial growth of *Colletotrichum lindemuthianun* was obtained with 91%-deacetylated chitosan while a 20% reduction was obtained with 66%-deacetylated form (Stössel and Leuba, 1984). Due to the process (described above) by which chitosan is produced, the degree of deacetylation is very variable in commercially available

chitosans. Thus, experimental results with chitosan may be inconsistent even using the same pathosystem.

Three hypotheses have been proposed regarding the mechanism by which chitosan inhibits the growth and affects the development of fungi:

1) Chitosan causes the leakage of intracellular electrolytes and proteinaceous constituents by interfering with the negatively charged residues of macromolecules exposed on the fungal cell wall (Bautista et al., 2006; Leuba and Stoseel, 1986).
2) Interaction of diffused hydrolysis products with the microbial DNA causes the inhibition of transcription of mRNA and protein synthesis (Hadwiger et al., 1986, Bautista et al., 2006).
3) Chitosan acts as chelator of metal and essential nutrients from spores, affecting the normal growth and development of the fungi (Cuero et al., 1991).

None of these hypotheses has been fully elucidated yet, but some progress has been made using *Neurospora crassa* as a model (Palma et al., 2009). Membrane permeabilization, cell death and lysis of spores of *N. crassa* treated with chitosan have been reported (Palma et al., 2009). This result suggests a possible interaction of chitosan with the membrane causing the leakage of intracellular electrolytes and essential nutrients, resulting in the lysis and death of the spores.

Chitosan has been tested for management of fruit-decay fungi *in vitro* and *in vivo* (El Ghaouth et al., 1992; El Ghaouth et al., 1994). In culture, chitosan inhibited spore germination, germ tube elongation and radial growth of two postharvest pathogens of

strawberry fruits, *B.* and *Rhizopus stolonifer* (El Ghaouth et al., 1992). However, decay of fruits was not reduced by chitosan treatments. Bell pepper fruits treated with chitosan exhibited a restriction in the colonization of *B. cinerea* and change in the ultrastructure of infected plant cells (El Ghaouth et al., 1994). Chitogel, a formulated chitosan solution, produced a reduction in disease development in grapes caused by *B. cinerea* and morphological alterations in the pathogen (Ait et al., 2004). Hyphae of *B. cinerea* treated with chitosan showed increased accumulation of small vesicles. Similar results were observed in spores of *Trichoderma harzianum* in which "intracellular lipid depositions" were observed in spores treated with chitosan. However, why these vesicles form following a treatment with chitosan is still unclear. In carrots a reduction of 72% and 76% in foliar disease severity caused by *B. cinerea* and *Alternaria radicina*, respectively, was observed after chitosan treatment (Jayaraj et al., 2009).

Experiments to test the effect of chitosan against members of the genus *Fusarium* and other soil-borne pathogens have indicated some efficacy of chitosan for managing these fungi (Bell et al., 1998; Abd-El-Karem et al., 2006). A significant reduction in disease severity caused by *Fusarium oxysporum* in celery was achieved when chitosan was applied as root dip to a disease-tolerant celery cultivar (Bell et al., 1998). However, chitosan amendments into soil did not reduce populations of *F. oxysporum*. These results suggest that the disease-control activity of chitosan is also dependent on application method. In tomato all the causal agents of tomato root rot (*Rhizoctonia solani*, *Fusarium solani* and *Sclerotium rolfsii*) showed a reduction in radial growth *in vitro* and reduced disease incidence under greenhouse conditions (Abd-El-Karem et al., 2006).

Role of chitosan in plant disease resistance

Enhanced resistance to plant pathogens in plants treated with chitosan or its derivatives has been observed in several crops including pearl millet, grapes, coconut, carrots, beans, tomato, rice and cucumbers (Manjunatha et al., 2009; Aziz et al., 2006; Ben-Shalom et al., 2003; Jayaraj et al., 2099; Lizama-Uc et al., 2007; Benhamou et al., 1998; Agrawal et al., 2002; Iriti and Faoro, 2008). However, the specific pathway involved in the induction of resistance by chitosan is still under investigation.

Increased accumulation of enzymes that play important roles in the defense mechanisms employed by plants against fungi has been observed following treatments with chitosan. Grape leaves treated with chitosan produced increased levels of chitinases and β-1-3 glucanases (Aziz et al., 2006). In coconut, β-1-3 glucanase activity increased after the imbibition by coconut calli of chitosan in solution (Lizama-Uc et al., 2007). In the same studies with coconut, the activation of a 46 kDa MAPK-like protein after chitosan treatment was demonstrated, and it remained activated for at least 80 min.

Increased peroxidase activity has been also demonstrated in cucumber plants treated with chitosan and inoculated with *B. cinerea* (Ben-Shalom et al., 2003). However, no reduction of gray mold caused *B. cinerea* was obtained in spite of the increased activity of the enzyme. Contrary to this result, carrot plants inoculated with *A. radicina* and *B. cinerea* and previously treated with chitosan showed increased accumulation of H_2O_2 at the site of infection and lower disease development compared with the control plants (Jayaraj et al., 2009).

Constitutive expression of pathogenesis-related proteins like PR-1 and PR-5 following pathogen inoculation in chitosan-treated has been detected in pearl millet and

carrot (Manjunatha et al., 2009; Jayaraj et al., 2009). In rice, northern blot analyses revealed potent accumulation of two pathogenesis-related proteins (*OsPR5* and *OsPR10*) on seedling leaves treated with chitosan (Agrawal et al., 2002). In addition, chitosan elicited the production of anti-fungal phytoalexins like the flavonoid sakuranetin and the diterpenoid lactone momilactone A. Increased accumulation of phytoalexins has been also detected in grapevine leaves treated with chitosan (Aziz et al., 2006). These results showed the possible role of chitosan in initiating multiple events linked with defense/stress response in plants.

Recently, nitric oxide was reported as being involved in chitosan-induced resistance to downy mildew disease in pearl millet (Manjunatha et al., 2009). Pretreatment with nitric oxide scavenger and nitric oxide synthase inhibitor before pathogen inoculation reduced the ability of chitosan to protect pearl millet plants against downy mildew. These authors reported that the maximum resistance against downy mildew after chitosan treatment was obtained four days after the initial treatment and it persisted for an additional seven days, consistent with induction of host resistance as a mechanism contributing to chitosan's effect on disease control.

In addition to the activation of all of these proteins, enzymes and components of the machinery of the defense response in the plant, structural changes at the cellular level that may restrict pathogen colonization have been observed following chitosan treatment (Iriti and Faoro, 2008; Benhamou et al., 1998). Enhanced callose deposition has been observed in bean leaf fragments treated with 0.15% chitosan (Iriti and Faoro, 2008). In pearl millet callose deposition was also detected two hours after pathogen inoculation in chitosan-treated plants but not in control plants (Manjunatha et al., 2009).

Some results suggest that chitosan activates plant defense genes through the octadecanoid pathway (Doares et al., 1995). Early on, soluble chemical derivatives of chitin and chitosan were found to possess proteinase inhibitor-inducing activities in tomato plants (Walker-Simmons and Ryan, 1984). Then, increases in jasmonic acid levels in leaves of tomato plants were observed two hours after supplying the polypeptide systemin, oligogalacturonides, or chitosan to the plants through their cut stems (Doares et al., 1995). Despite this suggestive evidence of the possible involvement of jasmonic acid in chitosan-mediated resistance, little progress has been made in this topic in recent years.

All of these results provide further evidence for the possible role of chitosan as a signal to activate plant defense responses. However, more research has to be done to determine the pathway or components of resistance used by chitosan.

Chitin

NaOH (40%)

Chitosan

Figure 1.2. Transformation of chitin into chitosan via deacetylation.
(Picture modified from Rabea et al., 2003).

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CHAPTER 2

IN VITRO AND *IN VIVO* EVALUATION OF ESSENTIAL OILS, BICARBONATE SALTS AND OMRI-CERTIFIED MATERIALS AGAINST *COLLETOTRICHUM ORBICULARE*

INTRODUCTION

Cucumbers (*Cucumis sativus* L.) and others members of the Cucurbitaceae family are affected by foliar fungal diseases that reduce yields and affect fruit marketability. *Colletotrichum orbiculare* (Berk. & Mont.) Arx (syn. *Colletotrichum lagenarium* (Pass) Ellis & Halst.) causes anthracnose disease of cucurbits, affecting primarily watermelons, melons and cucumbers (Zitter et al., 1996). Leaves, stems and fruits may become affected by *C. orbiculare* (Monroe et al., 1997; Anonymous, 1996), producing circular light brown or reddish lesions where the conidia are produced. Losses of 30% or more can occur in susceptible crops where control practices are not followed (Yeop et al., 2005). In the U.S., the disease is most severe in states in the humid midwestern, eastern and southeastern regions, causing yield losses of up to 60% (Monroe et al., 1997; Thompson and Jenkins, 1985).

Resistant varieties and fungicide applications, as well as certified disease-free seed, are used extensively on conventional farms for management of the disease (Rowell et al., 2010-11). The use of partially resistant and susceptible cucumber cultivars often necessitates the use of preventive fungicides (Rowell et al., 2010-2011) to reduce the impact of the disease on yields. Growers rely on repeated applications of fungicides in order to achieve acceptable levels of disease control (Monroe et al., 1997). In the United States, approximately 70% of the cultivated area of watermelon receives fungicides

applications to control foliar fungal disease (Keinath, 2000). The most intensive management of this disease is through the use of protectant and eradicant fungicides such as azoxystrobin, chlorothalonil and mancozeb (Pernezny et al., 2006). In Kentucky, these fungicides, as well as fixed coppers, form part of the recommendations to manage cucurbit diseases (Rowell et al., 2010-11).

The application of synthetic fungicides is not an option for organic farms according to the National Organic Program (NOP), which defines an organic food as “Food produced without using most conventional pesticides; fertilizers made with synthetic ingredients or sewage sludge; bioengineering; or ionizing radiation” (USDA, 2011). These regulations, the continually increasing interest in preserving the environment, and public concern about pesticides residues in food, have resulted in increasing interest in organic products for plant disease control. Among these, essential oils, plant extracts, biological agents, soil extracts, and bicarbonate salt are acceptable materials for disease and pest management under the standards of the National Organic Program (NOP). Thus, the objective of this research was to investigate fungitoxicity and potential disease control obtained with essential oils, bicarbonate salts, and natural, organically certifiable spray materials against *C. orbiculare in vitro* and *in vivo*.

MATERIALS AND METHODS

Fungal isolate

Colletotrichum orbiculare was isolated from cucumber foliage collected at a commercial farm in Harrison County, Kentucky in 2007. The fungus was cultured on 39% (w/v) potato dextrose agar (PDA; Difco Lab., Detroit, MI) under continuous

fluorescent light at 22˚C. Single-conidium cultures were obtained as follows. After 10 days of incubation, a sterile spatula was used to scrape the pinkish mass of conidia from a culture plate flooded with 10 ml of sterile distilled water. Fifty microliters were spread on 20% (w/v) water agar (Difco Lab; Detroit, MI) and incubated for 12 hours at 22˚C. Single germinated conidia were transferred to PDA and incubated for 5 days. After the incubation time the pathogen was transferred and grown on green bean agar (453 g of caned green bean/L mixed with 17g of agar) under continuous fluorescent light at 22˚C to induce sporulation (Jeun et al., 2003). One isolate was chosen for identification by pathogenicity tests and morphological features of conidia (100 conidia) and appressoria (Appendix 1). The ITS (Internal Transcribed Spacer) sequence of the isolate was also obtained via direct sequencing using primers ITS1 and ITS4 (White et al., 1990), and species determination was verified via a Genbank BLAST search. Spore suspensions at a concentration of 10^5 and 10^6 conidia/ml were used for *in vitro* and *in vivo* experiments, respectively.

Dilutions and preparations of materials for *in vitro* and *in vivo* experiments

Essential oils. Undiluted essential oils (100%) were purchased from Wild Oats Markets, Inc. in Lexington, KY. The scientific names of source plants, the common names of essential oils, their major compounds and chemical structures are listed in Table 2.1. A stock solution of 1000 µg/ml was prepared by dissolving 1 µl of the essential oil in 999 µl of 0.1 % Tween 20 (Soylu et al., 2006). The solution was vigorously agitated to form an emulsion and sterilized through a disposable membrane filter of 0.22 µm pore size (Millipore, Bedford, MA) (Soylu et al., 2006). All concentrations used in these

assays were adjusted from the stock solution to obtain final concentrations ranging from 0 to 500 µg/ml (Daferera et al., 2000). *In vivo* all essential oils were tested at 1000 µg/ml (Herath and Abeywickrama, 2008). Tween 20 at 0.1 % was a control in all experiments.

Bicarbonate salts. Ammonium bicarbonate (NH_4HCO_3), potassium bicarbonate ($KHCO_3$) and sodium bicarbonate ($NaHCO_3$) salts were purchased from Sigma Aldrich (St. Louis, Mo). Filter-sterilized stock solutions of 1 M were prepared, and three concentrations (0.1, 0.25 and 0.5 M) were evaluated *in vitro* for each salt (Hervieux et al., 2002; Olivier et al., 1999). Bicarbonate salts were tested *in vivo* at 0.25 M (Hervieux et al., 2002), as concentrations higher than 0.25 M showed severe plant toxicity.

OMRI-certified materials. Ten commercial OMRI-certified materials were evaluated *in vitro* and *in vivo.* These were divided in three major groups: plant-based extracts which included Trilogy®(a.i. neem oil) and Regalia® SC (a.i. extracts of giant knotweed); biological-based products which included Sonata® (a.i. *Bacillus pumilis*), Serenade Max® (a.i. *Bacillus pumilis*), Actinovate® (a.i. *Streptomyces lydicus*) and SoilGard 12G® (a.i. *Gliocladium virens*); and, commercial fungicides, including copper-based products, Kocide® 2000 (a.i. copper hydroxide) Kocide® 3000 (a.i. copper hydroxide), Bordeaux mixture (a.i. copper sulfate mixed with hydrated lime) and horticultural lime sulfur (HLS) (a.i. calcium polysulfide). A complete description of each commercial product is presented in Table 2.2. All OMRI-certified materials were dissolved in ddiH_2O and filter-sterilized. *In vitro* four concentrations were tested, 100, 150, 200 and 250 µg/ml, and *in vivo* all materials were evaluated at 1000 µg/ml. Although Regalia® SC is labeled only as a plant defense inducer, it was evaluated as a

protectant fungicide as well as a plant defense inducer because of its antifungal activity observed *in vitro* in our experiments.

***In vitro* measurement of antifungal effects on mycelial growth**

The antifungal activity of essential oils, bicarbonate salts and OMRI-certified material was determined *in vitro* using a colorimetric method, which uses the absorbance (OD 450) of cultures in a microtiter plate to assess mycelial growth. This protocol was modified from procedures described previously (Daeschel 1992; Shad and Reddy, 2000; Wilson et al., 1997). The assay was conducted as follows: 100 µl of conidial suspension (10^6 conidia/ml) were added to 400 µl of potato dextrose broth (PDB, Difco Lab., Detroit, MI), and to this was added the amount required for each test material plus sterile ddH2O to achieve the desired concentration of the test material in a final volume of one ml. The mixture was vortexed for 5-10 seconds, and one hundred microliters of the mixture were added into each of eight wells (replicates for each concentration) of a 96-well, sterile, flat bottom microplate (Nunc-Immuno™ Plate, USA). Conidial suspensions which were not treated or treated with Quadris® 50 WG (azoxystrobin (AZ)) at 2 µg ai/ml (Syngenta, Greensboro, NC) served as negative and positive controls, respectively. For all materials tested, identical mixtures without conidia were prepared and added to the same plate to serve as controls in order to test for changes in absorbance due to chemical transformations rather than suppression of mycelial growth. The 96-well plate was then sealed with a sterile adhesive polyester film (VWR Scientific, Scientific Industries, Inc., Bohemia, NY) and incubated under continuous light during 48 h at 22˚C. After the incubation period, the fungal mycelial density ($OD_{450\ nm}$) was measured with a ELx800™ Universal Microplate Reader (Bio-Tek™, Princeton, NJ) equipped with Delta-Soft 3™

kinetics and endpoint software. To obtain the net fungal growth, the mean absorbance values of the mixtures without conidia were subtracted from the absorbance of each replicate of the mixture with conidia. This corrected absorbance value was transformed to percent of mycelial growth inhibition (mgi) relative to the control using the follow formula: mgi = $(1- A_n/A_0)*100$; where A_n is the absorbance of the sample and, A_0 is the mean absorbance of the control (non-treated conidia). The experiment was performed twice with eight replications per treatment.

In vivo evaluation of essential oils, bicarbonate salts and OMRI-certified materials

Organic seeds of cantaloupe (*Cucumis melo* cultivar 'Edens Gem OG') were obtained from Jonny's Selected Seeds (Maine, USA). Plants were grown in 10-cm diameter plastic pots filled with Pro-Mix® (Quakertown, PA) under greenhouse conditions at 27˚C and 23˚C, day and night respectively. Plants were fertilized weekly using fish emulsion 5-1-1 (Ferti·lome®, Bonham, Texas) at rate of 0.3 oz/liter of water. The fertilizer had a formulation of 0.5% ammoniacal nitrogen, 4.5% other water-soluble nitrogen, 1% available phosphate (P_2O_5) and 1% soluble potash. Ten day-old seedlings with cotyledons fully expanded were sprayed until runoff with the materials to be evaluated using a hand sprayer (VWR International, Cat. No. 23609-182). Plants spray with water and spray with Quadris® 50 WG were used as negative and positive control, respectively. After the plants dried, 3 ml of a 10^6 spore/ml suspension of *C. orbiculare* were sprayed over the cotyledons using a hand sprayer. Inoculated plants were placed in a moist chamber (100% relative humidity) for 24 h (Jeun et al., 2003). After incubation, plants were removed from trays and randomized in the greenhouse using a completely

randomized design. Disease symptoms were scored 4-6 days post-inoculation by harvesting cotyledons and capturing digital images of these using a *Nikon* COOLPIX L110. Each picture was analyzed for disease severity using Assess Image Analysis Quantification Software 2.0. Ten plants per treatment were used and the experiments were done twice.

Statistical analysis

All results were analyzed using the INFOSTAT Statistical Software Version 2004 (InfoStat, FCA, Córdova Argentina). Analysis of variance (ANOVA) was performed, and inferences were made at the significance level of $P<0.05$.

RESULTS

Essential oils. No more than 20% mycelial inhibition *in vitro* was obtained with any of the essential oils (Fig. 2.1). *In vivo*, some of the essential oils did not provide consistent results between repetitions. During the first experiment, basil, lemon eucalyptus, rosemary and tea tree significantly reduced ($P<0.05$) cotyledon necrosis compared to the Tween 20-treated plants (Fig. 2.2). Despite the reduction in disease severity observed, all plants had more than 45% of the cotyledon area affected. During the second experiment none of the essential oils provided a significant reduction ($P>0.05$) in disease severity.

Bicarbonate salts: *In vitro* studies showed that NH_4HCO_3, $KHCO_3$ and $NaHCO_3$ provided >75% inhibition of mycelial growth of *C. orbiculare* at all concentrations tested (Fig. 2.3). *In vivo,* the three bicarbonate salts reduced cotyledon necrosis ($P<0.05$) compared with water-treated plants (Fig. 2.4). $KHCO_3$ and $NaHCO_3$ showed the lowest

cotyledon necrosis with less than 20% damage with symptoms and were not significantly difference (P>0.05) from the conventional pesticide (AZ) used as a control. For all three salts, concentrations higher than 0.25 M produced phytotoxicity (necrosis of the plant tissue) (*data not shown*).

Plant-based extracts. *In vitro,* Trilogy® failed to inhibit the growth of *C. orbiculare* at all concentrations tested. At 100 and 150 µg/ml Trilogy® promoted mycelium growth rather than inhibiting it (Fig. 2.5A). Regalia®SC produced more than 40% inhibition at concentrations ≥100 µg/ml (Fig. 2.5B). At the highest concentration (250 µg/ml), Regalia®SC gave >75% inhibition of mycelial growth of *C. orbiculare.* *In vivo* experiments showed no effect on disease severity by either Trilogy® or Regalia®SC when applied shortly before inoculation (Fig. 2.6). Regalia®SC was also tested as a plant defense elicitor by treating plants and inoculating 48 h later, which resulted in significant reductions (P<0.05) in disease severity (Fig. 2.7).

Biological-based products: *In vitro,* Sonata® was the most fungitoxic OMRI-certified biological-based product against *C. orbiculare*. Sonata® showed no difference in mycelial growth inhibition compared with the positive control (AZ) at concentrations ≥200 µg/ml (Fig. 2.8A). More than 82% mgi was obtained with Sonata® at concentrations ≥150 µg/ml (Fig. 2.8A). Serenade Max® provided between 20-40% inhibition of mycelial growth at concentrations ≥100 µg/ml (Fig. 2.8B). Actinovate® was the least effective OMRI-certified biological-based product with less than 19% inhibition of mycelium growth at all concentrations tested (Fig. 2.8C).

In vivo, Sonata®, Serenade Max® and SoilGard 12G® produced a significant reduction in disease severity (Fig. 2.9-2.10), whereas Actinovate® failed to control disease symptoms compared with the water control (Fig. 2.10). As in the *in vitro* experiments, Sonata® was the most effective biological-based product controlling the disease *in vivo*.

Copper-based products and lime sulfur. *In vitro*, Kocide® 2000 and Kocide® 3000 showed inhibition of mycelial growth in a dose-dependent manner reaching more than 90% inhibition at 200 μg/ml (Fig. 2.11 A-B). Bordeaux mixture showed more than 50% inhibition of mycelium growth at concentrations ≥100 μg/ml (Fig. 2.11 C). Horticultural lime sulfur showed highly antifungal activity against *C. orbiculare*. At concentration ≥5 μg/ml, more than 90% inhibition of mycelium growth was achieved (Fig. 2.11D).

In vivo, a significant reduction in disease severity was obtained with all copper-based products and lime sulfur at concentrations of 500 and 1000 μg/ml (Fig. 2.12). At 500 μg/ml, Kocide® 3000 and HLS showed no statistically difference with the conventional fungicide (AZ). At 1000 μg/ml, all copper-based products and lime sulfur provided disease control equivalent to that obtained with AZ.

DISCUSSION

Among the materials tested *in vitro*, we found that numerous materials--all bicarbonate salts, Regalia®SC, Sonata®, Serenade Max®, all copper-based fungicides and horticultural lime sulfur--significantly inhibited mycelial growth of *C. orbiculare*. Using a colorimetric assay, the antifungal activity of all these products was assessed in an

accurate and rapid way. With the exception of Regalia®SC, all products that showed effectiveness against *C. orbiculare in vitro*, were effective controlling the disease *in vivo*. Essentials oils, Trilogy® and Actinovate® failed to inhibit mycelial growth of *C. orbiculare in vitro* as well as disease severity *in vivo*.

Essential oils failed to suppress *in vitro* growth of *C. orbiculare* and disease development in cantaloupe plants. All the essential oils tested here had been shown previously antifungal activity against plant pathogens like *Botrytis cinerea, Penicillium digitatum, Alternaria alternata*, *Phytophthora infestans, P. capsici, Fusarium verticillioides, Rhizoctonia solani*, and *Sclerotinia sclerotiorum* (Fandohan et al., 2004; Feng and Zheng, 2005; Müller-Riebau et al., 1995; Soliman and Badeaa, 2002; Soylu et al., 2006; Wilson et al., 1997; Daferera et al., 2000). The specific mechanisms underlying the inhibitory actions of essential oils against plant pathogenic fungi are as yet unclear but some possible modes of action have been proposed. First, essential oils, or their active components, contain hydroxyl groups which are highly antimicrobial and might be are responsible for their mode of action (Farag et al., 1989). The hydroxyl group might be bind the active site of enzymes and alter their activity (Ceylan and Fung, 2004), which in turn might alter fungal metabolism. Also, alcohols present in essential oils have been shown to have antimicrobial activity against bacteria, acting as protein denaturants (Ceylan and Fung, 2004). Essential oils exhibit different levels of antifungal activity depending on the type of oil, its concentration, and the species of phytopathogenic fungus evaluated (Wilson et al., 1997; Soliman and Badeaa, 2002). Thus, the results obtained here might be the effect of a combination of factors like type of oil and concentration which resulted in failure of basil, rosemary, fennel, lemon eucalyptus and tea tree to

inhibit mycelial growth of *C. orbiculare* and development of cucurbit anthracnose disease.

Bicarbonate salts, NH_4HCO_3, $KHCO_3$ and $NaHCO_3$, displayed high antifungal activity against *C. orbiculare in vitro* and *in vivo*. These compounds are among the most common bicarbonate salts tested *in vitro* and *in vivo* against plants pathogens (Palmer et al., 1997; Homa et al., 1981; Horst et al., 1992; Ziv and Zitter, 1992). Previous *in vitro* studies performed by Ziv and Zitter (1992), showed that NH_4HCO_3, $KHCO_3$ and $NaHCO_3$ inhibit growth of *C. orbiculare* and other cucurbit pathogens. *In vivo* experiments with bicarbonate salts are more limited (Ziv and Zitter, 1992; Hervieux et al., 2002), but in one previous study these three bicarbonate salts produced significant reductions in disease development caused by *Alternaria cucumerina*, *Didymella bryoniae* and *Ulocladium cucurbitae* in cucumber (Ziv and Zitter, 1992). These results are comparable to the results presented here, where all salts were effective controlling anthracnose disease on cantaloupe. $KHCO_3$ and $NaHCO_3$ were the most effective, providing more than 80% control over the non-treated plants under greenhouse conditions (Fig. 4).

One obvious limitation of the use of these bicarbonate salts as foliar treatments is the phytotoxicity caused at concentrations above 0.25 M. Similar results were observed on cucumber cotyledons treated with $KHCO_3$ at concentrations higher than 1.25% (Ziv and Zitter, 1992). Since no damage was observed at concentrations at or below 0.25 M this problem may be avoidable by not exceeding this dosage.

Among the OMRI-certified, plant-based extracts tested, only Regalia®SC significantly inhibited mycelial growth of *C. orbiculare in vitro*. However, *in vivo*

experiments Regalia® SC and Trilogy® showed no control of anthracnose disease. Regalia® SC is labeled for managing foliar diseases not by acting directly on the pathogen but by inducing resistance in the treated plant. In our experiments we observed direct antifungal activity on the several species of *Colletotrichum* using Regalia® SC. While this suggests that this plant-based extract may have direct antifungal activity that has not been previously reported, when we tested this product *in vivo* to evaluate its possible efficacy as a preventive fungicide directly affecting the pathogen, no reduction in disease was observed. In contrast, the evaluation of Regalia® SC as a plant inducer (=application 48 h prior to inoculation) resulted in a reduction in disease severity caused by *C. orbiculare*. Thus, our results suggest that the best use of this product is as a plant defense inducer as is indicated on the product label.

Trilogy® (ai. neem oil), a product labeled to manage anthracnose preventively, failed in both the *in vitro* and *in vivo* experiments. Moreover, Trilogy actually promoted mycelial growth *in vitro*. While the data available on the efficacy of Trilogy® for managing diseases in cucurbits is limited, in other crops this fungicide provided no disease control, as seen in our experiments. For example, neem oil failed to suppress spot anthracnose on leaves of dogwood (Hagan and Akridge, 2007). In field trials with other crops such as tomato, Trilogy® failed to suppress foliar diseases caused by *Alternaria solani* and *Septoria lycopersici* (Seamen et al., 2004; Wszelaki and Miller, 2005). These results, along with our own studies, suggest that Trilogy® is not as effective as other OMRI-certified materials for managing necrotrophic fungal foliar disease like anthracnose. Evaluation of Trilogy® on cucurbits under field conditions is highly

encouraged in order to validate results obtained in more controlled but artificial conditions.

With respect to the biological-based products evaluated, Sonata®, SoilGard 12G and Serenade Max® were effective against cucurbit anthracnose while Actinovate®SP was not. None of the three fungicides are currently labeled for cucurbit anthracnose control, but use of Sonata Max or SoilGard 12G against other, labeled diseases on organic farms may provide control of anthracnose. Moreover, SoilGard 12G is a fungicide labeled for management of "Damping-off" and rot root pathogens of ornamental and fruit crop plants by soil applications; yet, unexpectedly, we found that foliar applications of SoilGard 12G suppressed anthracnose disease in cantaloupe. From the limited literature available on the efficacy of these biological-based products for managing anthracnose, Gleason et al., (2004) found that Serenade WP apply in a 7-day interval in muskmelon suppressed anthracnose disease compared with the non-treated control (Gleason et al., 2004). Their findings support the data obtained here from *in vitro* and greenhouse experiments. These biological-based products are among the most attractive materials to manage pest and diseases by organic growers and here we provided evidence that these three products, Sonata®, SoilGard™12G and Serenade Max®, might provide control of anthracnose disease when these are used to manage other diseases described on the labels.

All copper-based materials as well as horticultural lime sulfur were highly effective at inhibiting the growth of *C. orbiculare in vitro* and disease development *in vivo*. In numerous trials testing the efficacy of certified organic antifungal materials, copper-based product have provided the best results in terms of disease management

(Seamen et al., 2004; Joslin and Taber, 2003; Wszelaki et al., 2003). However, there are two main concerns with the use of copper-based products for disease management. First, it can be toxic to beneficial microorganisms, soil fauna, and to the plant by continuous application; and second, its use is highly regulated by the National Organic Program and OMRI (Van Zwieten et al., 2004; Streit, 1984; Wszelaki and Miller, 2005; EPA).

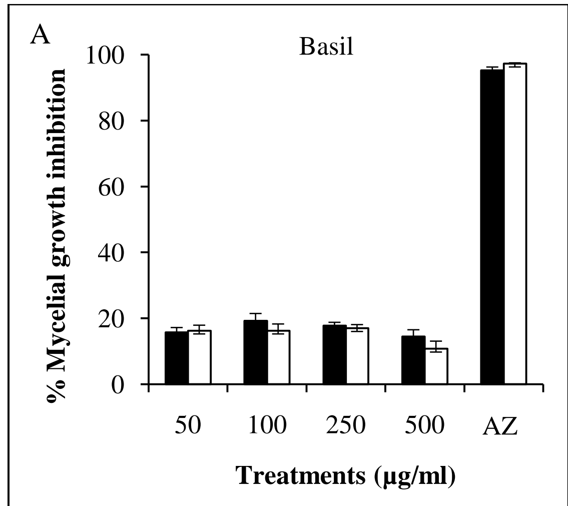
Given the limited data in terms of the efficacy of disease-control products available for organic growers, our results reported here provide an extensive evaluation of products that may have potential for managing anthracnose disease in cucurbits. However, all of the promising materials identified in this report must be thoroughly tested under field conditions under an organic production system in order to have high-confidence conclusions as to the efficacy of these products for managing anthracnose disease caused by *C. orbiculare*.

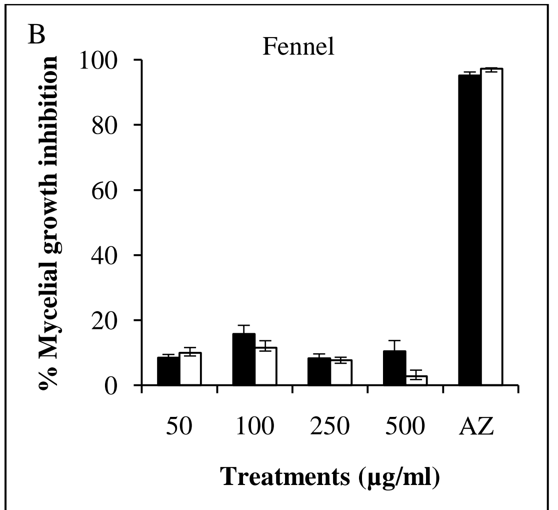
Table 2.1. Commercial essential oils tested for antifungal activity.

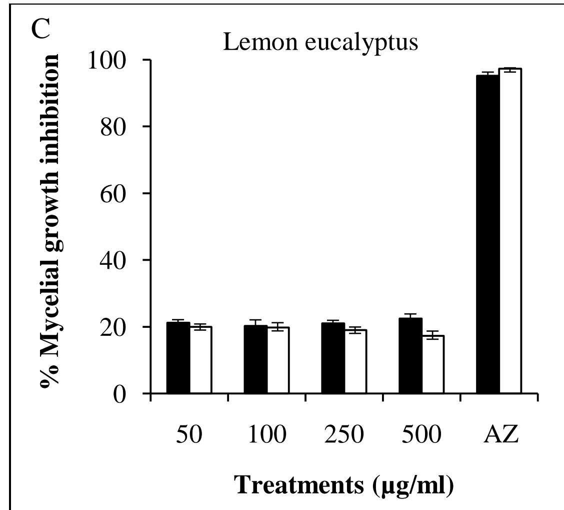
Scientific name of aromatic plants	**Common name of essentials oils**	**Major component(s) of essential oils**	**Structure of the major component(s)**
Foeniculum vulgare Mill	Fennel	Anethole ($C_{10}H_{12}O$)	
Rosmarinus officinalis L.	Rosemary	Borneol ($C_{10}H_{18}O$)	
Ocimum basillicum L.	Basil	Estragole ($C_{10}H_{12}O$)	
Melaleuca alternifolia L.	Tea tree	Terpinen-4ol ($C_{10}H_{18}O$)	
Eucalyptus citriodora	Lemon eucalyptus	Citronellal ($C_{10}H_{18}O$)	

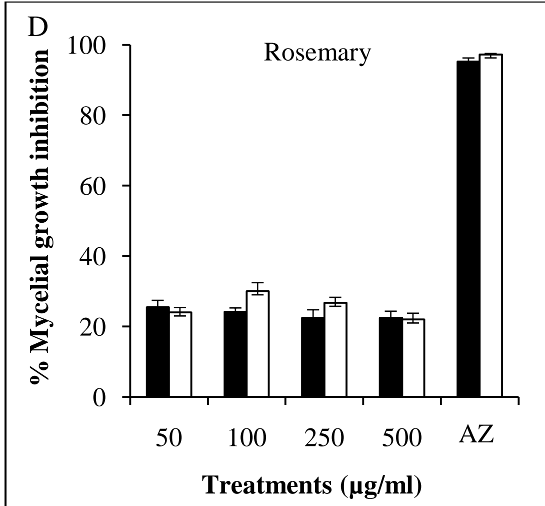
Table 2.2. OMRI-certified materials evaluated *in vitro* and *in vivo*.

Material tested	**Active Ingredient**	**Manufacture company**
Sonata®	1.38 % *Bacillus pumilis*	AgraQuest™
Serenade Max®	14.6 % *Bacillus subtilis*	AgraQuest™
Actinovate® SP	0.037 % *Streptomyces lydicus*	Natural Industries, Inc.
SoilGard™ 12G	12% *Gliocladium virens*	Certis, USA
Kocide® 2000	53.8 % Copper hydroxide	DuPont™
Kocide® 3000	46.1 % Copper hydroxide	DuPont™
Bordeaux mixture	12.5 % copper hydroxide, copper sulfate	Hi-Yield®
Trilogy®	70 % neem oil	Certis, USA
Regalia® SC	5 % giant knotweed	Marrone®Bio Innovations
Lime sulfur	26 % calcium polysulfide	Hi-Yield®
Quadris® 50 WG	50 % azoxystrobin	Syngenta


A
Basil
100
80
60
40
20
0
% Mycelial growth inhibition
50
100
250
500
AZ
Treatments (µg/ml)


B
Fennel
100
80
60
40
20
0
% Mycelial growth inhibition
50
100
250
500
AZ
Treatments (µg/ml)


C
Lemon eucalyptus
100
80
60
40
20
0
% Mycelial growth inhibition
50
100
250
500
AZ
Treatments (µg/ml)


D
Rosemary
100
80
60
40
20
0
% Mycelial growth inhibition
50
100
250
500
AZ
Treatments (µg/ml)

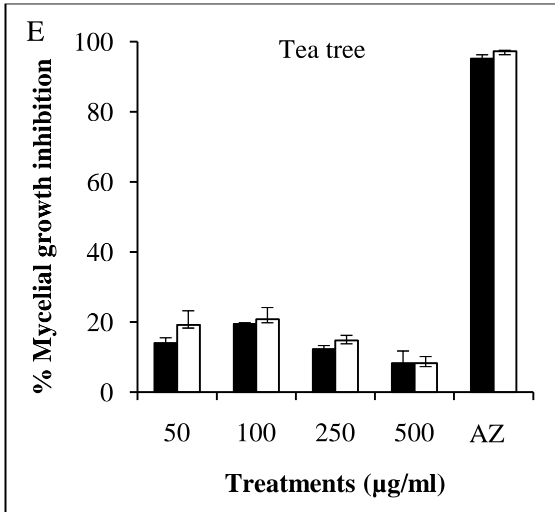
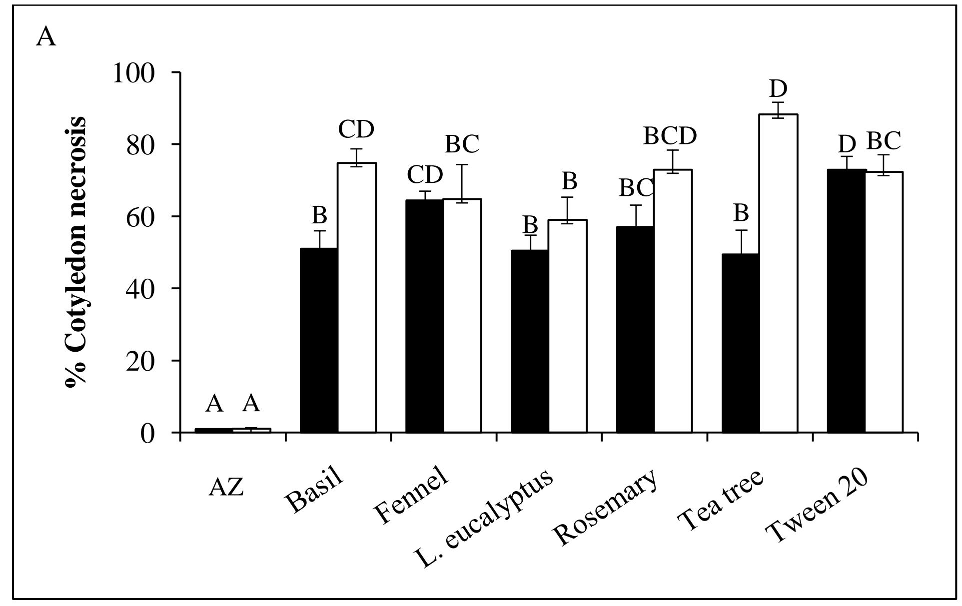

E
Tea tree
100
80
60
40
20
0
% Mycelial growth inhibition
50
100
250
500
AZ
Treatments (µg/ml)

Figure 2.1. *In vitro* antifungal activity of essential oils. A) Basil, B) fennel, C) lemon eucalyptus, D) rosemary and E) tea tree. Solid bars are experiment 1 and empty bars are experiment 2. Quadris® (AZ) was used as a positive control in all experiments at concentration of 2 µg ai/ml. The percent of mycelial growth inhibition (mgi) related to the negative control (Tween 20-treated conidia) was determined using the absorbance ($OD_{450\ nm}$) after 48 h of incubation. The mgi was calculated using the following formula: mgi = $(1- A_n/A_0)*100$; where A_n is the absorbance of each treatment and, A_0 is the absorbance of the control (non-treated conidia). Error bars indicate standard errors.



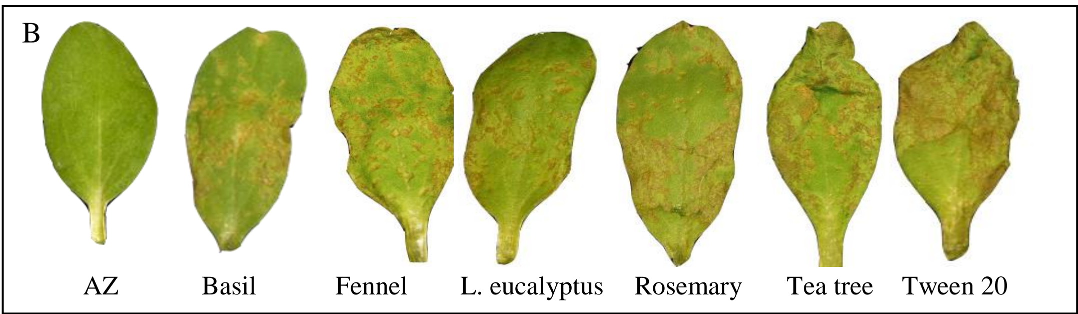
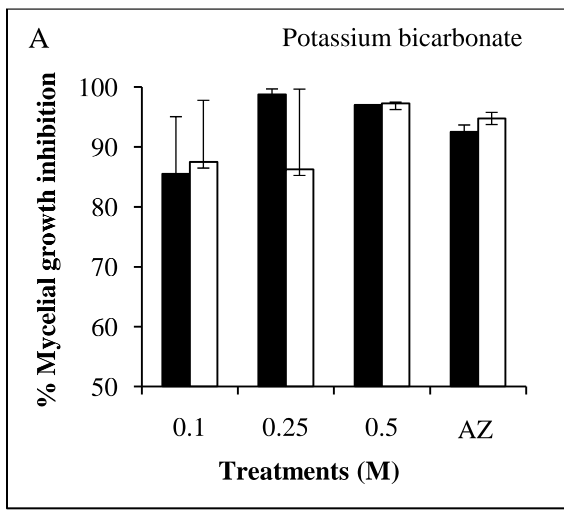
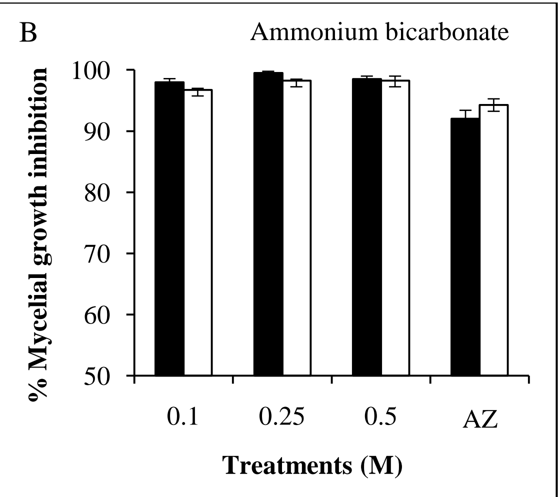


Figure 2.2. ***In vivo* evaluation of essential oils.** A) Percentage of cotyledon necrosis and B) representative pictures of cotyledons from experiment 2. Solid bars are experiment 1 and empty bars are experiment 2. Cantaloupe plants were sprayed with the essential oils, allowed to dry, and inoculated with 10^6 conidia/ml of *Colletotrichum orbiculare*. Five days post-inoculation the percentage of cotyledon necrosis was determined. All essential oils were evaluated at 1000 µg/ml. Bars indicate standard errors. Means values followed by the same letter do not differ significantly according to Fisher's least significant difference ($P = 0.05$).





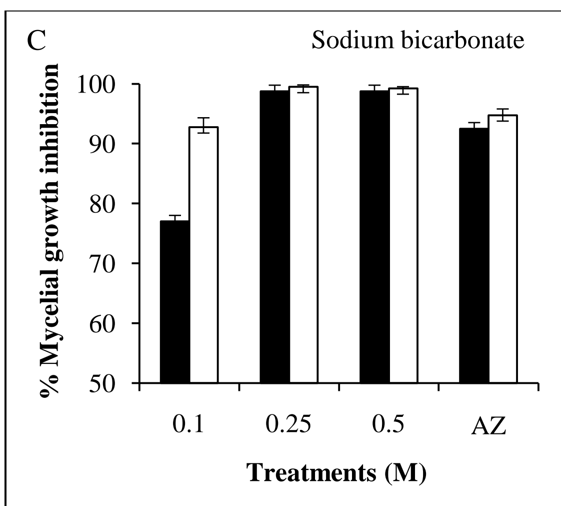
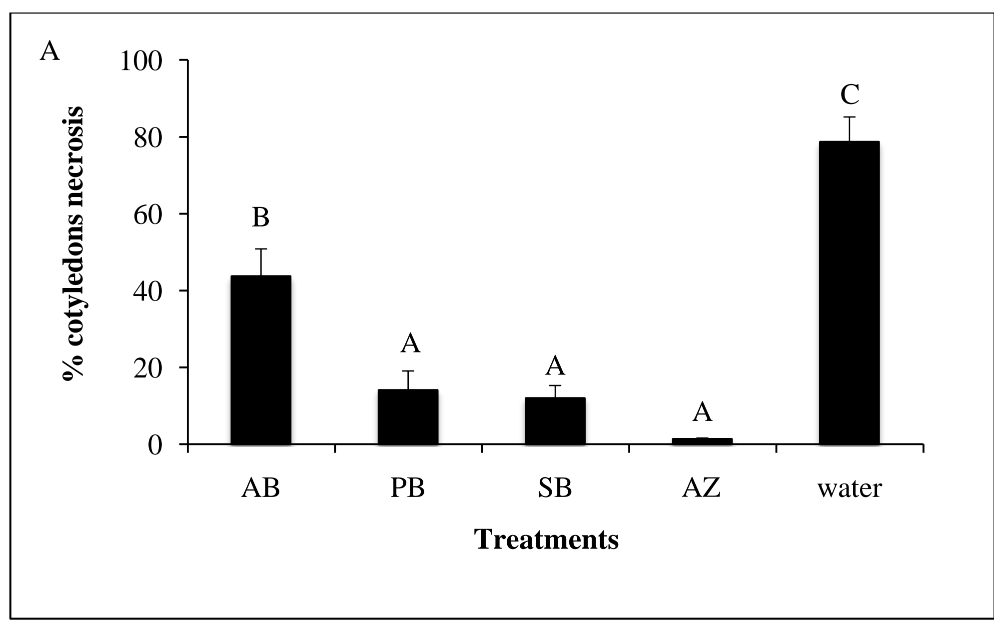


Figure 2.3. *In vitro* antifungal activity of bicarbonate salts. A) Potassium bicarbonate B) ammonium bicarbonate and, C) sodium bicarbonate. Solid bars are experiment 1 and empty bars are experiment 2. Quadris® (AZ) was used as a positive control in all experiments at concentration of 2 µg/ml. The percent of mycelial growth inhibition (mgi) related to the control (non-treated conidia) was determined using the absorbance ($OD_{450\ nm}$) after 48 h of incubation. The mgi was calculated using the following formula: mgi = $(1 - A_n/A_0)*100$; where A_n is the absorbance of each treatment and, A_0 is the absorbance of the control (non-treated conidia). Error bars indicate standard errors.



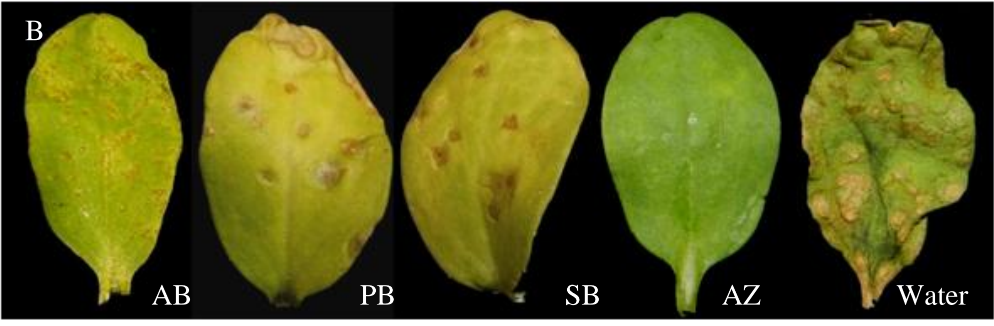
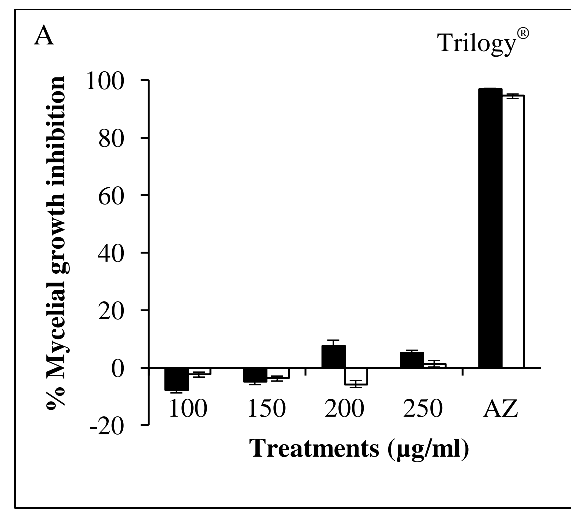


Figure 2.4. ***In vivo*** **evaluation of bicarbonate salts.** A) Percentage of cotyledon necrosis and B) representative pictures of cotyledons. Solid bars are experiment 1 and empty bars are experiment 2. Cantaloupe plants were sprayed with the bicarbonate salts and later inoculated with 10^6 conidia/ml of *Colletotrichum orbiculare*. Five days post-inoculation the percentage of cotyledons necrosis was determined. All bicarbonate salts were evaluated at 0.25 M. Bars indicate standard errors. Mean values followed by the same letter do not differ significantly according to Fisher's least significant difference (P = 0.05). AB=Ammonium bicarbonate, PB=Potassium bicarbonate, SB=Sodium bicarbonate and AZ (Quadris).



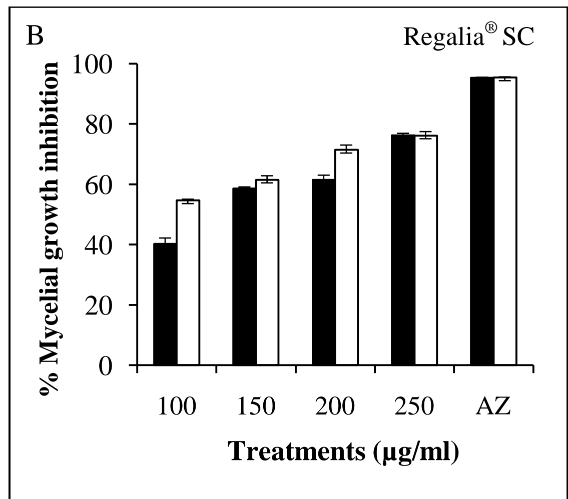


Figure 2.5. *In vitro* antifungal activity of plant extract-based products. A) Trilogy®, and B) Regalia® SC. Solid bars are experiment 1 and empty bars are experiment 2. Quadris® (AZ) was used as a positive control in all experiments at concentrations of 2 µg/ml. The percentage of mycelial growth inhibition (mgi) related to the control (non-treated conidia) was determined using the absorbance ($OD_{450\ nm}$) after 48 h of incubation. The mgi was calculated using the following formula: mgi = $(1- A_n/A_0)*100$; where A_n is the absorbance of each treatment and, A_0 is the absorbance of the control (non-treated conidia). Error bars indicate standard errors.

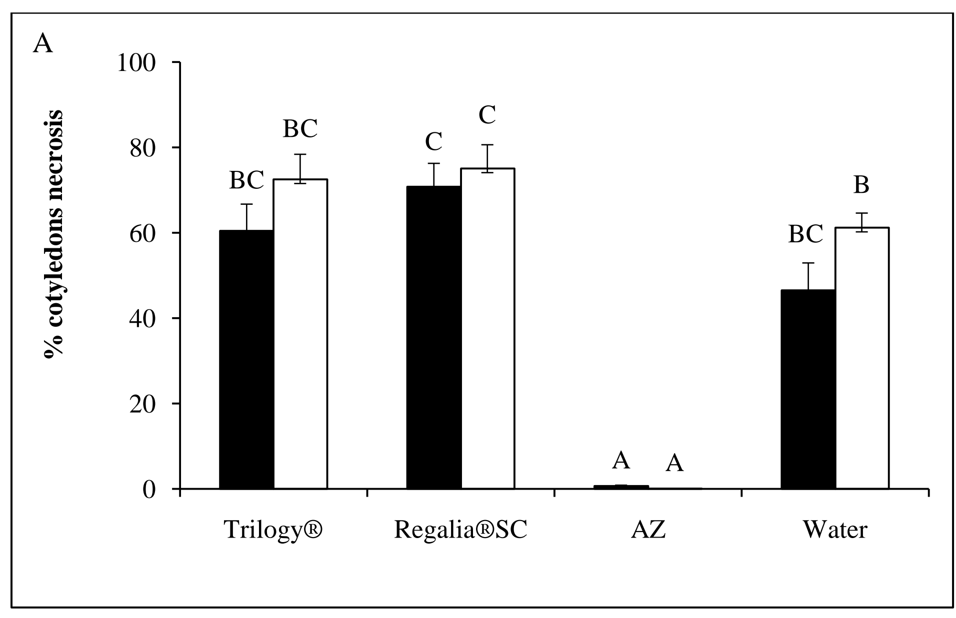


Figure 2.6. *In vivo* evaluation of plant extract-based products. A) Percentage of cotyledon necrosis and B) representative pictures of cotyledons from experiment 1. Solid bars are experiment 1 and empty bars are experiment 2. Cantaloupe plants were sprayed with the Trilogy® and Regalia® SC and later inoculated with 10^6 conidia/ml of *Colletotrichum orbiculare*. Five days post-inoculation the percentage of cotyledon necrosis was determined. Error bars indicate standard errors. Means values followed by the same letter do not differ significantly according to Fisher's least significant difference ($P = 0.05$).

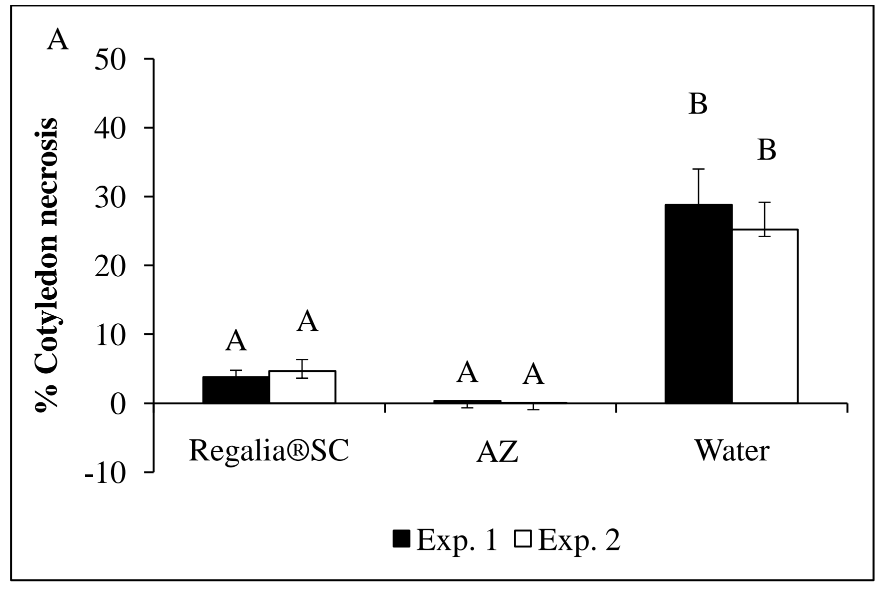
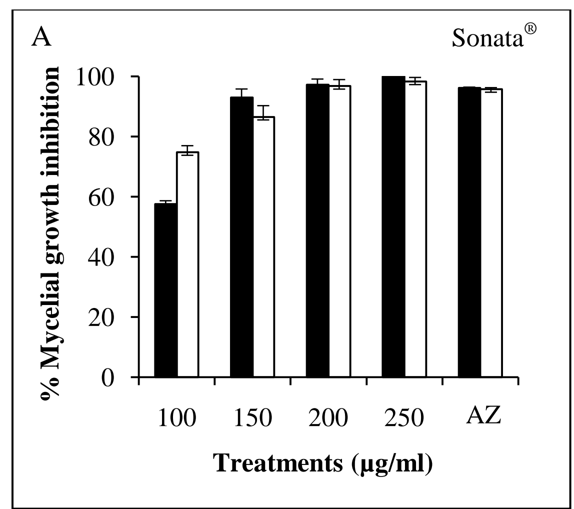
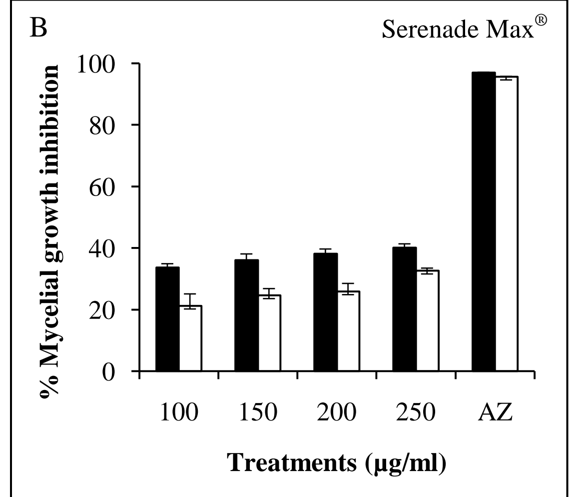


Figure 2.7. *In vivo* evaluation of defense-elicitor effect of Regalia® SC applied two days prior to inoculation. A) Percentage of cotyledon necrosis and B) representative pictures of cotyledons from experiment 1. Solid bars are experiment 1 and empty bars are experiment 2. Cantaloupe plants were sprayed with Regalia® SC and later inoculated with 10^6 conidia/ml of *Colletotrichum orbiculare*. Five days post-inoculation the percentage of cotyledons necrosis was determined. Error bars indicate standard errors. Means values followed by the same letter do not differ significantly according to Fisher's least significant difference ($P = 0.05$).


A
Sonata®
% Mycelial growth inhibition
0
20
40
60
80
100
100
150
200
250
AZ
Treatments (µg/ml)


B
Serenade Max®
% Mycelial growth inhibition
0
20
40
60
80
100
100
150
200
250
AZ
Treatments (µg/ml)

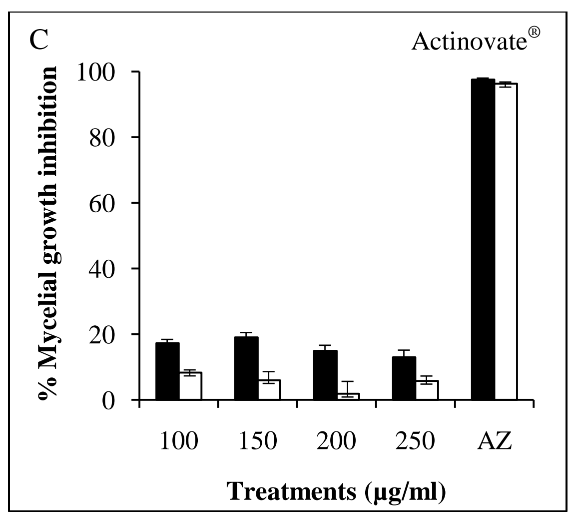

C
Actinovate®
% Mycelial growth inhibition
0
20
40
60
80
100
100
150
200
250
AZ
Treatments (µg/ml)

Figure 2.8. *In vitro* antifungal activity of biological-based products. A) Sonata® (*Bacillus pumilus* QST 2808), B) Serenade Max® (*Bacillus subtilis* QST 713) and C) Actinovate® *Streptomyces lydicus* (WYEC 108). Solid bars are experiment 1 and empty bars are experiment 2. Quadris® (AZ) was used as a positive control in all experiments at concentration of 2 µg/ml. The percent of mycelial growth inhibition (mgi) related to the control (non-treated conidia) was determined using the absorbance ($OD_{450\ nm}$) after 48 h of incubation. The mgi was calculated using the following formula: mgi = $(1-A_n/A_0)*100$; where A_n is the absorbance of each treatment and, A_0 is the absorbance of the control (non-treated conidia). Error bars indicate standard errors.

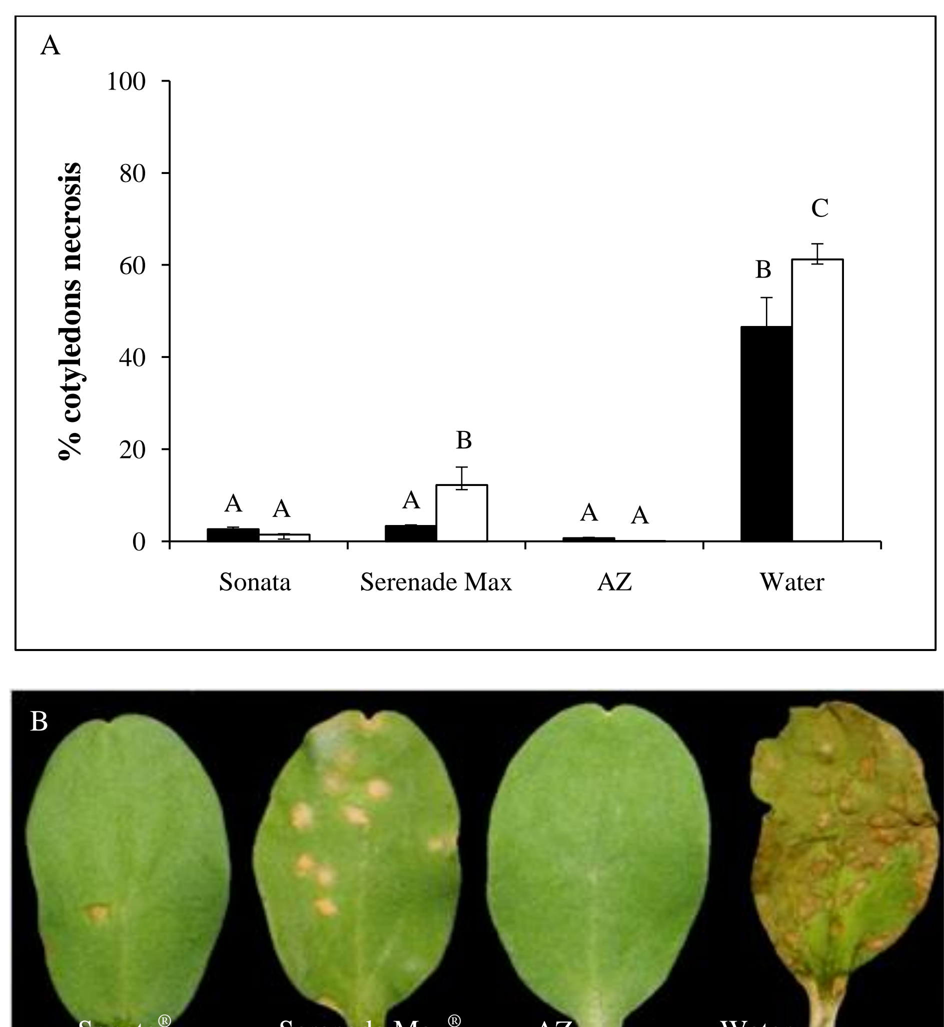


Figure 2.9. *In vivo* evaluation of plant biological-based extracts. A) Percentage of cotyledon necrosis and B) representative pictures of cotyledons from experiment 2. Solid bars are experiment 1 and empty bars are experiment 2. Cantaloupe plants were sprayed with Sonata® (*Bacillus pumilus* QST 2808) and Serenade Max® (*Bacillus subtilis* QST 713) and later inoculated with 10^6 conidia/ml of *Colletotrichum orbiculare*. Five days post-inoculation the percentage of cotyledon necrosis was determined. Error bars indicate standard errors. Mean values followed by the same letter do not differ significantly according to Fisher's least significant difference ($P = 0.05$).

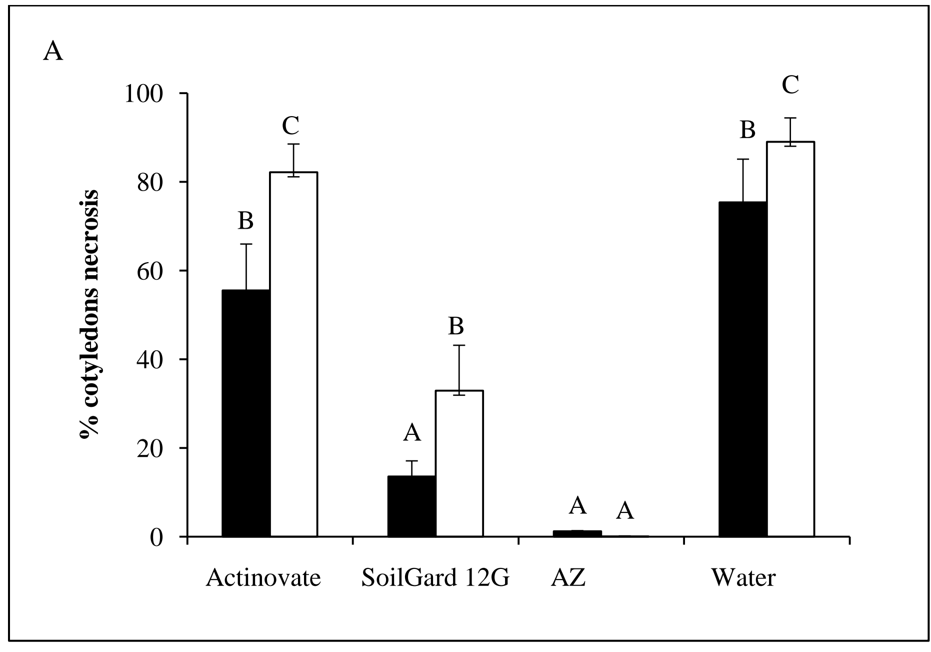
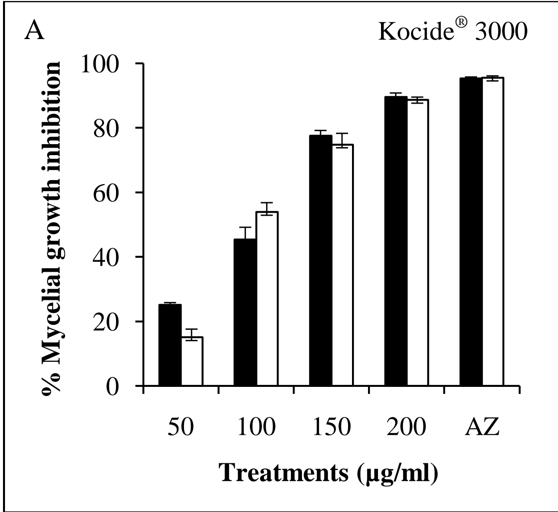
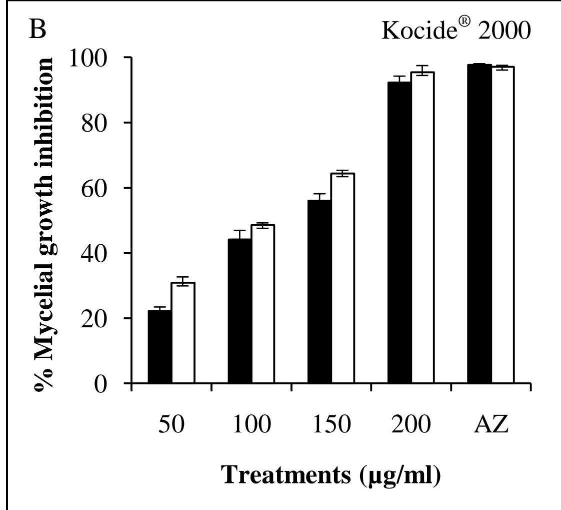
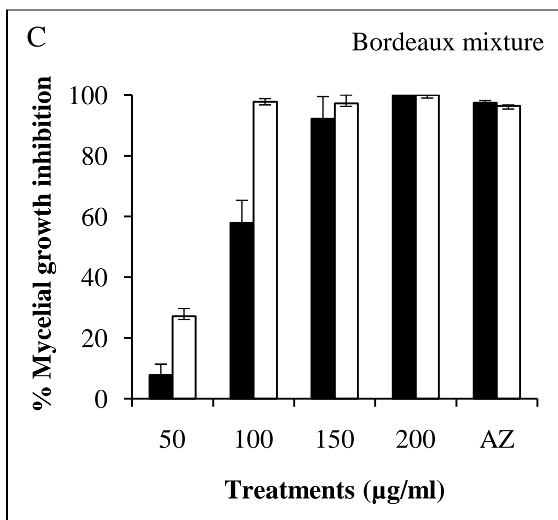


Figure 2.10. *In vivo* evaluation of plant biological-based extracts. A) Percentage of cotyledon necrosis and B) representative pictures of cotyledons from experiment 1. Solid bars are experiment 1 and empty bars are experiment 2. Cantaloupe plants were sprayed with Actinovate® and SoilGard™12G® and later inoculated with 10^6 conidia/ml of *Colletotrichum orbiculare*. Five days post-inoculation, the percentage of cotyledon necrosis was determined. Error bars indicate standard errors. Mean values followed by the same letter do not differ significantly according to Fisher's least significant difference ($P = 0.05$).







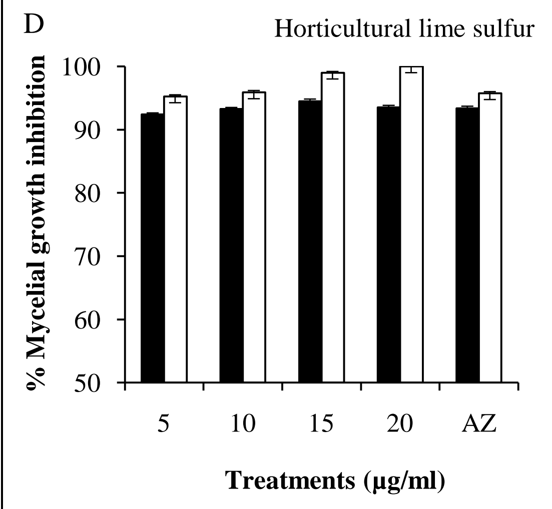


Figure 2.11. *In vitro* antifungal activity of copper-based products and horticultural lime sulfur. A) Kocide® 3000, B) Kocide® 2000, C) Bordeaux mixture and, D) horticultural lime sulfur. Solid bars are experiment 1 and empty bars are experiment 2. Quadris® (AZ) was used as a positive control in all experiments at concentration of 2 µg/ml. The percent of mycelial growth inhibition (mgi) related to the control (non-treated conidia) was determined using the absorbance ($OD_{450\ nm}$) after 48 h of incubation. The mgi was calculated using the following formula: mgi = $(1- A_n/A_0)*100$; where A_n is the absorbance of each treatment and, A_0 is the absorbance of the control (non-treated conidia). Error bars indicate standard errors.

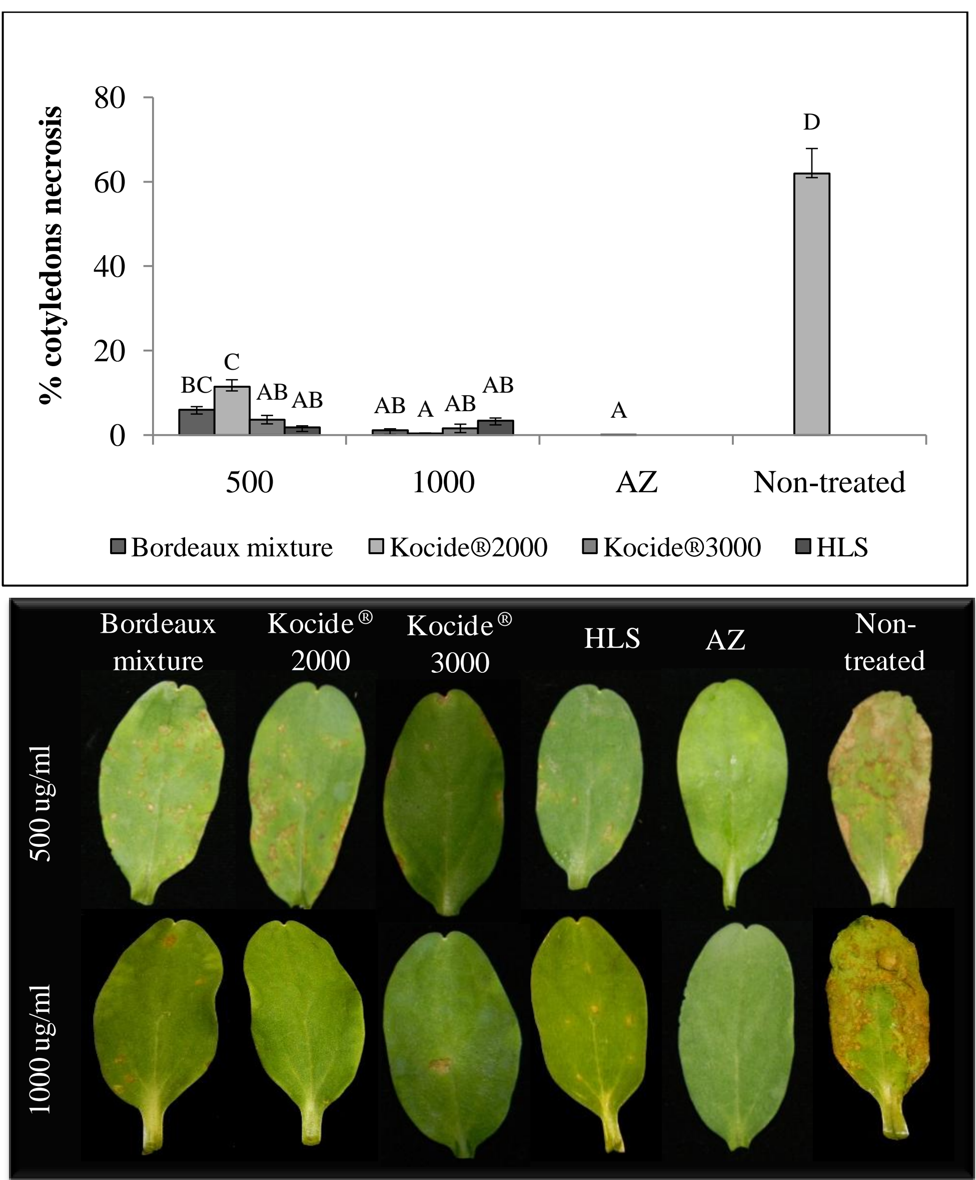


Figure 2.12. *In vivo* evaluation of copper-based products and horticultural lime sulfur. A) Percentage of cotyledon necrosis of plants treated with Kocide® 3000, Kocide® 2000, Bordeaux mixture and horticultural lime sulfur and B) representative pictures of cotyledons from experiment 1. Cantaloupe plants were sprayed with the copper-based products and horticultural lime sulfur and later inoculated with 10^6 conidia/ml of *Colletotrichum orbiculare*. Five days post-inoculation the percentage of cotyledons necrosis was determined. Means values followed by the same letter do not differ significantly according to Fisher's least significant difference (P = 0.05).

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CHAPTER 3

EVALUATION FOR SYNERGISTIC INTERACTIONS OF CHITOSAN AND OMRI-CERTIFIED MATERIALS AGAINST *COLLETOTRICHUM ORBICULARE*

INTRODUCTION

Three responses are possible when two or more pesticides are combined: synergism, antagonism and additivity. Synergism is defined as the combined action of two or more pesticides in which the control provided by their joint application is greater than the predicted control (Couch and Smith, 1991; Gisi, 1996). The control provided by the joint application of two or more pesticides which is less than the predicted control is defined as antagonism, while the control provided by the co-operation of two or more pesticides which is equal to the response predicted is defined as additivity (Couch and Smith, 1991). Synergistic interactions are affected by the sensitivity of the target organism to the fungicides, the ratio of the components in the mixture and their modes of action (Gisi, 1996). Synergism has been detected between fungicides with similar as well as different modes of action (De Waard, 1996; Burpee and Latin, 2008). Combinations of fungicides with different modes of action may affect the target organism at different stages of the life cycle or at different biochemical pathways, which might result in a synergistic interaction of the mixture (Gisi, 1996).

Reports of fungicide synergism for control of vegetable diseases using certified organic fungicides are limited. Combinations of copper sulfate and chitosan showed moderate control of late blight on detached leaves compared with untreated leaves, but whether the response was synergistic or additive was not addressed in that paper

(Hadwiger and McBride, 2006). In field experiments, combinations of Sonata® with Kocide® 2000 showed more early blight disease severity than Sonata® or Kocide® 2000 alone (Wszelaki and Miller, 2005). This suggests the possibility of antagonistic interactions between these two OMRI-certified materials extensively used by organic growers to manage fungal disease in vegetables, although those experiments were not designed to test for synergism or antagonism.

With the increased concern about the potential buildup of copper in the soil to levels that are potentially toxic to plants, soil fauna, and soil microbiota as well as to aquatic ecosystems receiving runoff from treated fields (Van Zwieten et al., 2004; Streit, 1984; Wszelaki and Miller, 2005; EPA, 2006), testing for synergism to reduce the amount copper applied to organic and conventional farms is a much-needed area of investigation. Chitosan, a deacetylated form of chitin, is biodegradable, environmentally safe and non-toxic to higher animals (Guerrero et al., 2007), and has shown antifungal activity against several plant pathogens (Muñoz et al., 2009; Stössel and Leuba, 1984; El Ghaouth et al., 1992; El Ghaouth et al., 1994). Thus, the objective of this research was to test for synergism between chitosan and copper-based products and OMRI-certified materials *in vitro* using a colorimetric assay.

MATERIALS AND METHODS

Materials selected for synergistic interactions assessment

Consistent with published guidelines (Couch and Smith, 1991; Burpee and Latin, 2008; Gisi, 1996), all materials were tested at concentrations that provided less than 70 % of mycelial growth inhibition (mgi) in the *in vitro* studies previously performed (Chapter

2). The colorimetric assay described in Chapter 2 was used to measure the mycelial growth of *Colletotrichum orbiculare* (Berk. & Mont.) Arx (syn. *C. lagenarium* (Pass) Ellis & Halst.) in each combination tested.

Combinations tested *in vitro* included:

1. Kocide® 2000 and chitosan #2
2. Kocide® 3000 and chitosan #2
3. Regalia® SC and chitosan #2
4. Sonata® and Kocide® 2000
5. Sonata® and Kocide® 3000
6. Regalia® SC and Sonata®
7. Regalia® SC and Kocide® 2000
8. Regalia® SC and Kocide® 3000
9. Two bicarbonate salts, $KHCO_3$ and $NaHCO_3$

Combinations tested *in vivo* included:

1. Kocide® 2000 and chitosan #2
2. Sonata® and Trilogy®.

Assessment of fungicide synergism

Using the colorimetric assay described in Chapter 2 and one isolate of *C. orbiculare*, experiments were performed to test for synergistic suppression of mycelial growth with mixtures of selected materials. The Abbott formula, % control exp = A + B – (AB/100) in which A and B are the control levels given by the single fungicides, was used to estimate the expected efficacy of the mixtures (Gisi 1996). The actual percentage control was determined using the following formula: mgi = 100*[1- (A_n/A_0)]; where A_n is

the absorbance of the sample and A_0 is the absorbance of the control (non-treated conidia suspended in potato dextrose broth [PDB]). For the *in vivo* experiments, cantaloupe (*Cucumis melo* cultivar 'Edens Gem OG') plants were grown and fertilized as described in Chapter 2. Ten day-old seedlings with cotyledons fully expanded were sprayed until runoff with the materials to be evaluated using a hand sprayer. After the plants dried, 3 ml of a 10^6 spore/ml suspension of *C. orbiculare* were sprayed over the cotyledons using a hand sprayer. Inoculated plants were placed in a moist chamber (100% relative humidity) for 24 h (Jeun et al., 2003). After incubation, plants were removed from trays and randomized using a completely randomized block design in the greenhouse. Disease symptoms were scored 4-6 days post-inoculation by harvesting cotyledons and capturing digital images of these using a *Nikon* COOLPIX L110. Each picture was analyzed for disease severity using Assess Image Analysis Quantification Software 2.0. Ten plants per treatment were used and the experiments were done twice.

Analysis of variance and comparisons between the actual and expected control were performed using INFOSTAT Statistical Software Version 2004 (InfoStat, FCA, Córdova Argentina). *In vitro* experiments were performed twice with four replicates per treatment. For the *in vivo* experiments, ten plants per treatment were used and the experiments were done twice.

RESULTS

The principal response obtained in mixtures between Chitosan #2 and OMRI-certified materials was antagonism. Combinations between Chitosan #2 and the two copper-based products, Kocide® 2000 and Kocide® 3000, showed consistent antagonistic

effects in both experiments at chitosan concentrations of 50 μg/ml (Tables 3.1 and 3.2). At chitosan concentrations of 20 μg/ml, results were variable between experiments, although none were synergistic. Similarly, combinations of Chitosan #2 and Regalia® SC showed antagonistic responses at chitosan concentrations of 50 μg/ml in both experiments, whereas at chitosan concentrations of 20 μg/ml, results varied across experiments, although none were synergistic (Table 3.3).

Additivity was the main response obtained in combinations of Sonata® with Kocide® 2000 and Kocide® 3000, but antagonism and one instance of synergism were also observed (Table 3.4 and 3.5). Synergism was observed between Sonata® at 20 μg/ml combined with Kocide® 3000 at 10 μg/ml, although not consistently between experiments. Antagonism was obtained when Sonata® was combined with Kocide® 2000 at rates of 10 μg/ml + 20 μg/ml, 20 μg/ml + 10 μg/ml and 20 μg/ml + 20 μg/ml in the second experiment, whereas additivity was the response obtained with the same concentrations in the first experiment.

Combinations of Sonata® and Regalia® SC showed antagonistic, additive and synergistic responses (Table 3.6). Synergism was obtained at combination rate of 20 μg/ml + 50 μg/ml (Sonata® and Regalia® SC, respectively) in both experiments, the only product/rate combination in our studies where synergism occurred in both repetitions of the experiment.

Antagonism was the principal response obtained when Regalia® SC was combined either with Kocide® 2000 or Kocide® 3000 (Tables 3.7 and 3.8). Synergism was observed between Regalia® SC and Kocide® 3000 at the two combination rates (20

μg/ml + 20 μg/ml) and (20 μg/ml + 50 μg/ml) in the first experiment while in the second experiment the same rates in combination produced antagonism.

The only response obtained by combinations of bicarbonate salts ($KHCO_3$ and $NaHCO_3$) was antagonism. The response was consistent in both experiments performed (Table 3.9).

Results of both repetitions of the *in-vivo* experiments were similar, thus results of only one representative experiment are presented. The combination of Chitosan #2 and Kocide® 2000 was antagonistic when tested *in vivo*. No difference in disease severity was observed between Kocide® 2000 and the mixture of Chitosan #2/Kocide® 2000 (Fig. 3.1). However, the combination of Chitosan #2 and Kocide® 2000 showed less disease than Chitosan #2 and the water control. *In vivo* results from the combination of Sonata® and Trilogy® showed an antagonistic response (Fig. 3.2).

DISCUSSION

Synergism is a valuable phenomenon when it occurs in fungicide mixtures, but in the present study the main response obtained with mixtures of materials suitable for disease control on organic farms was antagonism. Only a limited number of mixtures showed synergistic interactions but even in those cases, the effect was rarely consistent between experiments.

Combinations between chitosan the copper-based products Kocide® 2000 and Kocide® 3000 showed antagonistic interactions in almost all combinations tested (Table 1-2). The nature of this antagonistic response may be due to the chelating properties of chitosan with respect to metals. Chitosan is a complex of highly basic polysaccharides

with reactive amino groups and reactive hydroxyl groups which are involved in several chemical processes (Dutta et al., 2004). It has been established that the most stable interaction of Cu^{2+} with chitosan is at the amino site (Terreaux et al., 2006), resulting in more ordered supramolecular structures (Kolyadina et al., 2005). One of the hypotheses to explain the mode of action of chitosan against fungi is that the positively charged amino group of chitosan interacts with the negatively charged residues of macromolecules exposed on the fungal cell wall, causing leakage of intracellular electrolytes and proteinaceous constituents from the cells (Bautista et al., 2006; Stoseel and Leuba, 1986). Therefore, we can hypothesize that if chitosan is mixed with copper, it will bind to the amino groups in the chitosan molecule and the ability of chitosan to interact with negatively charged residues in the fungal cell wall will be affected, resulting in a reduction in the antifungal properties of chitosan as seen in the present study. In addition it has been proposed that copper ions are capable of penetrating fungal spores and inhibiting enzymatic reactions, causing membrane damage on the fungal cell wall that results in leakage of metabolites from fungal cells (Vidhyasekaran, 2004). If Cu^{2+} ions are bound to the amino groups of chitosan, its antifungal properties might also be reduced by not been available to penetrate the fungal cell. The *in vitro* and *in vivo* results presented here clearly show antagonism between chitosan and copper, but our experiments were not designed to test this hypothesis as to mechanism of action. Future investigations on the detailed mechanism of these combinations against fungal pathogens are needed.

Mixtures between Chitosan #2 and Regalia® SC also resulted in antagonistic responses. Regalia® SC is a plant-based extract from the giant knotweed plant

(*Reynoutria sachalinensis*) which, according to the label, activates the plant's natural defense mechanism against fungi. In a previous chapter we showed that this material also has antifungal properties against *Colletotrichum orbiculare*. How these two products interact to result in an antagonistic response is unknown and requires more detailed study.

There are limited data regarding fungicide synergism between OMRI-certified materials. *In vivo*, applications of Sonata® with Kocide® 2000 to manage *Alternaria solani* in tomato had shown more disease in plants treated with this mixture than with either fungicide alone, which suggest an antagonistic interaction (Wszelaki and Miller, 2005). Our *in vitro* results of combinations of Sonata® and Kocide® 2000 showed variable results of additivity and antagonism between experiments. In these combinations we were unable to generate reproducible results, thus more investigation and evaluation of this interaction *in vivo* is necessary to understand the interaction between these fungicides.

The interaction between Sonata® and Regalia® SC (20 µg/ml + 50 µg/ml, respectively) was the only treatment for which synergism was documented in both experiments (Table 6). To our knowledge this is the first time that these fungicides have been shown to interact synergistically. Thus, further evaluation of this combination *in vivo* is necessary to confirm the *in vitro* results presented here.

Combinations between several OMRI-certified materials (Sonata, Kocide 2000, Kocide 3000 and Regalia SC) showed some cases of additivity as well as synergism and antagonism. Several mechanisms may be involved in the responses obtained in our experiments. It has been established that synergistic interactions decrease rapidly with increasing control levels of one of the single components (Samoucha and Cohen, 1984;

Samoucha and Cohen, 1988). This may explain the response obtained in some of the combinations like Regalia® SC and Kocide® 3000, where the single action of Kocide® 3000 is higher than Regalia® SC. It has been proposed that the presence of one fungicide might affect the translocation of the other fungicide or enhance the degradation of the other component in the mixture (Gisi, 1996). Studies on tomato plants revealed a degradation of cymoxanil fungicide in plants treated with a mixture of oxadixyl and cymoxanil that was not observed on plants treated with cymoxanil alone (Cohen and Gisi, 1993). More studies are necessary to understand the possible mechanism involved in the fungicide interactions.

Results of this study suggest that organic farmers may not be able to obtain fungicide synergism between OMRI-certified materials to control *C. orbiculare*. To our knowledge this is the most complete and one of the few research efforts done to evaluate OMRI-certified materials for synergistic interactions. However, these results should be confirmed through *in vivo* experiments to draw more accurate conclusions and make further recommendations. Thus, this research will provide the starting point for further investigations in this direction.

Table 3.1. Efficacy of Chitosan #2 and Kocide® 3000 alone and in combination against mycelial growth of *Colletotrichum orbiculare in vitro*.

Treatments	Rate	% control exp. 1 [a]		% control exp. 2 [a]	
		Act.	**Exp.**	**Act.** [a]	**Exp.**
Chitosan	20 µg/ml	15.0	…	7.8	…
	50 µg/ml	59.0	…	59.8	…
Kocide® 3000	20 µg/ml	2.0	…	8.0	…
	50 µg/ml	15.0	…	4.7	…
Chitosan + Kocide® 3000	20 + 20	8.5*	16.9	-3.5ns	15.3
	20 + 50	16.0*	27.4	5.4ns	11.9
	50 + 20	22.0*	60.8	16.4*	63.0
	50 + 50	22.4*	65.3	18.3*	61.7

[a] Percentage control determined using mgi = 100*[1- (A_n/A_0)] where A_n is the absorbance of the sample and A_0 is the absorbance of the control (non-treated conidia suspended in PDB) and expected value using the Abbott formula (%C exp = A + B – (AB/100)) where A and B are the control levels given by the single fungicides. Actual values followed by an * are significantly different (*t* test, $P<0.05$) from expected values. Actual values significantly lower or higher than expected reflect antagonism and synergism, respectively. Additivity is represented by ns (= not significant).

Table 3.2. Efficacy of Chitosan #2 and Kocide® 2000 alone and in combination against *Colletotrichum orbiculare in vitro*.

Treatments	Rate	% control exp. 1 [a]		% control exp. 2 [a]	
		Act.	**Exp.**	**Act.**	**Exp.**
Chitosan	20 ug/ml	14.6	…	1.6	…
	50 ug/ml	58.6	…	61.6	…
Kocide® 2000	20 ug/ml	18.1	…	7.2	…
	50 ug/ml	31.3	…	18.7	…
Chitosan + Kocide® 2000	20 + 20	15.1*	30.0	-2.4 ns	9.0
	20 + 50	16.5*	41.5	12.4*	20.1
	50 + 20	26.3*	66.1	9.6*	64.0
	50 + 50	27.5*	71.5	9.0*	68.7

[a] Percentage control determined using mgi = 100*[1- (A_n/A_0)] where A_n is the absorbance of the sample and A_0 is the absorbance of the control (non-treated conidia suspended in PDB) and expected value using the Abbott formula (%C exp = A + B – (AB/100)) where A and B are the control levels given by the single fungicides. Actual values followed by an * are significantly different (*t* test, $P<0.05$) from expected values. Actual values significantly lower or higher than expected reflect antagonism and synergism, respectively. Additivity is represented by ns (= not significant).

Table 3.3. Efficacy of Chitosan #2 and Regalia® SC alone and in combination against *Colletotrichum orbiculare in vitro*.

Treatments	**Rate**	**% control exp. 1** [a]		**% control exp. 2** [a]	
		Act.	**Exp.**	**Act.**	**Exp.**
Chitosan	20 ug/ml	17.7	…	8.0	…
	50 ug/ml	51.8	…	69.8	…
Regalia® SC	20 ug/ml	-6.1	…	1.0	…
	50 ug/ml	2.9	…	16.1	…
Chitosan + Regalia® SC	20 + 20	1.9*	12.8	7.8ns	8.0
	20 + 50	-0.6ns	20.2	12.3*	22.9
	50 + 20	5.7*	53.2	22.3*	74.7
	50 + 50	4.2*	48.9	20.1*	70.0

[a] Percentage control determined using mgi = 100*[1- (A_n/A_0)] where A_n is the absorbance of the sample and A_0 is the absorbance of the control (non-treated conidia suspended in PDB) and expected value using the Abbott formula (%C exp = A + B – (AB/100)) where A and B are the control levels given by the single fungicides. Actual values followed by an * are significantly different (*t* test, $P<0.05$) from expected values. Actual values significantly lower or higher than expected reflect antagonism and synergism, respectively. Additivity is represented by ns (= not significant).

Table 3.4. Efficacy of Sonata® and Kocide® 3000 alone and in combination against *Colletotrichum orbiculare in vitro*.

		% control exp. 1 [a]		**% control exp. 2** [a]	
Treatments	**Rate**	**Act.**	**Exp.**	**Act.**	**Exp.**
Sonata®	10 ug/ml	-0.9	…	-7.4	…
	20 ug/ml	4.2	…	-1.8	…
Kocide® 3000	10 ug/ml	16.7	…	-3.5	…
	20 ug/ml	18.3	…	-2.3	…
Sonata® + Kocide® 3000	10 + 10	14.9[ns]	15.6	4.8[ns]	-10.8
	10 + 20	11.1[*]	17.2	6.3[ns]	-9.9
	20 + 10	16.3[*]	1.4	1.4[ns]	-5.4
	20 + 20	22.6[ns]	22.2	5.8[ns]	-4.1

[a] Percentage control determined using $mgi = 100*[1- (A_n/A_0)]$ where A_n is the absorbance of the sample and A_0 is the absorbance of the control (non-treated conidia suspended in PDB) and expected value using the Abbott formula (%C exp = A + B – (AB/100)) where A and B are the control levels given by the single fungicides. Actual values followed by an * are significantly different (*t* test, $P<0.05$) from expected values. Actual values significantly lower or higher than expected reflect antagonism and synergism, respectively. Additivity is represented by ns (= not significant).

Table 3.5. Efficacy of Sonata® and Kocide® 2000 alone and in combination against *Colletotrichum orbiculare in vitro*.

Treatments	Rate	% control exp. 1 [a]		% control exp. 2 [a]	
		Act.	**Exp.**	**Act.**	**Exp.**
Sonata®	10 ug/ml	-11.7	…	2.7	…
	20 ug/ml	-20.0	…	5.2	…
Kocide® 2000	10 ug/ml	-0.2	…	8.7	…
	20 ug/ml	2.9	…	20.2	…
Sonata® + Kocide® 2000	10 + 10	-4.7*	-11.8	13.6ns	10.1
	10 + 20	-9.8ns	-8.4	9.6*	22.2
	20 + 10	-3.8ns	-20.6	9.7*	13.7
	20 + 20	2.4ns	-16.7	11.0*	24.7

[a] Percentage control determined using mgi = 100*[1- (A_n/A_0)] where A_n is the absorbance of the sample and A_0 is the absorbance of the control (non-treated conidia suspended in PDB) and expected value using the Abbott formula (%C exp = A + B – (AB/100)) where A and B are the control levels given by the single fungicides. Actual values followed by an * are significantly different (*t* test, *P*<0.05) from expected values. Actual values significantly lower or higher than expected reflect antagonism and synergism, respectively. Additivity is represented by ns (= not significant).

Table 3.6. Efficacy of Sonata® and Regalia® SC alone and in combination against *Colletotrichum orbiculare in vitro*.

		% control exp. 1[a]		% control exp. 2[a]	
Treatments	**Rate**	**Act.**	**Exp.**	**Act.**	**Exp.**
Sonata®	10 µg/ml	4.0	…	12.5	…
	20 µg/ml	5.5	…	14.5	…
Regalia® SC	20 µg/ml	-8.8	…	1.3	…
	50 µg/ml	2.8	…	14.9	…
Sonata® + Regalia® SC	10 + 20	-11.5*	-4.5	9.5*	13.7
	10 + 50	4.3ns	6.7	25.0ns	25.5
	20 + 20	11.2ns	-2.8	25.8*	15.5
	20 + 50	32.5*	8.2	50.3*	27.1

[a] Percentage control determined using mgi = 100*[1- (A_n/A_0)] where A_n is the absorbance of the sample and A_0 is the absorbance of the control (non-treated conidia suspended in PDB) and expected value using the Abbott formula (%C exp = A + B – (AB/100)) where A and B are the control levels given by the single fungicides. Actual values followed by an * are significantly different (*t* test, $P<0.05$) from expected values. Actual values significantly lower or higher than expected reflect antagonism and synergism, respectively. Additivity is represented by ns (= not significant).

Table 3.7. Efficacy of Regalia® SC and Kocide® 2000 alone and in combination against *Colletotrichum orbiculare in vitro*.

		% control exp. 1 [a]		**% control exp. 2** [a]	
Treatments	**Rate**	**Act.**	**Exp.**	**Act.**	**Exp.**
Regalia®SC	20 ug/ml	-17.5	…	30.9	…
	50 ug/ml	20.8	…	24.8	…
Kocide® 2000	20 ug/ml	9.0	…	20.3	…
	50 ug/ml	14.1	…	32.3	…
Regalia®SC + Kocide® 2000	20 + 20	-5.1[ns]	-7.0	17.0[*]	45.0
	20 + 50	12.6[ns]	-1.0	23.2[*]	53.2
	50 + 20	-4.0[*]	27.9	18.9[*]	40.2
	50 + 50	16.0[*]	31.9	24.9[*]	48.9

[a] Percentage control determined using mgi = 100*[1- (A_n/A_0)] where A_n is the absorbance of the sample and A_0 is the absorbance of the control (non-treated conidia suspended in PDB) and expected value using the Abbott formula (%C exp = A + B – (AB/100)) where A and B are the control levels given by the single fungicides. Actual values followed by an * are significantly different (*t* test, $P<0.05$) from expected values. Actual values significantly lower or higher than expected reflect antagonism and synergism, respectively. Additivity is represented by ns (= not significant).

Table 3.8. Efficacy of Regalia® SC and Kocide® 3000 alone and in combination against *Colletotrichum orbiculare in vitro*.

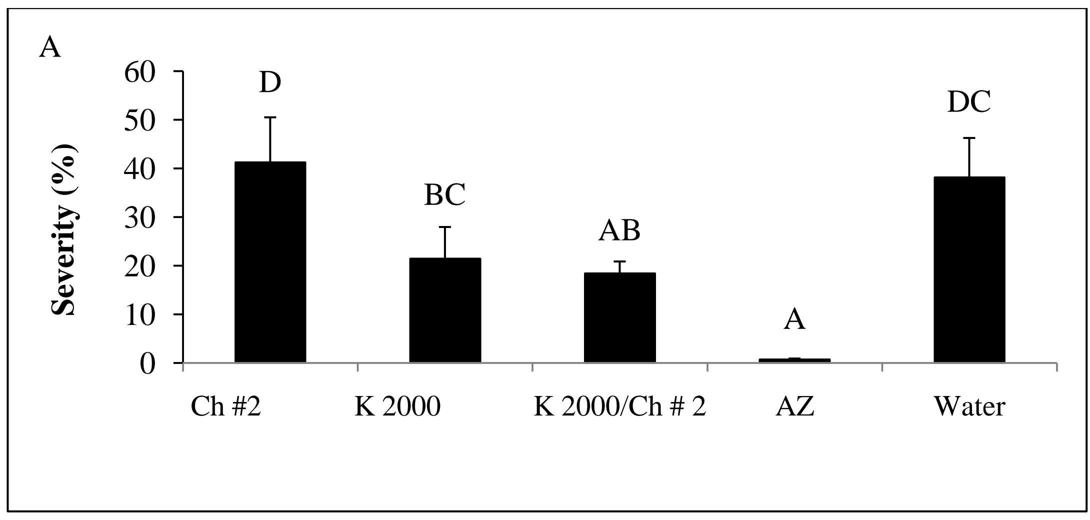
Treatments	Rate	% control exp. 1[a]		% control exp. 2[a]	
		Act.	Exp.	Act.	Exp.
Regalia®SC	20 ug/ml	-6.1	…	8.6	…
	50 ug/ml	8.2	…	12.7	…
Kocide® 3000	20 ug/ml	10.6	…	32.7	…
	50 ug/ml	18.7	…	31.3	…
Regalia®SC + Kocide® 3000	20 + 20	10.8*	5.1	26.1*	38.5
	20 + 50	16.8*	13.9	29.0*	37.1
	50 + 20	9.3*	17.8	24.6*	41.3
	50 + 50	21.6*	25.2	29.2*	40.0

[a] Percentage control determined using mgi = 100*[1- (A_n/A_0)] where A_n is the absorbance of the sample and A_0 is the absorbance of the control (non-treated conidia suspended in PDB) and expected value using the Abbott formula (%C exp = A + B – (AB/100)) where A and B are the control levels given by the single fungicides. Actual values followed by an * are significantly different (*t* test, $P<0.05$) from expected values. Actual values significantly lower or higher than expected reflect antagonism and synergism, respectively. Additivity is represented by ns (= not significant).

Table 3.9. Efficacy of potassium bicarbonate and sodium bicarbonate alone and in combination against *Colletotrichum orbiculare in vitro*.

		% control exp. 1[a]		% control exp. 2[a]	
Treatments	**Rate**	**Act.**	**Exp.**	**Act.**[a]	**Exp.**
Potassium bicarbonate (PB)	0.025 M	-16.3	…	-15.8	…
	0.050 M	44.3	…	-35.7	…
Sodium bicarbonate (SB)	0.025 M	44.3	…	43.9	…
	0.050 M	64.8	…	69.9	…
PB + SB	0.025 + 0.025	24.9*	41.0	-20.0*	33.0
	0.025 + 0.050	55.1*	61.3	-27.0*	64.6
	0.050 + 0.025	18.9*	63.8	-10.0*	24.9
	0.050 + 0.050	45.3*	79.0	35.3*	59.2

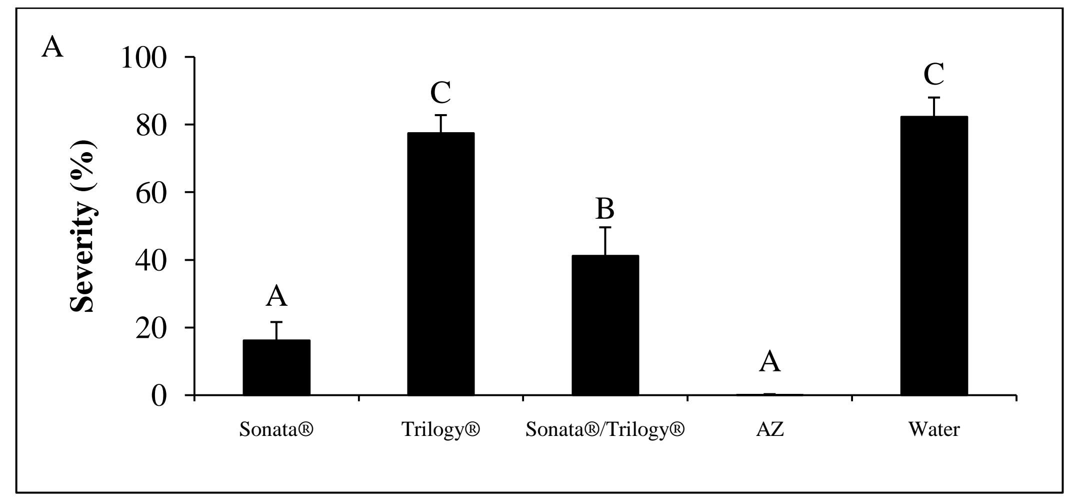
[a] Percentage control determined using mgi = 100*[1- (A_n/A_0)] where A_n is the absorbance of the sample and A_0 is the absorbance of the control (non-treated conidia suspended in PDB) and expected value using the Abbott formula (%C exp = A + B – (AB/100)) where A and B are the control levels given by the single fungicides. Actual values followed by an * are significantly different (*t* test, $P<0.05$) from expected values. Actual values significantly lower or higher than expected reflect antagonism and synergism, respectively. Additivity is represented by ns (= not significant).



B Treatment	Control %	
	Actual[a]	Expected[b]
Kocide® 2000	44.0	…
Chitosan # 2	-8.0	…
Kocide® 2000/Chitosan # 2	18.5	25.2*

[a] Percent control determined using mgi = 1- (A_n/A_0)*100 and expected value using the Abbott formula (%C exp = A + B – (AB/100)).
[b] According to a *t* test, expected values followed by * are statically significant from actual values at $P<0.05$.

Figure 3.1. Anthracnose control by the combination of Chitosan #2 and Kocide® 2000 *in vivo*. A) Percentage cotyledon necrosis, B) assessment of synergism among Chitosan #2 and Kocide® 2000 and, C) representative pictures of cotyledons. Cantaloupe plants were sprayed with Chitosan #2 and Kocide® 2000 and later inoculated with 10^6 conidia/ml of *Colletotrichum orbiculare*. Chitosan #2 and Kocide® 2000 were tested at 100 µg/ml. Five days post-inoculation the percentage of cotyledon necrosis was determined. Bars indicate standard errors. Means values followed by the same letter do not differ significantly. K 2000 = Kocide® 2000, Ch #2 = Chitosan #2, AZ = axozystrobin.



B Treatment	Control %	
	Actual[a]	Expected[b]
Sonata®	80.4	…
Trilogy®	5.8	…
Sonata®/Trilogy®	49.9	79.2*

[a] Percent control determined using mgi = 1- (A_n/A_0)*100 and expected value using the Abbott formula (%C exp = A + B – (AB/100)).
[b] According to a *t* test, expected values followed by * are statically significant from actual values at $P<0.05$.

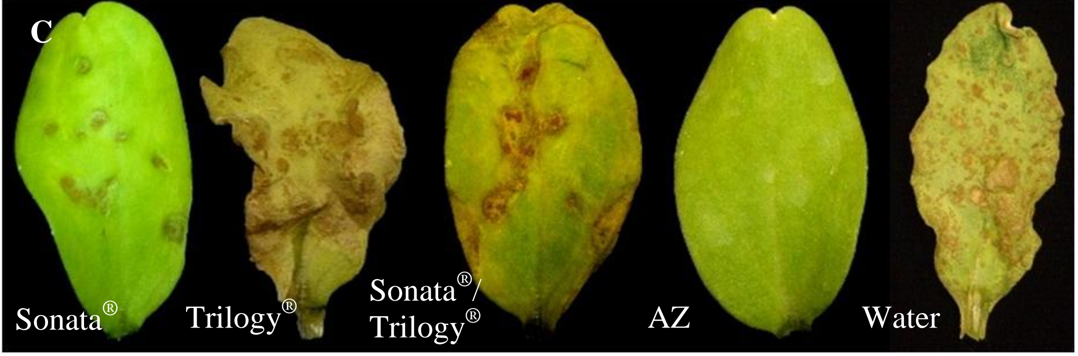


Figure 3.2. Anthracnose disease control by the combination of Sonata® and Trilogy® *in vivo*. A) Percentage of cotyledon necrosis, B) assessment of synergism among Sonata® and Trilogy® and, C) representative pictures of cotyledons. Cantaloupe plants were sprayed with Sonata® and Trilogy® and later inoculated with 10^6 conidia/ml of *Colletotrichum orbiculare*. Sonata® and Trilogy® were tested at 100 µg/ml. Five days post-inoculation the percentage of cotyledons necrosis was determined. Bars indicate standard errors. Means values followed by the same letter do not differ significantly.

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CHAPTER 4

EFFICACY OF OMRI-CERTIFIED FUNGICIDES AND CHITOSAN FOR MANAGING EARLY BLIGHT AND SEPTORIA LEAF SPOT IN TOMATO

INTRODUCTION

In Kentucky, fresh-market tomatoes are among the most valuable vegetables grown, with approximately 1,000 acres cultivated for wholesale and farmers' markets (Coolong at al., 2009). Given this region's warm and humid summers, foliar fungal diseases are among the main production constraints favored by such weather conditions. Early blight, caused by *Alternaria solani* (Ellis & Martin) Jones & Grout, and Septoria leaf spot, caused by *Septoria lycopersici* Speg., are the most common and important foliar diseases of tomatoes (*Lycopersicon esculentum* Mill.) in Kentucky (Coolong et al., 2008). Both diseases are favored by humid, rainy weather and mild temperatures between 24-29˚C (Rotem, 1994; Jones et al., 1991; Coolong et al., 2008; Nash and Gardner, 1988). Early blight typically begins in the lower canopy and progresses to the middle and upper canopy as fruit set occurs while Septoria leaf spot usually appears on the lower leaves after the fruits are set (Rotem, 1994; Jones et al., 1991; Madden et al., 1978; Coolong et al., 2008).

The management of necrotrophic foliage-attacking fungi is a challenge due to their wide host ranges, capacity to growth as saprophytes and the ability to attack young, weak or senescent tissue (Schumann and D'Arcy, 2006). These necrotrophic fungi are controlled primarily through crop rotation, the use of resistant varieties, pathogen-free

seed, and fungicide applications (Agrios, 2005; Madden et al., 1978; Rowell et al., 2006-07, Jones et al., 1991). However, the majority of tomato cultivars currently grown are susceptible to Septoria leaf spot and early blight. Hence, management of these diseases often depends on the use of fungicides such as strobilurins and chlorothalonil to prevent yield losses (Rowell et al., 2010-11). For certified organic growers, these synthetic fungicides are not permitted. Only OMRI (Organic Material Review Institute) listed products are allow for use in certified organic farms for fresh market tomatoes. Several products are registered and approved for managing foliar disease in organic agriculture in the U.S. However, data on the efficacy of these products for managing foliar necrotrophic fungi like *A. solani* and *S. lycopersici* under field conditions are limited (Wszelaki and Miller, 2005; Zitter et al., 2005; Seaman, 2004). Also, experiments performed under one set of environmental conditions are often not predictive of results in other environments. With the rapid increase in organic food production and the relative lack of research on this topic, the objective of this project was to test the efficacy of OMRI-certified materials as well other potentially certifiable materials to manage early blight and Septoria leaf spot under field conditions.

MATERIALS AND METHODS

Field plots. Field trials were conducted at the University of Kentucky Horticultural Research Farm on a Maury silt loam soil type. Plots were established in land in transition from conventional to organic practices. The cultivar used was 'Paragon OG' a determinate type with resistance to *Fusarium* wilt races 1 and 2 and *Verticillium* wilt (Johnny's Selected Seeds, Winslow, ME). Five-week old seedlings were transplanted

into the field on 18 June 2009 and 2010 for experiments I and II, respectively, and on 5 July 2010 for experiment III. For all field experiments a randomized complete block with four replicates was used (Appendix 2 Figure 1). Plots consisted of single rows 6.1 m long covered with black plastic mulch and with 46 cm between plants and 3 m between rows, with drip irrigation and fertigation; plants were staked and trellised. Pre-plant fertilization was done with nitrogen at 5.5 kg N per acre. Fertigation was at 14-day intervals using ½ gallon/A of Phytamin® Fish Plus 5-1-1 (California Organic Fertilizers, Inc.™, Hanfor, CA.). All materials tested were applied in a spray volume of 40 gal/A and increased up to 100 gal/A through the season to increase foliar coverage as plants grew. All treatments were sprayed at 7-day intervals using a CO_2 backpack sprayer fitted with hollow-cone nozzles (R&D Sprayer, Bellspray, Inc.) operating at 276 kPa. The positive, Quadris® (azoxystrobin) was used in a 7-day interval to fit it in the spray program, taking in consideration the possible development of resistance. The sprayer was calibrated to deliver 0.2 liter in a 30.5 m^2 (plot size). A total of 0.4 liters were applied to each plot (0.2 liter on each side of the plot) during the first three applications using a single hollow-cone nozzle spraying a 45.7 cm swath. From the fourth application through the last, 0.8 liters were applied to each plot (0.4 liters per side) using two hollow-cone nozzles spraying a 66.0 cm swath and spaced at 45.7 cm. For all experiments, the initial application was made three weeks after transplanting and continued throughout the season for a total of 9 (experiments I and II) or 8 (experiment III) applications. Table 4.1 describes all treatments and the rates applied in each experiment (Appendix 2 Table 1). Weeds were controlled using a small tractor pulled field cultivator and by manual removal throughout the season in all experiments. Each plot consisted of thirteen to

fourteen plants from which the seven plants in the middle of the plot were selected for assessments of disease severity and yield. Harvest of fresh ripe tomatoes was manually done four times in experiments I and III and five times in experiment II. After harvest, tomato fruits were separated into marketable (free of rot, adequate size) and non-marketable categories and weighed. Yield data were analyzed by analysis of variance and the least significant differences (LSD) to separate means ($P < 0.05$) using INFOSTAT Statistical Software Version 2004 (InfoStat, FCA, Córdova Argentina).

Disease severity assessment and data analysis. Severity of foliar necrosis caused by early blight and Septoria leaf spot was evaluated in all experiments. Both diseases together were evaluated as foliar necrosis. Disease severity was assessed by canopy position (lower, middle and upper canopy) and then averaged for the whole plot. Disease severity was estimated visually (four times in experiment I and three times in experiment II and III) at each of three canopy positions in each plot. A scale from 0 to 7 (0= 0%, 1= 1 - 14 %, 2= 15 – 29 %, 3= 30 – 49 %, 4= 50 – 69 %, 5= 70 – 84 %, 6= 85 – 95 % and 7= >96%) was used to estimate disease severity (Little and Hills, 1978). The standardized area under the disease progress curve (sAUDPC) was determined to express the cumulative disease severity occurring over the 43-, 49-, and 74-day period of experiment I, II, and III, respectively, using the mid-point or trapezoidal method (Madden et al., 2007). The data for sAUDPC and final disease severity were analyzed by ANOVA and treatments were compared with the LSD test. Weather data (temperature, precipitation and relative humidity) was measured daily by the University of Kentucky Agricultural Weather Center (Table 4.2).

RESULTS

Field trial I. During the field trial of 2009 (experiment I), based on symptoms and microscopic observations, the predominant disease observed was Septoria leaf spot and less than 10% of the foliar necrosis observed on plants was caused by early blight. Septoria leaf spot symptoms were first detected seven weeks after transplanting, when the tomato fruits were already set, while early blight symptoms were initially observed 10 weeks after transplanting (Fig. 4.1A), when fruits were ripening. An epidemic of bacterial spot (*Xanthomonas campestris pv. vesicatoria*), confirmed by PCR (*data not shown*) using previously published primers (Obradovic et al., 2004), was observed during the final few weeks of the growing season, affecting mainly tomato fruits and therefore, marketable yields.

Among the treatments evaluated, Sonata®, ammonium bicarbonate, Trilogy®, and acid-soluble chitosan provided no disease control (Table 4.3). In contrast, copper-based products (Bordeaux mixture and Kocide® 2000), provided a significant (P = 0.0001) reduction in disease severity compared with the unsprayed control; efficacy was equivalent to that provided by the positive control (Quadris® 50WG). In this and the other trials reported below, plants treated with Bordeaux mixture had leaf curling symptoms that were not observed in any other treatments. This physiological effect has been reported previously on tomato plants treated with Bordeaux mixture and may be associated with the use of this material under warm conditions (Wszelaki and Miller, 2005). Regardless of the disease control provided by the copper-based products and the positive control, no difference (P = 0.7421) was observed in total yield or marketable yield among treatments. Across all treatments, more than 40 % the total yields were

classified as unmarketable due to symptoms of disease on fruit (predominantly bacterial spot).

Field trial II. In the second field trial, the overall disease pressure was lower than in the first trial (Table 4.4; Fig. 4.1B). The lower disease pressure may have been caused by the unfavorable weather conditions during the growing season (Table 4.2). Both diseases were initially detected after fruit set, 48 days after transplanting, in the lower canopy position, resulting in a 49-day epidemic. As in the first experiment, microscopic examination of symptomatic leaves indicated that Septoria leaf spot was the predominant disease, with early blight causing approximately 20% of the lesions observed on plants.

As observed in the first trial, none of the OMRI-certified treatments (Sonata®, Serenade Max®, Trilogy®, Regalia® and lime sulfur) except the copper-based products provided a significant (P < 0.05) decrease in disease severity (Table 4.4). Likewise, water-soluble chitosan failed to provide significant disease control. As in experiment I, even with a significant reduction in disease severity provided by the copper-based products, no effect on yield and marketable yield (P>0.05) between treatments was observed.

Field trial III. In the third field trial, both diseases (Septoria leaf spot and early blight) were detected four weeks after transplanting (Figure 4.1C), resulting in a 74-day epidemic. Both diseases were present in similar proportions on symptomatic leaves, but they progressed slowly and remained at relatively low levels throughout the growing season. Concentric brown rings characteristic of early blight and small water-soaked spots with dark brown margins characteristic of Septoria leaf spot were observed on the same leaves and in coalescent lesions.

In contrast to results obtained during the first two trials, none of the treatments including copper-based products, provided disease control over the unsprayed check in the third trial ($P>0.05$) (Table 4.5). Horticultural lime sulfur had a final disease severity and sAUDPC significantly higher ($P<0.05$) than the unsprayed control (Table 4.5; Appendix 2 Figure 4). Total yields were comparatively lower in this trial than in the previous trials, but marketable yields were higher than the first trial and similar to the second. An improvement in total yield and marketable yield was obtained with spray applications of Serenade Max®, Bordeaux mixture, Regalia®, water-soluble chitosan and lime sulfur compared with the non-treated plants ($P<0.05$).

DISCUSSION

Among the materials tested, we found that copper-based fungicides were the only effective treatments for managing Septoria leaf spot and early blight of tomato in an organic production system. Despite the disease control obtained by copper-based fungicides, no yield improvement was obtained in any of the trials by these products. These copper products included Kocide® 2000, Kocide® 3000 and Bordeaux mixture. In numerous trials testing the efficacy of organic certified materials, copper-based products have provided the best results in terms of disease management but with inconsistent results in yield improvement. For example, in agreement with our results, Seaman et al., (2005) showed that the copper-based fungicide Champion® WP (a.i. copper hydroxide) was the only treatment that significantly reduced foliar symptoms caused by *S. lycopersici* and *A. solani* compared with untreated control in tomato. In that trial, none of the OMRI-certified materials (Plantshield, Mycostop, Trilogy®, CaCO3, SW-3 Seaweed,

Humega) produced a significant reduction in disease. Results similar to ours were reported in an Ohio study, where a significant reduction in disease severity was obtained with Champion® WP but no improvement in tomato yields was observed (Wszelaki et al., 2003). In a study conducted in Iowa, copper fungicide-treated plants had low disease severities, which resulted in a yield increase of over 60% more than the other negative control (Joslin and Taber, 2003). While copper fungicides have often provided better foliar disease control than other OMRI-certified materials in the field, there are instances where disease control provided by copper fungicides is not superior or is inconsistent. Inconsistent results have been obtained with Bordeaux mixtures in trials performed in consecutive years. In the Ohio study mentioned previously (Wszelaki and Miller, 2005), Bordeaux mixture did not provide a significant reduction in disease in 2002 whereas significant disease control was observed with the same product in 2003. In our own trials, no improvement in yields was obtained by Bordeaux mixture in any of the trials. As another example of trial to trial inconsistency, plants treated with Champion WP, which was effective in previous studies (Joslin and Taber, 2003; Wszelaki et al., 2003) showed no differences in disease control compared with the untreated plants (McGrath, 2009).

It is possible that in our trials the reduction in AUDPC and disease severity observed by copper-based fungicide was not sufficient to allow expression of yield benefits. The lack of effect of copper-based fungicides on yields might be related to the timing of the onset of the disease. In the first two trials, in which copper-based fungicides showed significant disease control, the initial symptoms of disease were detected after fruit-set. Previous field trials had showed no effect of fungicide applications for managing early blight or Septoria leaf spot on yields, regardless of disease control and

detection timing (Brammal, 1993; Ferrandino and Elmer, 1992). Septoria leaf spot (the predominantly disease in the first two trials) is commonly first detected after fruit set (Rotem, 1994; Jones et al., 1991; Madden et al., 1978; Coolong et al., 2008). This suggests that when necrotrophic foliar disease develops after fruit set, fungicide application may have little to no effect on yield. Although it is possible that the method used to measured yield didn't allow detect significant differences between treatments. Plants treated with copper based products (Bordeaux mixture, Kocide® 2000 and Kocide® 3000) produced bigger tomatoes compared with the unsprayed plants (*data not shown*). However, the size of the tomatoes was not considered as part of the yield assessment instead we measured the total pounds per plot. It is important to point out that these results might not apply to tomato cultivars with indeterminate growth, which continue growing and setting fruit throughout the growing season until frost, versus the determinate growth tomato used in the present study which produced all their fruit in a relatively short period of time. Furthermore, all three trials were conducted under weather conditions that were not as humid and/or rainy as the most extreme disease-conducive conditions possible in Kentucky's humid climate.

Copper-based products are recommended for managing fungal and bacterial foliar diseases in conventionally and organically produced tomatoes. However, one of the main concerns is the potential buildup of copper in the soil to levels that are potentially toxic to plants, soil fauna and soil microbiota as well as to aquatic ecosystems receiving runoff from treated fields and aquatic animals (Van Zwieten et al., 2004; Streit, 1984; Wszelaki and Miller, 2005; EPA, 2006). According to OMRI, copper fungicides are listed as synthetics and are permitted for use in organic crop production only in a manner that

minimizes copper accumulation in the soil. Nevertheless, the relatively poor efficacy of alternatives to copper may create conflicts for organics producers wanting to control fungal diseases of foliage while protecting their soil from copper accumulation.

None of the materials tested except the copper-based products provided a significant reduction in disease severity in any of the field trials. These include biological-based products (Sonata® and Serenade Max®), plant-based extracts (Regalia®SC and Trilogy®), ammonium bicarbonate, horticultural lime sulfur and chitosan. Biological-based products like Sonata® (ai. *Bacillus pumilis*) and Serenade Max® (ai. *Bacillus subtilis*) have been evaluated alone and in combination with other OMRI-certified materials resulting in no disease control (Wszelaki and Miller, 2005; McGrath and Moyer, 2003). Plants treated with combinations of Sonata® plus Kocide® 2000 showed more damage from early blight than the water control or than each fungicide used alone (Wszelaki and Miller, 2005). In the same trials, Sonata® applied alone showed more foliar damage caused by *A. solani* compared with the water control. In another trial, no effect on Septoria leaf spot severity was obtained by the application of Sonata® alone or in combination with compost tea, as compared with the untreated control (McGrath and Moyer, 2003). Field evaluations of Serenade Max® for disease control have also produced conflicting results. Combinations of Serenade Max® with Champion WP had provided a reduction in disease severity caused by *A. solani* and *S. lycopersici*, compared with the water control, but not using Serenade Max® and Champion WP alone (Wszelaki and Miller, 2005). In another trial, Serenade Max® applied with Kocide® 2000 showed the same levels of early blight as the untreated control (Lewis et al., 2004). A combination of Serenade Max® with Champion WP and

Biotune had no effect on disease severity of early blight (Zitter and Drennan, 2005). Trilogy®, an OMRI-certified material label as fungicide/miticide/insecticide was evaluated in three consecutive trials and showed no effect in disease control in any of the trials presented here. Our results are in agreement with Wszelaki et al., (2002) where Trilogy® showed no effect on early blight and Septoria leaf spot disease control.

In addition to the failure to provide disease control, yield improvement by some of the OMRI-certified materials described above has been lacking or very inconsistent. In the results presented here we were unable to establish any correlation between disease severity and yields. In a two-year study, Sonata® showed as much or more disease than the water control but surprisingly produced higher marketable yields than the water control, (Wszelaki and Miller, 2005). This suggests that Sonata® sometimes improves yields by a mechanism independent of disease control. However, this effect is inconsistent because in all three of our trials, Sonata® showed the same levels of disease as the unsprayed plants and no improvement of yield was obtained. Similarly, Trilogy® also failed to provide either disease control and/or yield improvement in our study or in those of others (Wszelaki and Miller, 2005; Zitter and Drennan, 2005; Lewis et al., 2004).

While some products showed no disease control or yield improvement, others improved yields by an unknown mechanism as seen in our third trial (Table 5). In the presence of low disease pressure during this trial, improvement in total yield and marketable yield was obtained with Serenade Max®, Bordeaux mixture, Regalia®SC, water-soluble chitosan and lime sulfur, although none actually provided measurable disease control. Regalia®SC is labeled as a "plant immune system booster" against fungi

and bacteria, but the role of this plant-based extract in yield improvement beyond disease control has not been investigated. Horticultural lime sulfur also improved yields even though disease severity was actually higher than in the unsprayed control, by some mechanism that we are unable to explain. Chitosan also provided no disease control in all three trials but did improved yield in the third trial. Chitosan (β 1,4-,linked glucosamine), a deacetylated form of chitin, is a natural compound derived from the outer shell of crustaceans which displays antimicrobial activity against fungi and bacteria (Liu et al., 2004; Park et al., 2002). Two biological roles have been attributed to this compound against fungal pathogens: first, antifungal activity at certain concentrations and second, acting as a potent elicitor enhancing plant resistance and also promoting plant growth (El Ghaouth et al., 1994; Shalom et al., 2003, Kim et al., 2005). This latter property of chitosan might be responsible for the observed increase in yields in our experiments in the absence of disease control. However, there is limited research done under field conditions to evaluate the effect of chitosan on yield improvement. In orchids, accelerated growth and development of meristemic tissue was observed in plants treated with chitosan (Uthairatanakij et al., 2007). Basil plants treated with chitosan showed increased growth proportional to the chitosan concentration (Kim et al., 2005). To our knowledge this is the first reports of tomato yield improvement by chitosan in U.S.

All of these results demonstrate the inconsistency in disease control between trials and/or failure to provide disease control or improved of yields by unknown mechanism by some of the OMRI-certified materials we tested here. This highlights the need for more research in order to develop more effective materials or to identify conditions that might reduce the efficacy of these materials. With the rapid growth of the U.S. organic

industry more data on the efficacy of these OMRI-certified materials is required to provide more tools and more effective options for organic growers.

The difference in disease pressure seen between 2009 and 2010 is likely the result of differences in weather conditions (Table 4.2). The environment plays a critical role in pathogen dispersal and disease development, insect reproduction, plant development and yield performance, as well as in the weathering of pesticide residues on the leaf surface (Agrios, 2005, Parker et al., 1995; Edelson and Magaro, 1988; Coakley et al., 1999). For a disease to occur, a susceptible host, a virulent pathogen, and a favorable environment must be present (Sbragia, 1975). The total precipitation accumulated in August of 2009 was 9.5 cm more than in the same month in 2010 (Table 2). A more marked reduction in precipitation was observed in September 2010, with 13.4 cm less than in the same month in 2009. The growing season of 2010 was dry, with a departure from the normal of -6.5 cm in precipitation by September 2010 which probably played a role in the low disease levels observed in the third trial (University of Kentucky Agricultural Weather Center). These drought conditions also resulted in a reduction in periods of relative humidity of ≥ 90% in 2010 compared with 2009 in these months (Table 4.2). During the first trial an explosive development of Septoria leaf spot was observed during September, during which weather conditions--mean temperature of 21˚C, 15 cm of total rain and 104 hours with relative humidity ≥ 90%--were favorable for this disease. Rain events play a critical role in the dispersal of S. *lycopersici* from infested plant to healthy plants (Parker et al., 1995) as well as providing leaf surface moisture favorable for infection and sporulation. Spores of *A. solani* are mainly dispersed by air movement, but rain splash can also disperse the spores from lesions or infested crop

residue (Rotem, 1994). Early blight development is highly favored by average temperatures >22˚C, >60 hours of relative humidity >90% and >2.5 cm of rain in a 7-day period (Madden et al., 1978). Despite the generally favorable conditions for early blight development during the first trial not much damage by this disease was observed, possibly due to low levels of primary inoculum at the site when we transplanted our first trial.

Table 4.1. Materials evaluated in field trials during 2009 and 2010.

Material or product tested [a]	**Active Ingredient**	**Rate of application**	**Experiment**
Sonata®	1.38 % *Bacillus pumilis*	4 qt/a	I, II, III
Serenade Max®	14.6 % *Bacillus subtilis*	3 lb/a	II, III
Bordeaux mixture	12.5 % Copper hydroxide, copper sulfate	4 oz/gal	I, II, III
Kocide®2000	53.8 % Copper hydroxide	2.3 lb/a	I
Kocide®3000	46.1 % Copper hydroxide	1 lb/a	II, III
Trilogy®	70 % Neem oil	2 % v/v	I, II, III
Regalia® SC	5% Giant Knotweed	2 qt/a	II, III
Ammonium bicarbonate	Ammonium bicarbonate	7.7 % v/v	I
Acid soluble chitosan	86 % chitosan	0.01 % w/v	I
Water-soluble chitosan	85 % chitosan	2 % w/v	II, III
Acetic acid	4 % acetic acid	0.5% v/v	I
Quadris® 50WG	50 % Azoxystrobin	3.2 oz/a	I, II, III
Lime sulfur	26 % Calcium polysulfide	3.3 fl oz/gal	II, III

[a] Materials applied in a 7-days interval.

Table 4.2. Weather conditions for the tomato trials site during the growing season of 2009 and 2010.

		Temperature (˚C)			Precipitation (cm)	Relative Humidity		
Months	**Year**	**Max**	**Min**	**Avg**	**Total Accum**	**Max**	**Min**	**# hours ≥ 90 %**
June	2009	28	18	23	13.1	86	50	40
	2010	30	20	25	11.7	87	48	53
July	2009	27	18	22	19.2	88	49	81
	2010	31	21	26	15.4	87	50	62
August	2009	28	18	23	11.5	90	51	76
	2010	32	19	26	2.0	86	41	31
September	2009	25	16	21	15.0	88	53	104
	2010	29	14	22	1.6	81	31	11
October	2009	16	8	12	14.7	89	53	68
	2010	22	8	15	3.2	74	27	0

Table 4.3. Effect of foliar fungicide application on severity of Septoria leaf spot and early blight and tomato yields, Experiment I.

Treatments[a]	**Final disease**[b] **severity (%)**	**sAUDPC**[c]	**Yields (kg/m^2)**[d]	**Marketable yields (kg/m^2)**[d]
Sonata®	86.7 bc[e]	26.9 bc	37.1 a	15.4 a
Bordeaux Mixture	29.0 a	11.1 a	37.9 a	15.7 a
Kocide®2000	23.6 a	8.9 a	36.1 a	16.3 a
Trilogy®	70.4 b	29.6 c	31.1 a	14.3 a
Ammonium bicarbonate	91.3 c	20.1 b	34.9 a	15.4 a
Acid-soluble chitosan	70.7 b	20.5 b	38.7 a	16.9 a
Acetic acid	76.8 bc	28.4 bc	34.1 a	14.5 a
Quadris® 50 WG	26.5 a	9.2 a	35.8 a	15.5 a
Unsprayed control	82.1 bc	25.1 bc	36.0 a	14.3 a
LSD Fisher		25.4		

[a] Treatments applied at 7-day interval. Acetic acid was included as negative control for acid-soluble chitosan; Quadris® 50 WG was included as positive control.
[b] Disease assessed 95 days after transplanting; foliar necrosis caused by *Septoria lycopersici* and *Alternaria solani* .
[c] Area under disease progress curve standardized for a 43-day epidemic.
[d] Values are the means (kg/m^2) of four replicate plots after four weeks of harvesting.
[e] Values are the means of four replicate plots. Means within a column followed by the same letter are not significantly different according to Fisher's least significant difference ($P = 0.05$).

Table 4.4. Effect of foliar fungicide application on severity of Septoria leaf spot and early blight and yields, Experiment II.

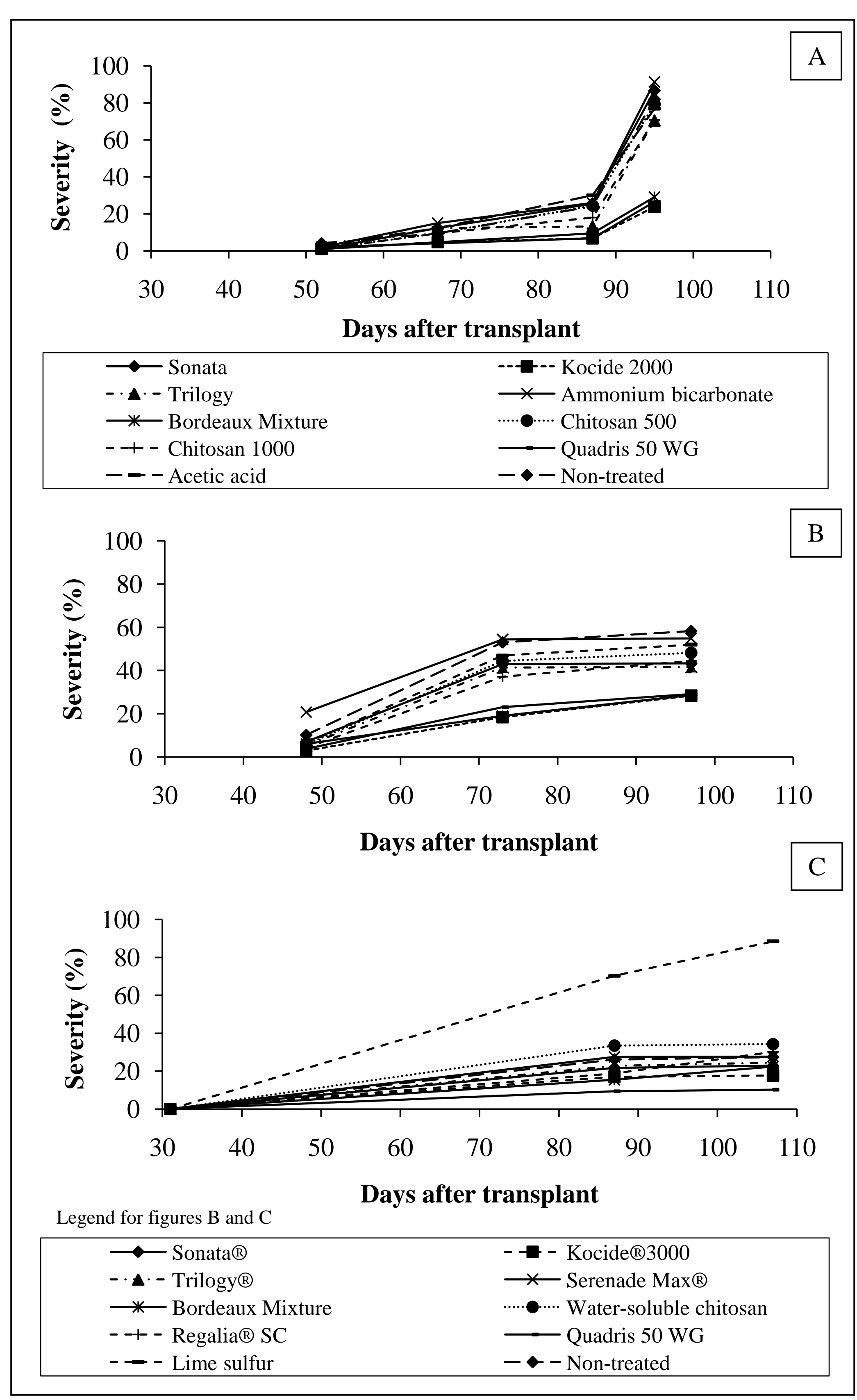
Treatments[a]	**Final disease**[b] **severity (%)**	**sAUDPC**[c]	**Yields (kg/m^2)**[d]	**Marketable yields (kg/ m^2)**
Sonata®	43.3 ab[e]	46.2 abcd	28.4 a	21.8 a
Serenade Max®	54.9 b	64.5 d	34.8 a	26.3 a
Bordeaux Mixture	28.6 a	24.3 ab	33.0 a	25.9 a
Kocide®3000	28.4 a	22.2 a	31.0 a	22.1 a
Trilogy®	41.6 ab	43.7 abcd	31.6 a	24.3 a
Regalia®SC	44.5 ab	40.7 abcd	33.5 a	25.2 a
Water-soluble chitosan	48.1 ab	47.8 bcd	29.0 a	21.7 a
Lime sulfur	52.0 b	50.9 cd	29.8 a	21.7 a
Quadris 50 WG	29.1 a	26.2 abc	34.9 a	25.9 a
Unsprayed control	58.8 b	58.9 d	31.6 a	24.3 a

[a] Treatments applied in a 7 days interval. Quadris® 50 WG was used as positive control.
[b] Disease assessed 97 days after transplant and caused by *Septoria lycopersici* and *Alternaria solani*
[c] Area under disease progress curve standardized for a 49-day epidemic.
[d] Values are the means (kg/m^2) for four replicate plots after four weeks of harvesting.
[e] Values are the means of four replicate plots. Means within a column followed by the same letter are not significantly different according to Fisher's least significant difference (P = 0.05).

Table 4.5. Effect of foliar fungicide application on severity of early blight and Septoria leaf spot and yields, Experiment III.

Treatments[a]	Final disease[b] severity (%)	sAUDPC[c]	Yields (kg/m^2)[d]	Marketable yields (kg/m^2)
Sonata®	21.7 abc[e]	18.7 ab	22.4 ab	19.2 abc
Serenade Max®	27.6 abc	22.7 ab	26.1 bcd	23.4 bcd
Bordeaux mixture	22.4 abc	13.9 ab	28.5 d	25.2 d
Kocide®3000	17.0 ab	14.4 ab	25.1 abcd	21.4 abcd
Trilogy®	24.5 abc	22.7 ab	23.0 abc	18.7 ab
Regalia®SC	30.1 bc	17.3 ab	28.7 d	25.3 d
Water-soluble chitosan	34.2 c	27.7 b	27.6 cd	24.0 cd
Lime sulfur	88.5 d	60.9 c	29.1 d	25.6 d
Quadris 50 WG	10.2 a	7.9 a	25.9 bcd	22.2 abcd
Unsprayed control	26.1 abc	22.4 ab	20.6 a	18.3 a

[a] Treatments applied on a 7-day interval. Quadris® 50 WG was used as positive control.
[b] Disease assessed 107 days after transplant and caused by *Alternaria solani* and *Septoria lycopersici*.
[c] Area under disease progress curve standardized for a 74-day epidemic.
[d] Values are the means (kg/m^2) of four replicate plots after four weeks of harvesting.
[e] Values are the means of four replicate plots. Means within a column followed by the same letter are not significantly different according to Fisher's least significant difference ($P = 0.05$).


A
Severity (%)
Days after transplant
Sonata
Kocide 2000
Trilogy
Ammonium bicarbonate
Bordeaux Mixture
Chitosan 500
Chitosan 1000
Quadris 50 WG
Acetic acid
Non-treated
B
Severity (%)
Days after transplant
C
Severity (%)
Days after transplant
Legend for figures B and C
Sonata®
Kocide®3000
Trilogy®
Serenade Max®
Bordeaux Mixture
Water-soluble chitosan
Regalia® SC
Quadris 50 WG
Lime sulfur
Non-treated

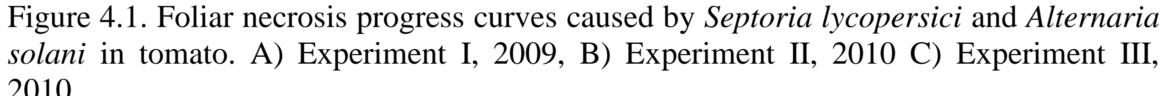


Figure 4.1. Foliar necrosis progress curves caused by *Septoria lycopersici* and *Alternaria solani* in tomato. A) Experiment I, 2009, B) Experiment II, 2010 C) Experiment III, 2010.

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CHAPTER 5

ANTIFUNGAL ACTIVITY OF CHITOSAN WITH DIFFERENT MOLECULAR WEIGHTS, PH, PERCENT DEACETYLATION AND SOLUBILITY AGAINST *COLLETOTRICHUM ORBICULARE IN VITRO* AND *IN VIVO*

INTRODUCTION

Colletotrichum orbiculare (Berk. & Mont.) Arx (syn. *C. lagenarium* (Pass) Ellis & Halst.) is the causal agent of cucurbit anthracnose (Zitter et al., 1996). The disease is controlled primarily through the frequent use of protectant and eradicant fungicides such as azoxystrobin, chlorothalonil and mancozeb (Pernezny et al., 2006). Regardless of the advantages in terms of disease control provided by these synthetic pesticides in agricultural commodities, in recent decades a marked increase interest in preserving the environment, as well as public concern about pesticides residues in food, has revived interest in organic products for disease control.

Its biodegradability, environmentally safety and non-toxicity to higher animals (Guerrero et al., 2007) are some of the characteristics that make chitosan an attractive and suitable natural compound for disease control. Chitosan is a deacetylated form of chitin derived from the outer shell of crustaceans. It is defined as a partially or completely deacetylated soluble form of chitin (Kasaai, 2009), a polymer composed of *ß*-1,4-linked subunits of glucosamine (2-amino-2-deoxy-ß-D-glucose) and lesser amounts of *N-acetylglucosamine* (Baños et al., 2006). Two biological roles have been attributed to this compound against fungal pathogens: first, antifungal activity by direct contact with the fungi at certain concentrations and second, acting as a potent elicitor enhancing plant resistance (El Ghaouth et al., 1994; Shalom et al., 2003). Antifungal activity of chitosan

has been demonstrated against powdery mildews, soilborne fungi, foliar pathogens, and postharvest fungi (Muñoz et al., 2009; Stössel and Leuba, 1984; El Ghaouth et al., 1992; El Ghaouth et al., 1994). The mechanism by which chitosan inhibits the growth and development of fungi has not been elucidated but three main hypotheses have been proposed. First, chitosan causes the leakeage of intracellular electrolytes and proteinaceous constituents by interfering with the negatively charged phospholipids of the fungal cell membrane (Bautista et al., 2006; Stöseel and Leuba, 1986). Second, diffused hydrolysis products of chitosan interact with the microbial DNA causing the inhibition of transcription and protein synthesis (Hadwiger et al., 1986, Bautista et al., 2006). Third, chitosan acts as a chelator of metals from the spore as well as essential nutrients affecting the normal growth and development of the fungi (Cuero et al., 1991). Changes in the morphology of the hypha and/or conidia of *Botrytis cinerea, Trichodema harzianum* and *Sphaeropsis sapinea* treated with chitosan have been observed (Vesentini et al., 2007; Ait et al., 2004; Clarete et al., 2007). However, the causal mechanisms of these changes was not adressed. Recently, studies using *Neurospora crassa* reported membrane permeabilization, cell death and lysis of conidia treated with chitosan (Palma et al., 2009).

Most commercially available chitosan preparations are more than 85 percent deacetylated, and have molecular weights between 100 kDa and 1000 kDa (Rhoades and Rastall, 2008). These are usually complexes with acids, such as acetic or lactic acids. Chitosan has a positive charge in acidic solutions, due to the presence of amines on the molecule that bind protons (Rhoades and Rastall, 2008). There are several factors, both intrinsic and extrinsic, that can affect the antimicrobial activity of chitosan. Some of these

are solubility, pH, molecular weight, temperature, salt concentration and degree of polymerization (Rhoades and Rastall, 2003). However, few investigations have taken these factors into account as important variables to study (Stoseel and Leuba, 1986; Vander et al., 1998; Fang et al., 2008; Romanazzi et al., 2009). In light of the possible direct effect of these variables on the antifungal activity of chitosan and the inconsistency of protocols for preparing chitosan, we tested the effect of two solvents in the antifungal activity of chitosan; determined the effect of dialysis, sterilization and pH on the antifungal activity of chitosan; determined the effect of chitosan on conidia germination; and evaluated the antifungal activity of chitosan *in vivo* using different methods of application.

MATERIALS AND METHODS

Fungal isolate

Colletotrichum orbiculare was isolated from cucumber foliage collected on a commercial farm in Harrison County, Kentucky in 2007. The fungus was cultured on 39% (w/v) Potato Dextrose Agar (PDA; Difco Lab., Detroit, MI) under continuous fluorescent light at 22˚C. Single-conidium cultures were obtained as follows. After 10 days of incubation, a spatula was used to scrape the pinkish mass of conidia from a culture plate flooded with 10 ml of sterile distilled water. Fifty microliters were spread on 20% (w/v) water agar (Difco Lab; Detroit, MI) and incubated for 12 hours at 22˚C. Single germinated conidia were transferred to PDA and incubated for 5 days. After the incubation time the pathogen was transferred and grown on green bean agar (453 g of caned green bean/L mixed with 17g of agar) under continuous fluorescent light at 22˚C to

induce sporulation (Jeun et al., 2003). One isolate was chosen for identification by pathogenicity tests and morphological features of conidia (100 conidia) and appressoria (Appendix 1). The ITS (Internal Transcribed Spacer) sequence of the isolate was also obtained via direct sequencing using primers ITS1 and ITS4 (White et al., 1990), and species determination was verified via a Genbank BLAST search. Spore suspensions at a concentration of 10^5 and 10^6 conidia/ml were used for *in vitro* and *in vivo* experiments, respectively.

***In vitro* evaluation of the antifungal activity of chitosan with different molecular properties**

With the objective of determining if the molecular weight, degree of deacetylation, concentration or solvent affected the antifungal activity of chitosan, seven chitosan products with different molecular properties (Table 5.1) were evaluated for their antifungal activity against *C. orbiculare in vitro*. All water-soluble chitosans were dissolved in sterile deionized water (ddH_2O). The acid-soluble chitosans were prepared with few modifications from a previously described procedure (Bautista et al., 2003). Briefly, chitosan was dissolved in 0.1% acetic acid by heating the solution on a hot plate for 30 minutes and then stirring continuously for 24 h at room temperature. For all *in vitro* experiments, solutions were filter-sterilized through a series of Milipore MF Membranes (0.8, 0.4 and 0.22 µm, Millipore, Ireland) aliquoted and stored at 4˚C until used. For all seven chitosans tested, five concentrations were evaluated, 0, 100, 150, 200 and 250 µg/ml (Vesentini et al., 2007). For acid-soluble chitosans, all concentrations were tested at a uniform concentration of acetic acid of 0.1%; acetic acid alone was used as a control in all experiments. The pH of

acid-soluble chitosans was adjusted to 5.6 with 1 N NaOH after dissolving in acetic acid (El Ghaouth, 1992). The pH of the acetic acid control was also adjusted to 5.6.

The antifungal activity of each chitosan was determined using a computerized colorimetric method, which uses the absorbance (OD) of cultures in a microtiter plate to estimate mycelial growth. This protocol was modified from procedures described previously (Daeschel 1992, Gul and Reddy, 2000; Wilson et al., 1997). The assay was conducted as follows: 100 µl of conidial suspension (10^6 conidia/ml) were added to 400 µl of potato dextrose broth (Difco Lab., Detroit, MI), and to this was added the amount required for each chitosan plus sterile ddH_2O to achieve the desired concentration of the test chitosan in a final volume of 1ml. The mixture was vortexed for 5-10 seconds and, 100 µl of the mixture were added into each of 8 wells (replicates for each concentration) of a 96-well, sterile, flat bottom microplate (Nunc-Immuno™ Plate, USA). Conidial suspensions which were non-treated and treated with Quadris® 50WG (AZ a.i. azoxystrobin) at 5 µg ai. azoxystrobin/ml (Syngenta, Greensboro, NC) were used as negative and positive controls, respectively. For all materials tested, identical mixtures without conidia were prepared and added to the same plate to serve as controls in order to test for changes in absorbance due to chemical transformations rather than suppression of mycelial growth. The 96-well plate was then sealed with a sterile adhesive polyester film (VWR Scientific, Scientific Industries, Inc., Bohemia, NY) and incubated under continuous light during 48 h at 22˚C. After the incubation period, the fungal mycelial density ($OD_{450\ nm}$) was measured with ELx800™ Universal Microplate Reader (Bio-Tek™, Princeton, NJ) equipped with Delta-Soft 3™ (kinetics and endpoint software). To obtain the net fungal growth, the mean absorbance of the mixtures without conidia was subtracted from the

absorbance of each replicate of the mixture with conidia. This corrected absorbance value was transformed to percent of mycelial growth inhibition (mgi) related to the control using the follow formula: mgi = (1- A_n/A_0) *100; where A_n is the absorbance of each treatment and A_0 is the absorbance of the control (non-treated conidia). The experiments were performed twice with eight replications per treatments.

***In vitro* evaluation of the effect of autoclaving on antifungal activity of chitosan**

It was previously demonstrated that autoclaved chitosan increased callose content and membrane permeability in soybean cells compared with non-autoclaved chitosan (Kohle et al., 1985), but the effect of this process on the antifungal activity of chitosan has not yet been determined. To test if sterilization by autoclaving the chitosan solution affected its antifungal activity, autoclaved and non-autoclaved chitosan solutions were evaluated. All chitosans were tested at 250 µg/ml and prepared as described in the previous section. Solutions were autoclaved for 30 minutes at 122˚C and 23 psi. The same colorimetric method was used to measure antifungal activity. Each treatment was replicated eight times and the experiment was performed twice.

***In vitro* evaluation of the effect of dialysis in the antifungal activity of chitosan**

Due to the fact that some, but not all, protocols for preparing chitosan include dialysis as a step in the process (El Ghaouth et al., 1992; Bell et al., 1998; Lizama-Uc et al., 2007; Vesentini et al., 2007), the antifungal activity of chitosan submitted to dialysis was compared to undialyzed chitosan. For this experiment, one water-soluble chitosan, chitosan #2, and one acid-soluble chitosan, chitosan 86, were used. Both were tested at 250 µg/ml

and dissolved as described in previous sections. To perform the dialysis, chitosan solution was poured into a Spectra/Por® 7 Dialysis Membrane MW kDa (Spectrum Laboratories, Inc. Rancho Dominguez, CA USA) and closed at each end with a 75 mm Spectra/Por® membrane closure (Spectrum Laboratories, Inc. Rancho Dominguez, CA USA). The membrane was placed inside a beaker and dialyzed against sterile ddH2O by constant stirring at 4˚C for 24 h. The colorimetric method previously described was used to measure the antifungal activity of dialyzed chitosan. Each treatment was replicated eight times and the experiment was done twice.

In vitro evaluation of the effect of pH changes in the antifungal activity of chitosan

In previous research it has been observed that the antifungal activity of chitosan can be affected by changes in pH (Stöseel and Leuba, 1986;). To test whether the antifungal activity of chitosan against *C. orbiculare* was affected by changes in pH, chitosan #2 and chitosan 86 at 250 µg/ml were evaluated over a pH range of 4 to 8. pH of chitosan solutions was adjusted using 1 N NaOH and 1 N HCL (El Ghaouth, 1992; Stöseel and Leuba, 1986). The colorimetric method previously described was used to measure the antifungal activity of chitosan adjusted to different pH. Each treatment was replicated four times and the experiment was done twice.

Evaluation of chitosan *in vivo*

Foliar application. Organic seeds of cantaloupe (*Cucumis melo* cultivar 'Edens Gem OG') were obtained from Johnny's Selected Seeds (Maine, USA). Plants were grown in 10-cm diameter plastic pots filled with Pro-Mix® (Quakertown, PA) under

greenhouse conditions at 27˚C and 23˚C, day and night respectively. Plants were fertilized weekly using Fish Emulsion 5-1-1 (Ferti·lome® Bonham, Texas) at rate of 9 ml/liter of water. All water-soluble chitosans were evaluated at 0.05%, 0.1% and 2% w/v chitosan as described previously (Bautista et al., 2003). Acid soluble-chitosans were tested at 0.1% (Vesentini et al., 2007) dissolved in 0.1% acetic acid and adjusted to 5.6 pH (El Ghaouth, 1992) because concentrations of chitosan higher than 0.1% were phytotoxic to cantaloupe seedling. Non-treated plants, plants treated with Quadris® 50WG (0.05%), and plants treated with acetic acid alone were used as controls. Twelve day-old plants with fully expanded cotyledons were sprayed until runoff using a hand sprayer (VWR® International, Cat. No. 23609-182). After plants dried, 3 ml of a 10^6 conidia/ml suspension of *C. orbiculare* were sprayed over the cotyledons using a hand sprayer. Inoculated plants were placed in a moist chamber (100% relative humidity) for 24 h (Jeun et al., 2008). After incubation, plants were transferred to the greenhouse for symptom development. Disease symptoms were scored 4-6 days post-inoculation by harvesting cotyledons and capturing digital images using a Nikon COOLPIX L110. Each image was analyzed for disease severity using Assess 2.0 Image Analysis Software for Disease Quantification (APS, PRESS). Ten plants per treatment were used and the experiments were performed at least twice.

Chitosan incorporated into the soil. To test if chitosan incorporated into the soil protected cantaloupe plants against *C. orbiculare,* we evaluated two of the water-soluble chitosan formulations (CH #2 and CH SG) sold as a fertilizer to improve seed germination, root development and general plant health. Each chitosan was mixed with Pro-Mix® (Quakertown, PA) to a final concentration of 300 µg/mg of soil. Non-

incorporated soil media was used as a negative control. Organic cantaloupe seeds were placed into soil media and grown under greenhouse conditions. Plants 12 days old with fully expanded cotyledons were spot-inoculated with a 20 µl-drop of a 10^6 conidia/ml suspension of *C. orbiculare*. Inoculated plants were placed in a moist chamber (100% relative humidity) for 24 h (Jeun et al., 2008). After incubation, plants were transferred to the greenhouse for symptom development. Disease symptoms were scored 4-6 days post-inoculation using a digital vernier caliper to measure lesion size. Ten plants per treatment were used and the experiment was repeated twice.

In vitro **quantitative assay of germination of conidia treated with chitosan**

To determine if the antifungal activity of chitosan observed in the *in vivo* experiments was due to inhibition of conidial germination, *in vitro* studies were done. Chitosan #1, chitosan #2, chitosan #3 and chitosan SG at 2% v/v, and chitosan 86 and chitosan 96 at 0.1% v/v (pH 5.6), were tested. Acetic acid at 0.1% (pH 5.6), water, and Quadris® 50WG at 0.05% were used as controls. Three 20-µl drops of each treatment of conidial suspension mixed with each chitosan were deposited onto untreated polystyrene Petri dishes (Becton, Dickinson, Franklin Lakes, NJ). These were placed inside a humidity chamber, which was incubated for 24 h at 23˚C, after which conidial germination was assessed microscopically. One hundred randomly selected conidia were assessed for germination for each drop. Each treatment was repeated four times and each experiment was performed at least two times.

In vitro evaluation of the antifungal activity of chitosan-amended media

PDA amended with chitosan #1, chitosan #2, chitosan #3 at 2% v/v and chitosan 86 and chitosan 96 at 0.1% v/v (pH 5.6) was used to test the antifungal activity of chitosan *in vitro* on the radial growth of *C. orbiculare*. PDA plates non-amended and amended with acetic acid at 0.1% (pH 5.6), and Quadris® 50WG at 0.05% were used as controls. An agar disk (5 mm diameter) from a pure culture of *C. orbiculare* was placed in the center of the PDA plates (Bautista et al., 2003) and incubated under continuous light at 23˚C. The radial growth was measured daily until the non-amended control reached the edge of the plate. The experiment was performed twice with three replicates per treatment.

Statistical analysis.

All results were analyzed using INFOSTAT Statistical Software Version 2004 (InfoStat, FCA, Córdova Argentina). Analysis of variance (ANOVA) was performed at the significance level of $P<0.05$. Fisher's least significance test (LSD, $P=0.05$) was performed to separate means.

RESULTS

In vitro evaluation of the antifungal activity of chitosans with different molecular properties

Water-soluble chitosan #2, #3 and #4, with molecular weights between 3 to 10 kDa, showed higher percentages of mgi than the other two water-soluble chitosans (#1 and SG) and the acid-soluble (86 and 96) (Figure 5.1-5.7). More than 90% mgi was

achieved with chitosan #2, #3 and #4 at 250 μg/ml. At this concentration (250 μg/ml), no differences were observed between chitosan #2, #3 and #4 and the positive control (AZ). Chitosan #1 and SG showed a stimulatory effect instead of inhibition at all the concentrations tested, failing to suppress mycelial growth of *C. orbiculare in vitro*. Both acid-soluble chitosans, with differing percent deacetylation but the same range of molecular weight, showed similar results (Fig. 5.6-5.7). More than 35% mgi was obtained with both acid-soluble chitosans at concentrations ≥200 μg/ml. Chitosan 86 showed an increase in the antifungal activity in a dose-dependent manner whereas, chitosan 96 showed similar values for percent mgi across all concentrations tested.

***In vitro* evaluation of the effect of autoclaving on antifungal activity of chitosan**

Results from the autoclaving assay showed no effect of autoclaving on the antifungal activity of chitosans #1, #2, #4, SG and 96 in experiment 1 (Fig. 5.8A). In this experiment, only autoclaved chitosan 86 showed a modest but significant difference ($P<0.0001$) in mgi compared with non-autoclaved chitosan. In the second experiment only CH SG showed significant differences in their antifungal activity between autoclaved and non-autoclaved solutions (Fig. 5.8B).

***In vitro* evaluation of the effect of dialysis on the antifungal activity of chitosan**

After dialysis, no change in pH was measured in any of the chitosan solutions and no change of color was observed. No effect of dialysis was observed on chitosan #2 compared with the non-dialyzed chitosan in both experiments (Fig. 5.9). Chitosan 86 showed a slight but significant ($P<0.0001$) decrease of approximately 10% in the

antifungal activity of dialyzed chitosan compared with the non-dialyzed in both experiments (Fig. 5.9).

In vitro evaluation of the effect of pH changes on the antifungal activity of chitosan

Solutions of chitosan #2 and 86 were adjusted to five different pHs (4, 5, 6, 7 and 8) to test the effect of pH on their antifungal activity. Chitosan #2 showed the maximum percent mgi at pH 6 (Fig. 5.10A). Chitosan 86 showed the maximum percentage of mgi at pH ranging from 4-7 in the first experiment and pH ranged from 5-7 in the second experiment (Fig. 5.10B). At pH 8 its antifungal activity sharply decreased and instead of inhibition, promotion of growth was observed (Fig. 5.10B).

Application and evaluation of chitosan *in vivo*

Foliar application. At concentrations of 0.05% and 0.1%, none of the water-soluble chitosans evaluated except CH #2 provided a significant reduction ($P<0.05$) in disease control compared to the negative control (Fig. 5.11A and 5.12A). At 2% (w/v), all water-soluble chitosans tested provided significant disease control compared with the negative control, with no more than 10% cotyledon necrosis (Fig. 5.11B and 5.12B). In experiment #2, all water-soluble chitosans provided disease control equal to the positive control. Symptoms of anthracnose were restricted to areas where visible chitosan residues from the spray application were not present (Fig. 5.12B).

Both acid-soluble chitosans were tested only at 0.1% because higher concentrations were highly phytotoxic to cantaloupe seedlings *(data not shown)*. CH 86 provided a significant reduction in anthracnose severity compared to the negative control

(AA) in both experiments (Fig. 5.13). However, CH 96 showed inconsistent results between experiments (Fig. 5.13).

Chitosan incorporated into the soil. No protection against *C. orbiculare* was achieved with CH SG incorporated into the soil in any of the experiments done (Fig. 5.14). Treatment with CH #2 resulted in smaller lesions ($P<0.05$) compared with the non-incorporated control in the second experiment while in the first experiment no difference was observed ($P>0.05$) (Fig. 5.14).

In vitro quantitative assay of germination of conidia treated with chitosan

All water-soluble chitosans except chitosan SG, and all acid-soluble chitosans, showed significantly ($P<0.05$) less spore germination than the negative control (Fig. 5.15A). The greatest suppression of spore germination was obtained with the acid-soluble chitosans 86 and 96. Conidia treated with acid-soluble chitosan showed morphological changes (Fig. 5.15B). No effect on the morphology of conidia of *C. orbiculare* was observed with water-soluble chitosans (*data not shown*).

In vitro evaluation of the antifungal activity of chitosan-amended media

Chitosan #1, #2 and #4 produced significant reductions ($P<0.05$) in the radial growth of *C. orbiculare* compared with non-amended media in both experiments (Fig. 5.16). No effect on radial growth of mycelium was observed by CH 86 and CH 96. Colonies of *C. orbiculare* growing on media amended with chitosan 86 and 96 showed a different phenotype characterized by greatly enhanced production of microstromata compared with the non-amended treatment or the media amended with acetic acid (Fig. 5.17).

DISCUSSION

We analyzed the antifungal activity of seven different chitosan-based products with different molecular properties against *C. orbiculare* and made a number of findings. First, we showed that water-soluble chitosans with molecular weight between 3 to 10 kDa, 80-and 85% deacetylated showed the highest *in vitro* antifungal activity among all chitosan-based products evaluated. These chitosan-based products were CH#2, CH#3 and CH#4. The two high molecular weight chitosans, CH 86 and CH 96, also showed inhibition of mycelial growth of *C. orbiculare in vitro*, but not as much as CH#2, CH#3 and CH#4. This suggests that there is a relationship between molecular weight and antifungal activity, in which the antifungal activity decreases with a molecular weight exceeding 10 kDa and is lost with molecular weight below 3 kDa (Fig. 1 Appendix 3). Although only one molecular weight of chitosan (4 kDa) was tested, previous research found antimicrobial activity of this chitosan against a wide array of organisms including *Escherichia coli*, *Pseudomonas aureofaciens*, *Enterobacter agglomerans*, *Bacillus subtilis*, *Candida kruisei* and *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Tikhonov et al., 2006). With a chitosan having that molecular weight and at concentration of 0.01 mg/ml, spore germination of *Fusarium oxysporum* f. sp. *radicis-lycopersici* was completely abolished. Using *Aspergillus niger* as model, it was previously demonstrated that the antifungal activity of chitosan was molecular weight-dependent (Fang et al., 2008). The highest antifungal activity of chitosan against *Aspergillus niger* was with a molecular weight of 50 kDa whereas chitosan with molecular weight of 800 kDa or more promoted fungal growth. These previous results are in agreement with our own data that

suggest a reduction in the antifungal activity as the molecular weight of chitosan is increased above some threshold value.

In vivo, only foliar applications of chitosan #2 showed significant disease control at concentrations of 0.05% and 0.1%. However, at higher concentrations (2%) all water-soluble chitosans provided a significant reduction in disease severity. In addition, the acid-soluble chitosan 86 showed some disease suppression *in vivo*. Thus, combining our *in vitro* and *in vivo* results, our data suggest that the antifungal activity of chitosan-based products is molecular weight- and concentration-dependent. Moreover, we concluded that, in addition to molecular weight and concentration, the antifungal activity of chitosan is also dependent on which fungal structure is targeted by the chitosan-based product. This was confirmed by the *in vitro* evaluation of chitosan on the radial growth of *C. orbiculare* and in the conidial germination assay. Conidia were sensitive to all chitosan-based products tested whereas only the water-soluble chitosan significantly decreased the radial growth of mycelium of *C. orbiculare.*

We tested the hypothesis that the antifungal activity of chitosan is affected by autoclaving and dialysis. Autoclaving the solution did not affect the antifungal activity of any of the chitosan-based products while dialysis slightly decreased de antifungal activity of acid-soluble chitosan but not water-soluble chitosan. None of these extrinsic factors caused major effects in the antifungal activity of chitosan. Thus, this suggests that these two processes are not critical parts of the protocols to prepare antifungal chitosan solutions.

Previously Stössel and Leuba (1984), established that the antifungal activity of chitosan depended on pH and demonstrated that chitosan is more effective against fungi

at pH 6 (when most amino groups are in the free base form) than at pH 7.5. Our pH experiments showed that CH #2 at pH 6 was more fungitoxic against *C. orbiculare* than at any other pH, whereas CH 86 was equally effective over a broader range of pH (4-7). A suboptimal pH might reduce the antifungal activity of water-soluble chitosans as well as acid-soluble chitosan, as seen in the results presented here. Therefore, optimization of the pH is a key step in the preparation of antifungal chitosan solutions.

Abnormal morphological changes in fungal hyphae and spores treated with chitosan have been commonly observed in *in vitro* assays (Ait et al., 2004; Vesentini et al., 2007; Palma et al., 2009). No effect on conidial morphology was observed with the water-soluble chitosan in our *in vitro* experiments. However, swelling of spores or formation of “giant spores” and “giant germ tubes” were observed in conidia treated with both (CH 86 and CH 96) acid-soluble chitosans. We don’t know whether the changes observed here are the result of direct action of chitosan on the fungal cell membrane. Nevertheless, in accordance with a previously established hypothesis, direct interaction of chitosan with the fungal membrane may cause cellular leakeage or lysis of the cell (Bautista et al., 2006; Stöseel and Leuba, 1986). If this is true, we would expected to see empty or lysed cells, but instead we observed swelling of the spores, suggesting interaction with fungal membranes is not an important mechanism of action. Clearly further work is need to determine how chitosan interacts with the conidia of *C.orbiculare* to cause the observed changes in morphology.

An important observation from this study was that the symptoms caused by *C. orbiculare* were restricted to areas where no visible chitosan residues were present. Chitosan has been used as preservative for coating fruits, because it forms a layer over

the fruit which prolongs shelf life (Jung et al., 2007). Also, tomato fruits coated with chitosan and later inoculated with *Colletotrichum* sp. showed a reduction in lesion size (Muñoz et al., 2009). This observation suggests that chitosan forms a layer that may hinder the penetration of the pathogen. An investigation of interest would be to test chitosan combined with a spreader-sticker or adjuvant to achieve uniform distribution over the leaf surface in order to test whether this improves disease control even at lower concentrations.

This research advances our understanding of the effect of several variables like molecular weight, pH and concentration on the antifungal activity of chitosan, which has potential as a natural fungicide. According to the US Environmental Protection Agency (2011), chitosan is an allowed active ingredient for use on crops as a foliar-applied plant growth enhancer, and as a substance that boosts the ability of plants to defend against fungal infections. For organic growers chitosan is a prohibited substance for crop pest, weed, and disease control (OMRI, 2011). According to the National Organic Program Rule: 205.601(m), chitosan may be used as either an adjuvant or inert ingredient in combination with active pesticidal substances (OMRI, 2011) but not as the active ingredient itself.

Table 5.1. Chitosan-based products evaluated on *in vitro* and *in vivo* experiments.

Chitosan	Molecular weight [a]	Solubility	Deacetylation (%) [b]	Company
Chitosan oligosaccharide (CH #1)	< 1 kDa	Water	85	LSK Biopartners, INC.
Chitosan oligosaccharide (CH #2)	3 - 4 kDa	Water	85	LSK Biopartners, INC.
Chitosan oligosaccharide (CH #3)	5 kDa	Water	80	LSK Biopartners, INC.
Chitosan oligosaccharide (CH #4)	10 kDa	Water	85	LSK Biopartners, INC.
Chitosan Super Growth (CH SG)	- [c]	Water	85	Super Growth-Plant Care
Chitosan from crab shell (CH 86)	190 - 375 kDa	1% Acetic acid	86	Sigma, Aldrich
Chitosan from crab shell (CH 96)	190 - 375 kDa	1% Acetic acid	96	Sigma, Aldrich

[a] Information provided by the manufacturer.
[b] Removal of acetyl groups (–$COCH_3$) from chitin.
[c] Not known

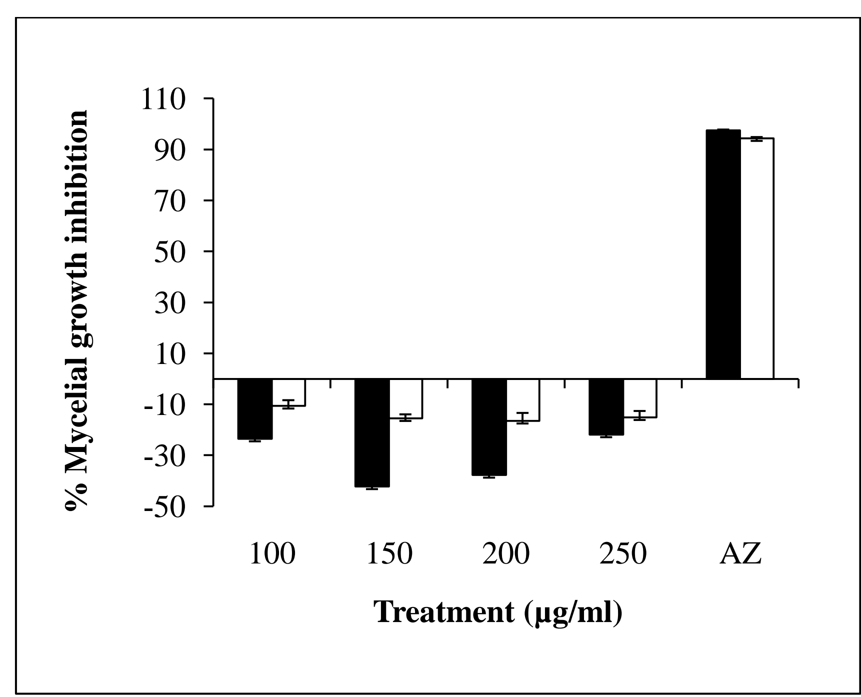


Figure 5.1. Antifungal activity of Chitosan #1 (< 1 kDa) measured using a colorimetric assay. Solid bars are experiment 1 and empty bars are experiment 2. Quadris® 50 WG (AZ) was used as a positive control at concentration of 2 µg/ml. The percent of mycelial growth inhibition (mgi) related to the negative control (non-treated conidia) was determined using the absorbance ($OD_{450\ nm}$) after 48 h of incubation. The mgi was calculated using the following formula: mgi = 100*[1- (A_n/A_0)]; where A_n is the absorbance of each treatment and, A_0 is the absorbance of the control (non-treated conidia). Error bars indicate standard errors.

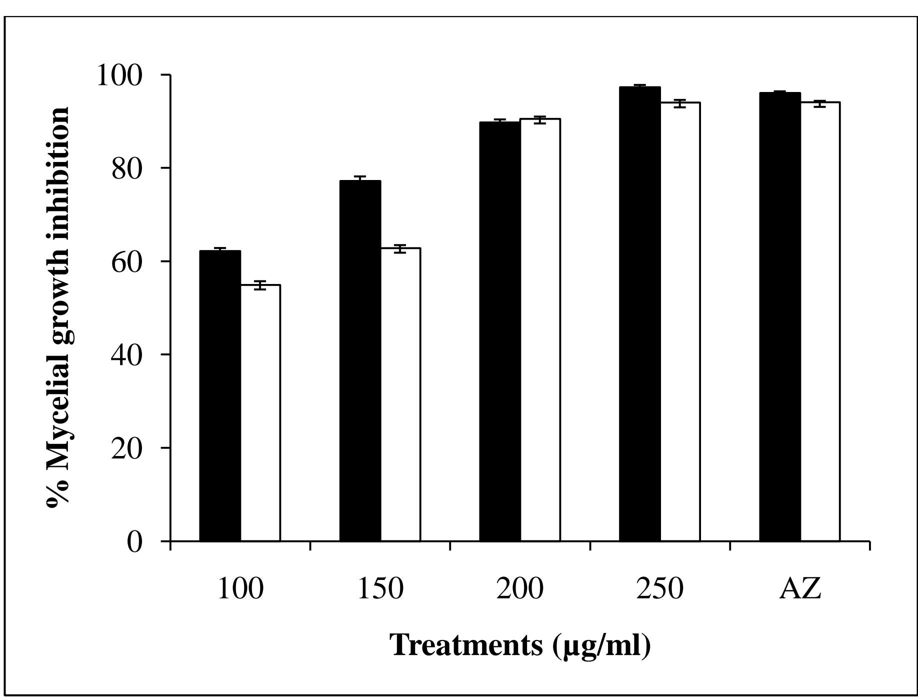


Figure 5.2. Antifungal activity of Chitosan #2 (3-4 kDa) measured using a colorimetric assay. Solid bars are experiment 1 and empty bars are experiment 2. Quadris® 50WG (AZ) was used as a positive control at concentration of 2 µg/ml. The percent of mycelial growth inhibition (mgi) related to the negative control (non-treated conidia) was determined using the absorbance ($OD_{450\ nm}$) after 48 h of incubation. The mgi was calculated using the following formula: mgi = $100*[1- (A_n/A_0)]$; where A_n is the absorbance of each treatment and, A_0 is the absorbance of the control (non-treated conidia). Error bars indicate standard errors.

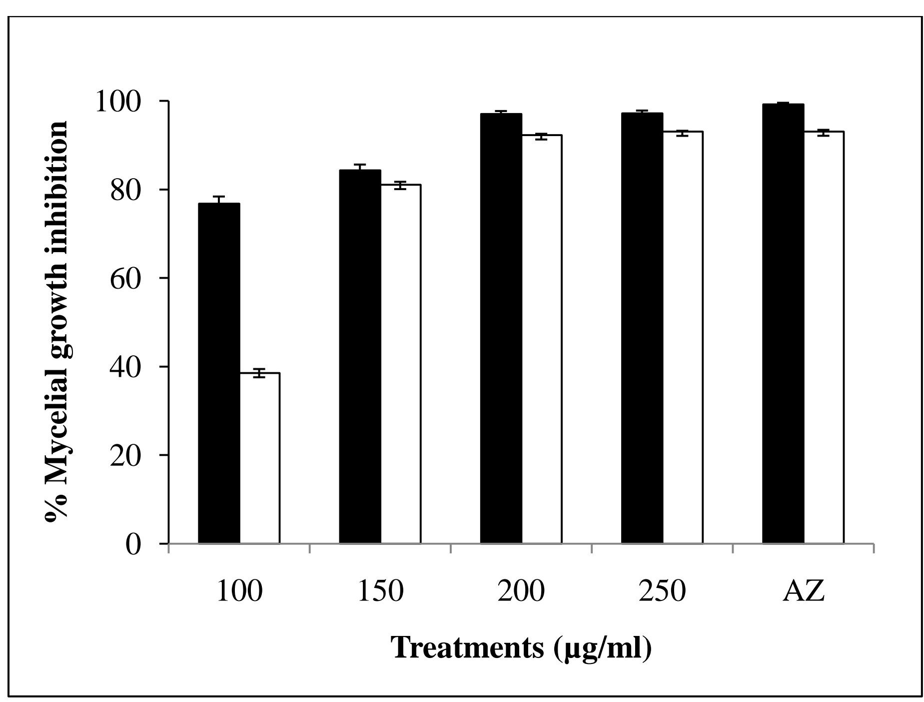


Figure 5.3. Antifungal activity of Chitosan #3 (5 kDa) measured using a colorimetric assay. Solid bars are experiment 1 and empty bars are experiment 2. Quadris® 50 WG (AZ) was used as a positive control at concentration of 2 µg/ml. The percent of mycelial growth inhibition (mgi) related to the negative control (non-treated conidia) was determined using the absorbance ($OD_{450\ nm}$) after 48 h of incubation. The mgi was calculated using the following formula: mgi = 100*[1- (A_n/A_0)]; where A_n is the absorbance of each treatment and, A_0 is the absorbance of the control (non-treated conidia). Error bars indicate standard errors.

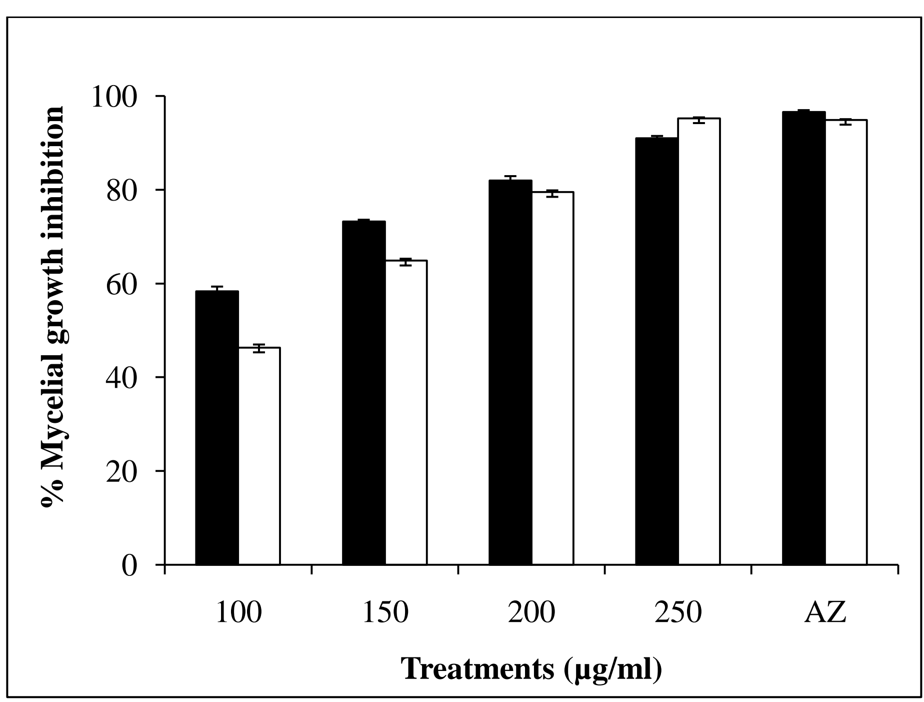


Figure 5.4. Antifungal activity of Chitosan #4 (10 kDa) measured using a colorimetric assay. Solid bars are experiment 1 and empty bars are experiment 2. Quadris® 50 WG (AZ) was used as a positive control at concentration of 2 µg/ml. The percent of mycelial growth inhibition (mgi) related to the negative control (non-treated conidia) was determined using the absorbance ($OD_{450\ nm}$) after 48 h of incubation. The mgi was calculated using the following formula: mgi = 100*[1- (A_n/A_0)]; where A_n is the absorbance of each treatment and, A_0 is the absorbance of the control (non-treated conidia). Error bars indicate standard errors.

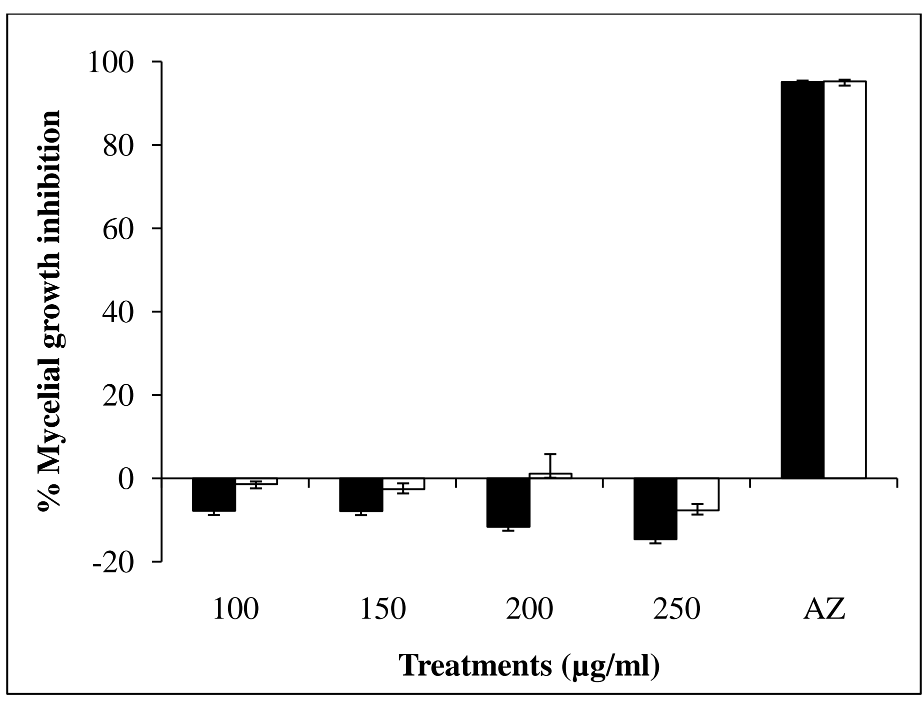


Figure 5.5. Antifungal activity of Chitosan SG measured using a colorimetric assay. Solid bars are experiment 1 and empty bars are experiment 2. Quadris® 50 WG (AZ) was used as a positive control at concentration of 2 µg/ml. The percent of mycelial growth inhibition (mgi) related to the negative control (non-treated conidia) was determined using the absorbance ($OD_{450\ nm}$) after 48 h of incubation. The mgi was calculated using the following formula: mgi = 100*[1- (A_n/A_0)]; where A_n is the absorbance of each treatment and, A_0 is the absorbance of the control (non-treated conidia). Error bars indicate standard errors.

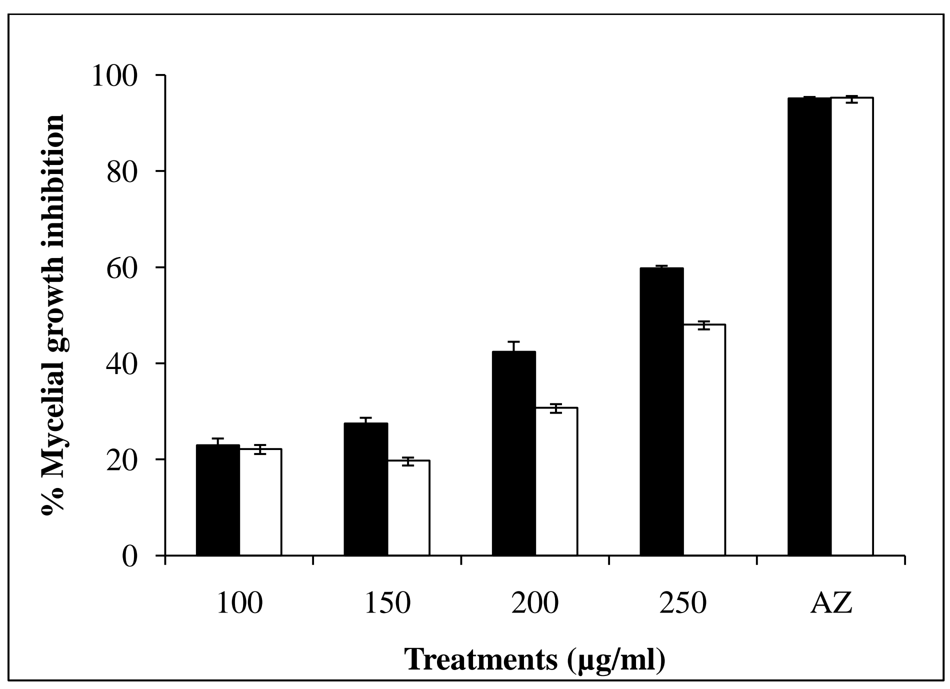


Figure 5.6. Antifungal activity of Chitosan 86 (190-375 kDa) measured using a colorimetric assay. Solid bars are experiment 1 and empty bars are experiment 2. Quadris® 50 WG (AZ) was used as a positive control at concentration of 2 µg/ml. The same concentration of acetic acid (AA) was kept equal throughout all concentrations and acetic acid was used as negative control. The percent of mycelial growth inhibition (mgi) related to the negative control (AA-treated conidia) was determined using the absorbance ($OD_{450\ nm}$) after 48 h of incubation. The mgi was calculated using the following formula: mgi = 100*[1- (A_n/A_0)]; where A_n is the absorbance of each treatment and, A_0 is the absorbance of the control (non-treated conidia). Error bars indicate standard errors.

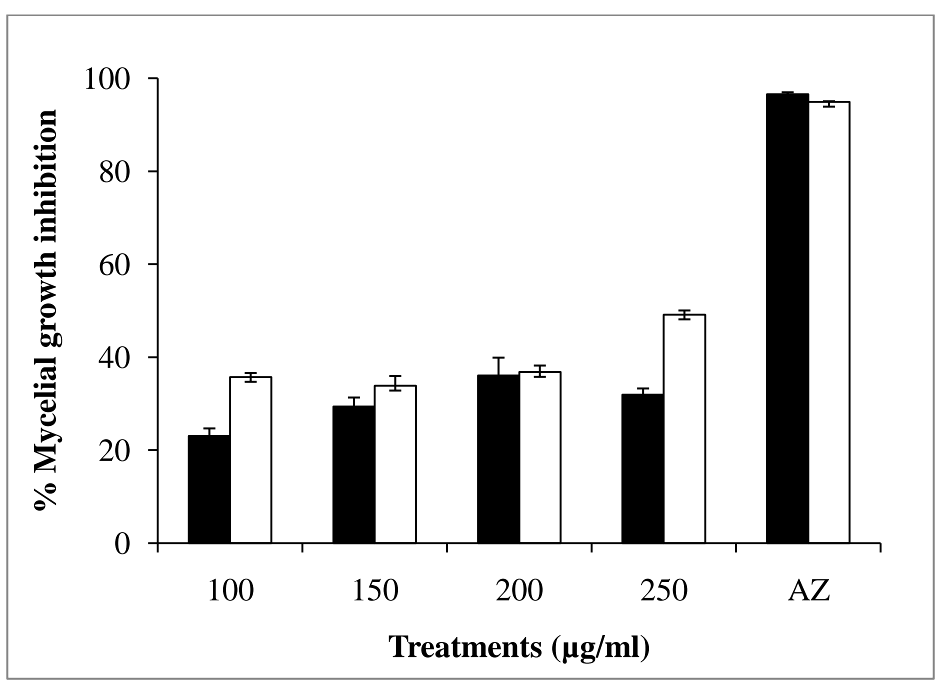
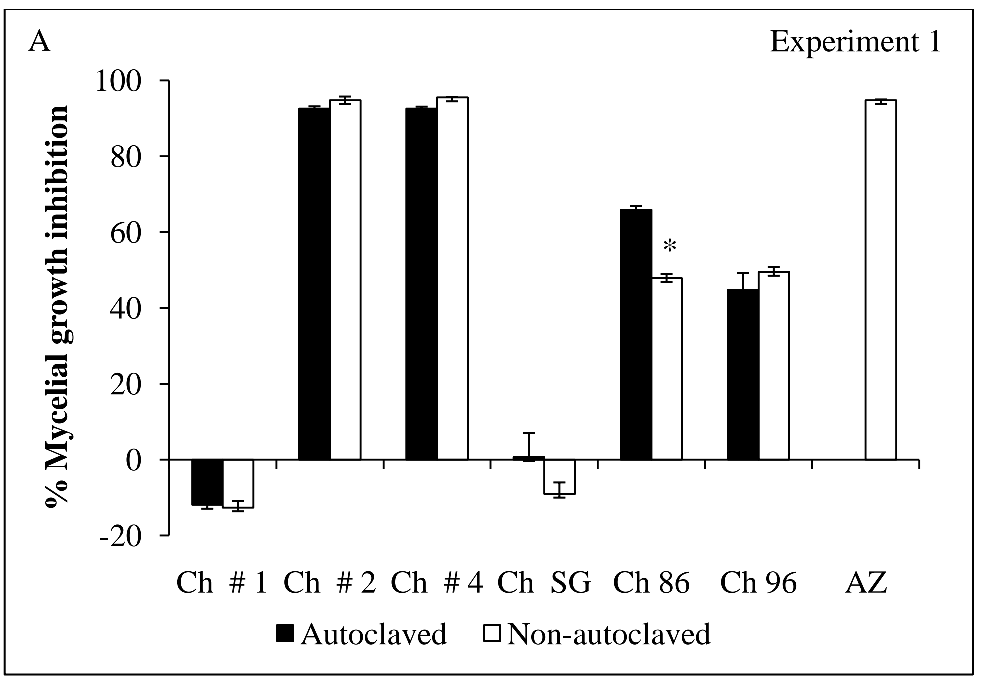


Figure 5.7. Antifungal activity of Chitosan 96 (190-375 kDa) measured using a colorimetric assay. Solid bars are experiment 1 and empty bars are experiment 2. Quadris® 50 WG (AZ) was used was used as a positive control at concentration of 2 µg/ml. The same concentration of acetic acid (AA) was kept equal throughout all concentrations and acetic acid was used as negative control. The percent of mycelial growth inhibition (mgi) related to the negative control (AA-treated conidia) was determined using the absorbance ($OD_{450\ nm}$) after 48 h of incubation. The mgi was calculated using the following formula: mgi = 100*[1- (A_n/A_0)]; where A_n is the absorbance of each treatment and, A_0 is the absorbance of the control (non-treated conidia). Error bars indicate standard errors.


A
Experiment 1
% Mycelial growth inhibition
100
80
60
40
20
0
-20
*
Ch # 1
Ch # 2
Ch # 4
Ch SG
Ch 86
Ch 96
AZ
Autoclaved
Non-autoclaved

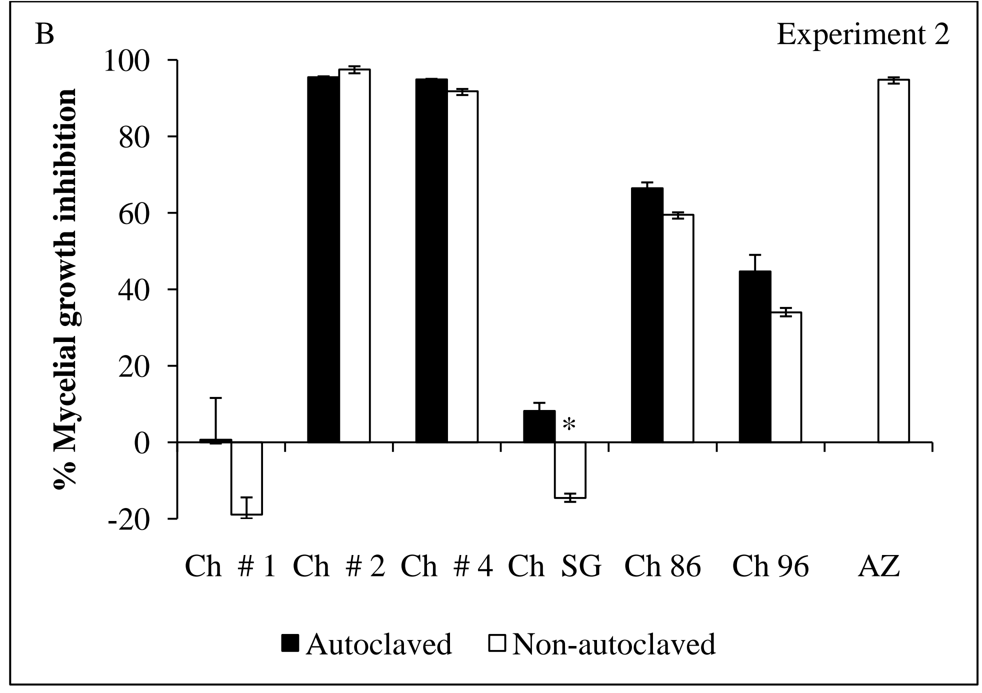
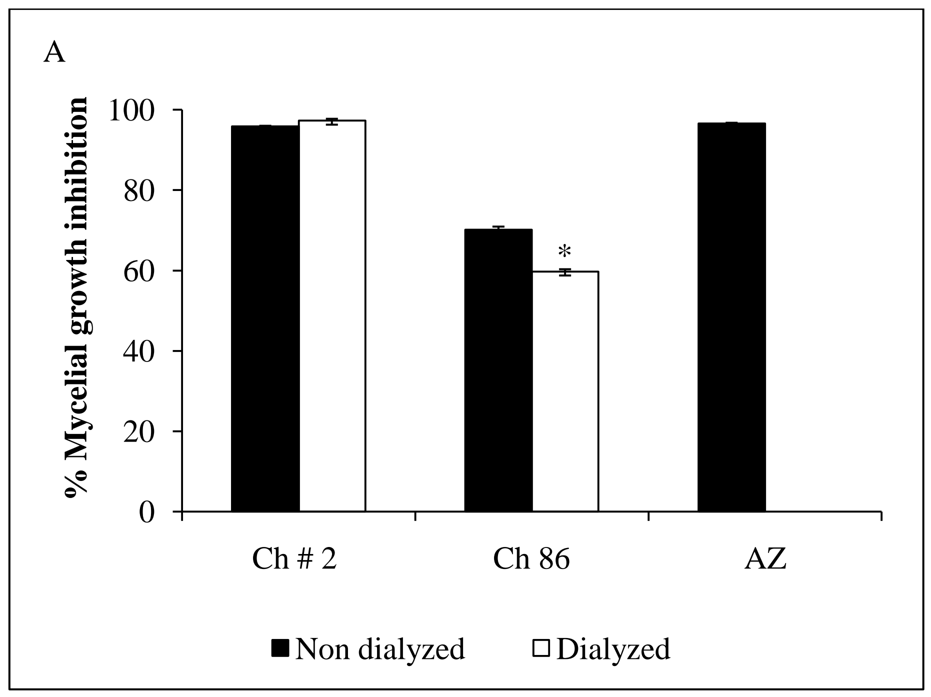

B
Experiment 2
% Mycelial growth inhibition
100
80
60
40
20
0
-20
*
Ch # 1
Ch # 2
Ch # 4
Ch SG
Ch 86
Ch 96
AZ
Autoclaved
Non-autoclaved

Figure 5.8. Antifungal activity of autoclaved solution of Chitosan #1 (< 1 kDa), Chitosan #2 (3-4 kDa), Chitosan #4 (5 kDa), Chitosan 86 (190-375 kDa) and Chitosan 96 (190-375 kDa). A) Experiment 1 and B) experiment 2. Solid bars are non-autoclaved chitosan and empty bars non-autoclaved chitosan. Both chitosan were evaluated at 250 µg/ml. Quadris® 50 WG (AZ) was used as a positive control at concentration of 2 µg/ml. The percent of mycelial growth inhibition (mgi) related to the negative control (non-treated conidia) was determined using the absorbance ($OD_{450\ nm}$). Acetic acid (AA) was used as a negative control to measure the mycelium growth inhibition of Chitosan 86 and 96. The mgi was calculated using the following formula: mgi = $100*[1- (A_n/A_0)]$; where A_n is the absorbance of each treatment and, A_0 is the absorbance of the control (non-treated conidia). Error bars indicate standard errors. *Statistically significant difference according to a *t* test ($P<0.05$).


A
% Mycelial growth inhibition
100
80
60
40
20
0
*
Ch # 2
Ch 86
AZ
Non dialyzed
Dialyzed

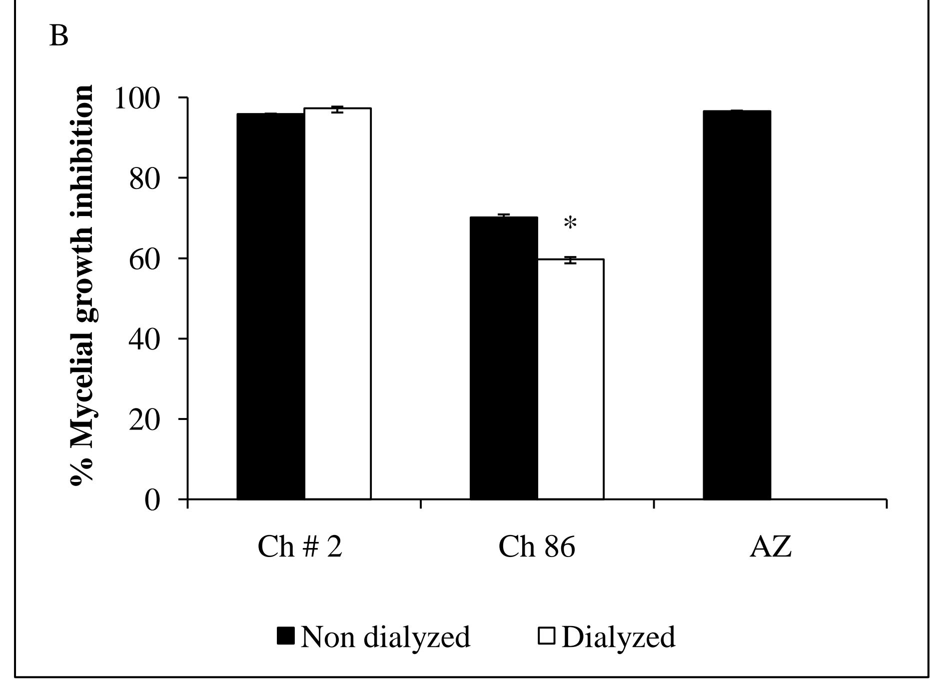
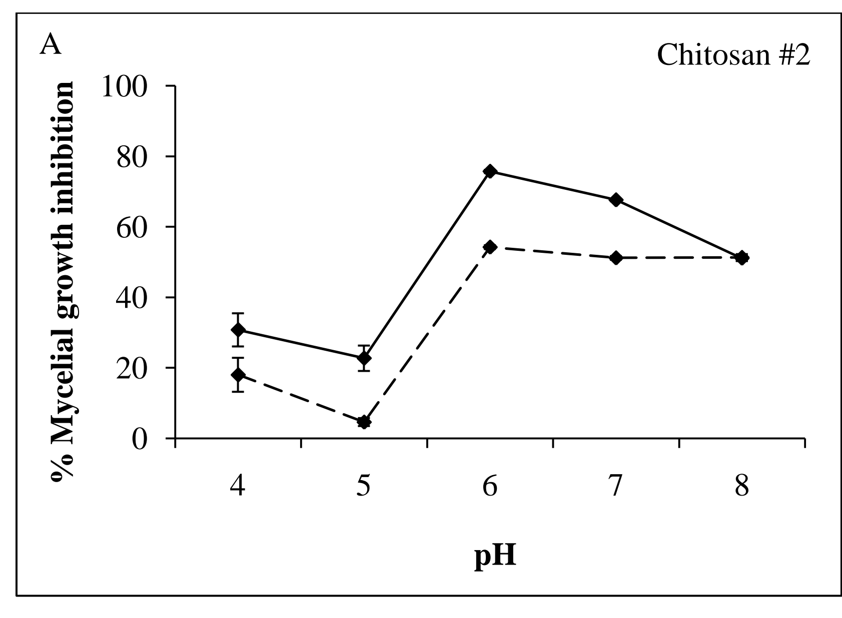

B
% Mycelial growth inhibition
100
80
60
40
20
0
*
Ch # 2
Ch 86
AZ
Non dialyzed
Dialyzed

Figure 5.9. Antifungal activity of dialyzed Chitosan #2 (3-4 kDa) and Chitosan 86 (190-375 kDa). A) Experiment 1 and B) experiment 2. Solid bars are non-dialyzed chitosan and empty bars dialyzed chitosan. Both chitosan were evaluated at 250 µg/ml. Quadris® 50 WG (AZ) was used as a as a positive control at concentration of 2 µg/ml. The percent of mycelial growth inhibition (mgi) related to the control (non-treated conidia) was determined using the absorbance ($OD_{450\ nm}$) after 48 h of incubation. Acetic acid (AA) was used as a negative control to measure the mycelium growth inhibition of Chitosan 86. The mgi was calculated using the following formula: mgi = 100*[1-(A_n/A_0)]; where A_n is the absorbance of each treatment and, A_0 is the absorbance of the control (non-treated conidia). Error bars indicate standard errors. *Denotes statically significant differences according to a *t* test ($P \leq 0.05$).



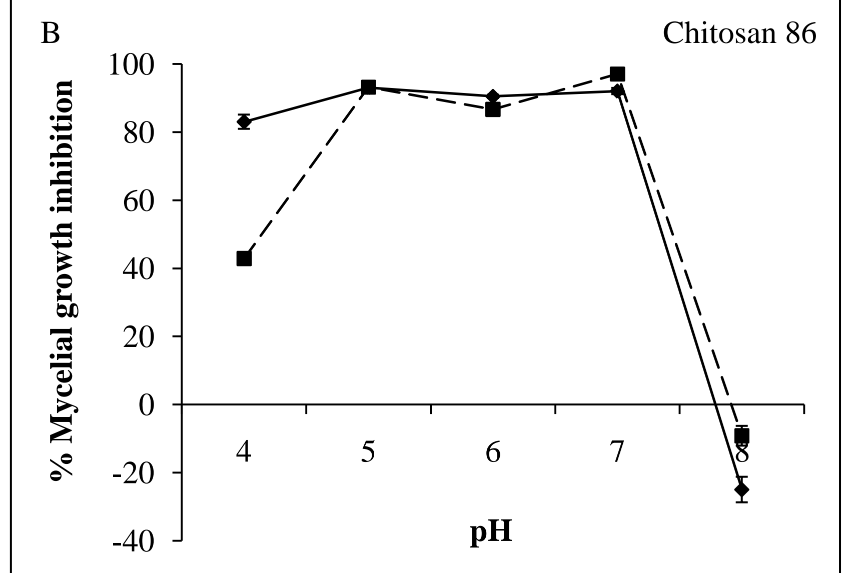
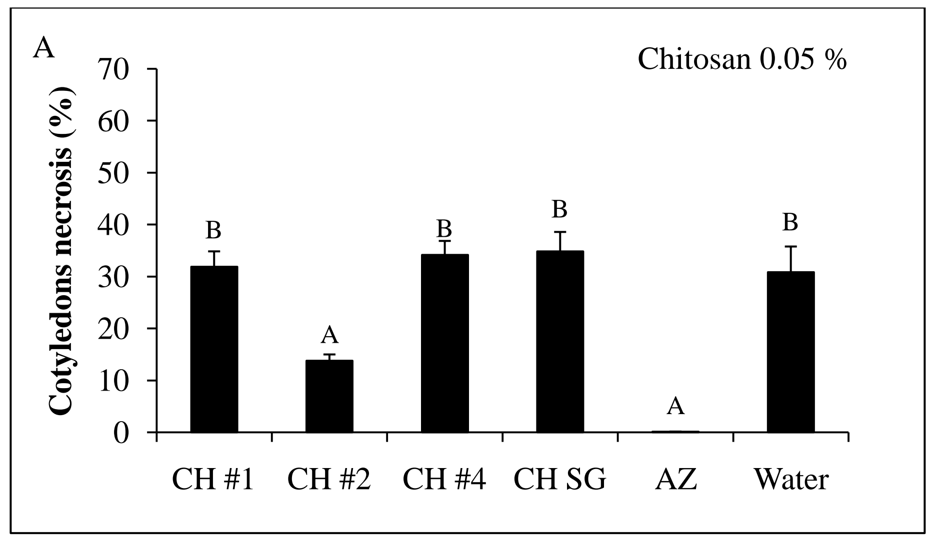
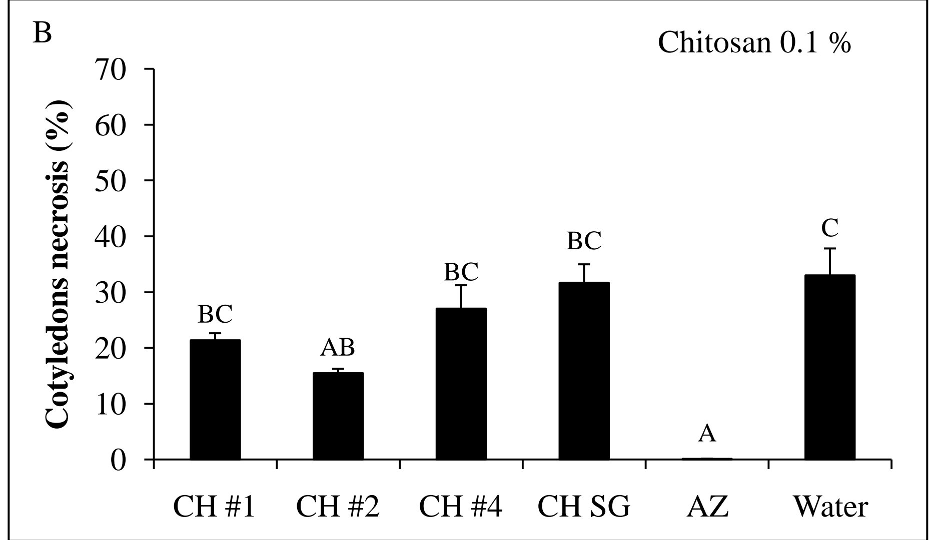


Figure 5.10. Antifungal activity of chitosan #2 and 86 at different pH. A) Chitosan #2 and B) chitosan 86. Solid lines are experiment 1 and dash lines are experiment 2. The percent of mycelial growth inhibition (mgi) related to the negative control (non-treated conidia) was determined using the absorbance ($OD_{450\ nm}$) after 48 h of incubation. Acetic acid (AA) was used as a negative control to measure the mycelium growth inhibition of Chitosan 86. The mgi was calculated using the following formula: mgi = $100*[1-(A_n/A_0)]$; where A_n is the absorbance of each treatment and, A_0 is the absorbance of the control (non-treated conidia). Error bars indicate standard errors.


A
Chitosan 0.05 %
Cotyledons necrosis (%)
70
60
50
40
30
20
10
0
B
A
B
B
A
B
CH #1
CH #2
CH #4
CH SG
AZ
Water


B
Chitosan 0.1 %
Cotyledons necrosis (%)
70
60
50
40
30
20
10
0
BC
AB
BC
BC
A
C
CH #1
CH #2
CH #4
CH SG
AZ
Water

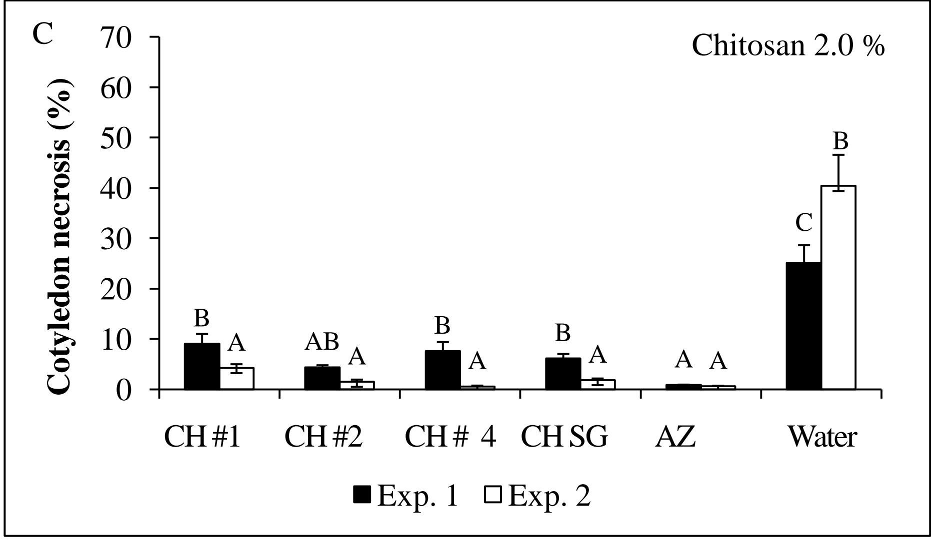

C
Chitosan 2.0 %
Cotyledon necrosis (%)
70
60
50
40
30
20
10
0
B
A
AB
A
B
A
B
A
A
A
C
B
CH #1
CH #2
CH # 4
CH SG
AZ
Water
Exp. 1
Exp. 2

Figure 5.11. Disease protection provided by water-soluble chitosan on cantaloupe. A) Plants treated with chitosan at 0.05%, B) plants treated with chitosan at 0.1%, and C) plants treated with chitosan at 2.0%. Cantaloupe plants were sprayed with the water-soluble chitosan solutions and later inoculated with 10^6 conidia/ml of *Colletotrichum orbiculare*. Five days post-inoculation the percentage of cotyledons necrosis was determined. Error bars indicate standard errors. Within a concentration, mean values followed by the same letter do not differ significantly (Fisher's LSD, P=0.05). CH #1 (chitosan #1), CH #2 (chitosan # 2), CH #4 (chitosan #4), CH SG (chitosan SG), AZ (Quadris).

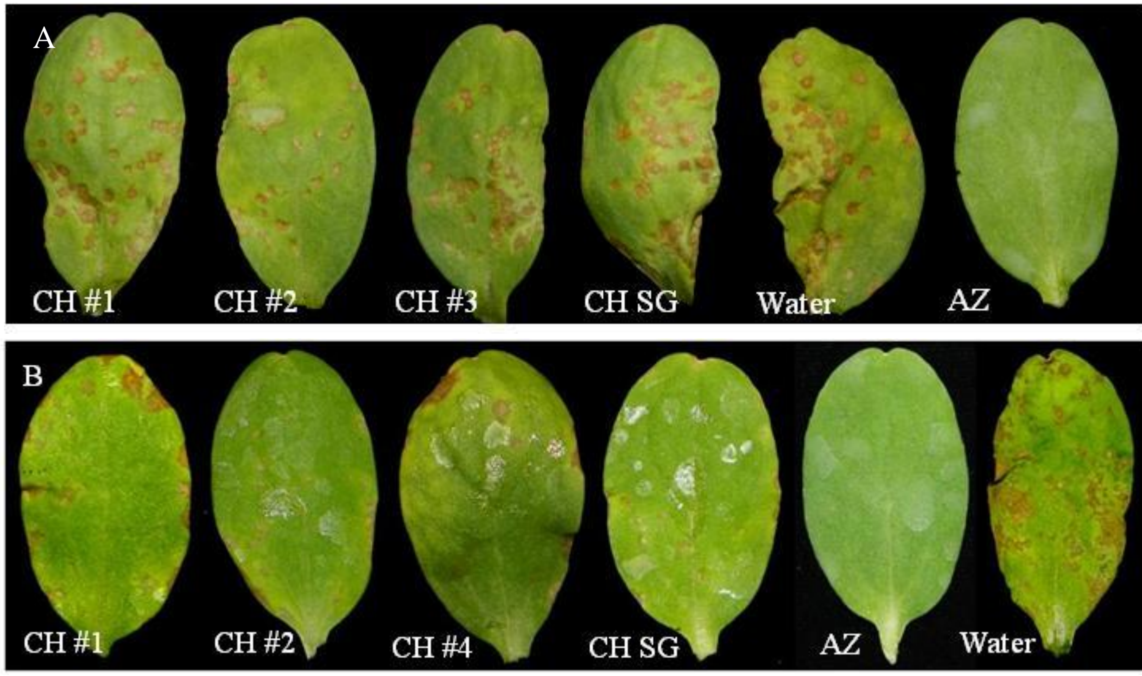
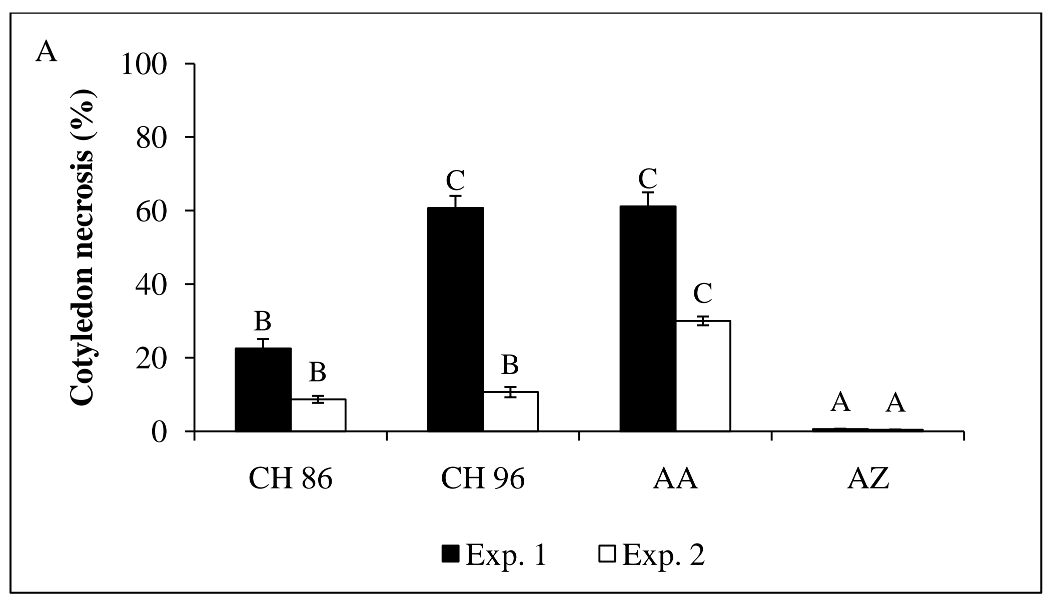


Figure 5.12. Representative cotyledons treated with water-soluble chitosan. A) Representative pictures of cotyledons treated with water soluble chitosan 0.05% and B) representative pictures of cotyledons treated with chitosan at 2%. Cantaloupe plants were sprayed with the water-soluble chitosan solutions and later inoculated with 10^6 conidia/ml of *Colletotrichum orbiculare*. Five days post-inoculation the percentage of cotyledons necrosis was determined. CH #1 (chitosan #1), CH #2 (chitosan # 2), CH #3 (chitosan #3), CH SG (chitosan SG), AZ (Quadris).



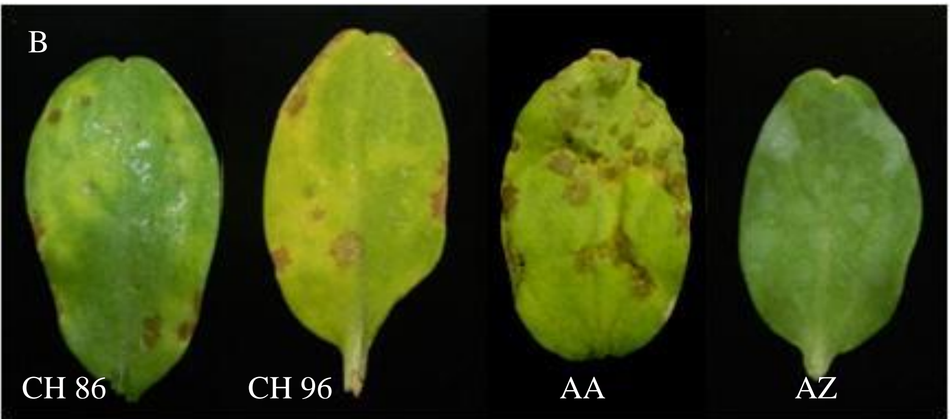


Figure 5.13. Evaluation of disease control provided by acid-soluble chitosan. A) Percentage of cotyledon necrosis and B) representative pictures of cotyledons. Solid bars are experiment 1 and empty bars are experiment 2. Cantaloupe plants were sprayed with the acid soluble chitosan solutions and later inoculated with 10^6 conidia/ml of *Colletotrichum orbiculare*. Five days post-inoculation the percentage of cotyledons necrosis was determined. All acid soluble chitosan were evaluated at 0.1%. Error bars indicate standard errors. Means values within an experiment followed by the same letter do not differ significantly (Fisher's LSD, *P*=0.05). CH 86 (chitosan #1), CH 96 (chitosan # 2), AA (Acetic acid), AZ (Quadris).

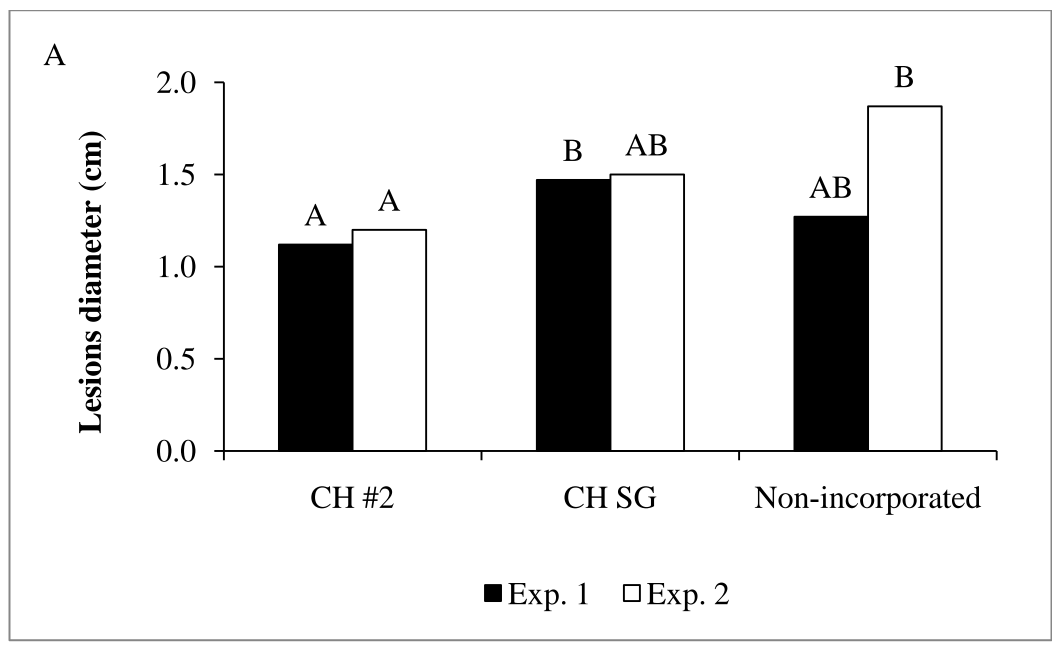
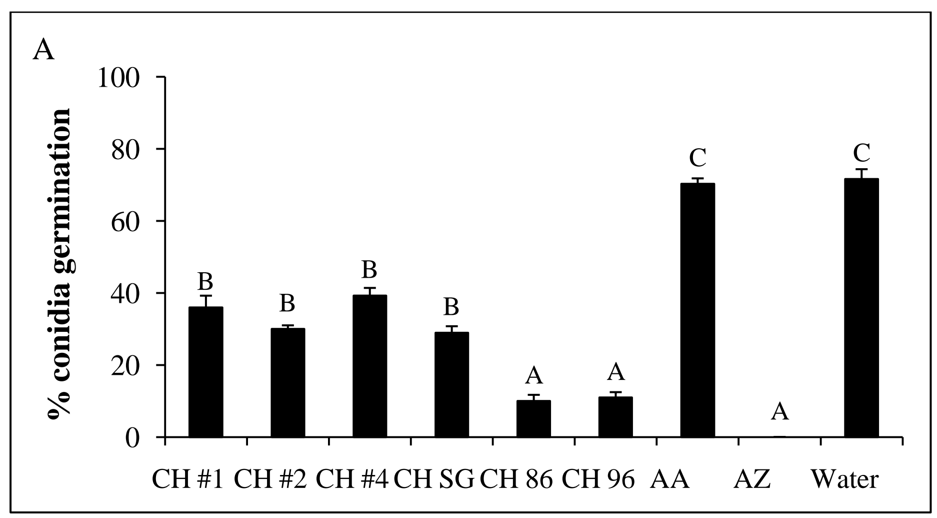


Figure 5.14. Induction of resistance by chitosan incorporated into the soil. A. Lesion diameter and B) representative pictures of cotyledons from experiment 2. Solid bars are experiment 1 and empty bars are experiment 2. Means values within an experiment followed by the same letter do not differ significantly (Fisher's LSD, *P*=0.05). CH #2 (chitosan # 2), CH SG (chitosan SG).



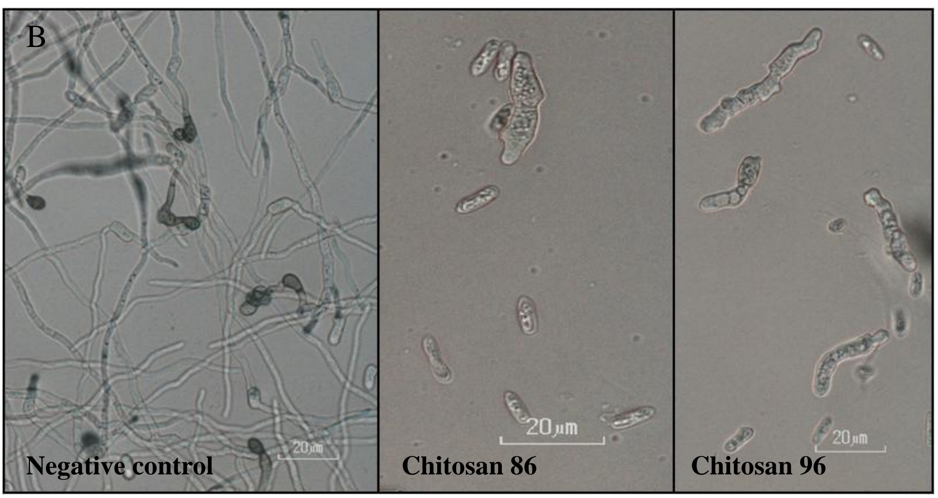


Figure 5.15. Effect of chitosan on conidial germination of *Colletotrichum orbiculare*. A) The percentage of conidia germination was determined 24 h after incubation with chitosan solutions (2% v/v) and B) representatives pictures of the negative control and conidia treated with chitosan 86 and chitosan 96. Quadris® 50 WG was used as a positive control at concentration of 2 µg ai/ml. Error bars indicate standard errors. Means values followed by the same letter do not differ significantly (Fisher's LSD, P=0.05). CH #1 (chitosan #1), CH #2 (chitosan # 2), CH #3 (chitosan #3), CH SG (chitosan SG), AA (acetic acid), AZ (Quadris).

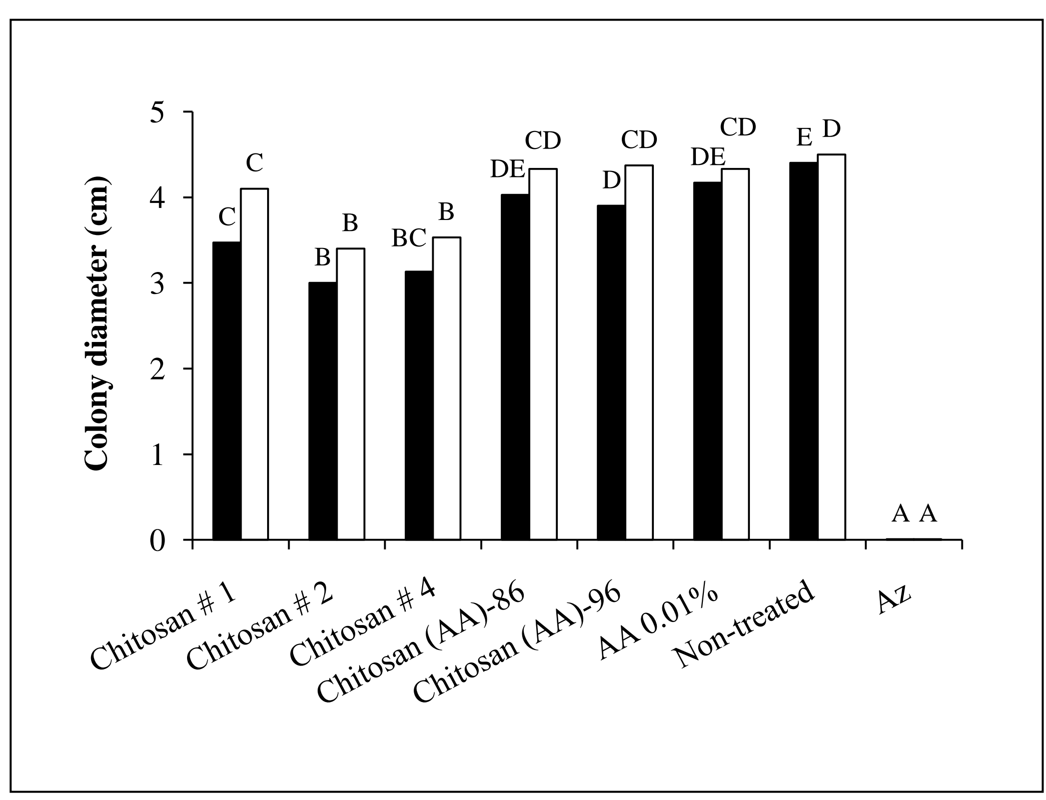


Figure 5.16. Antifungal activity of chitosan incorporated into culture media. Solid bars are experiment 1 and empty bars are experiment 2. All water soluble-chitosans were tested at 2% and the acid-soluble chitosans at 0.1%. Quadris® 50 WG (Az) was used as a positive control at concentration of 2 µg/ml. Error bars indicate standard errors. Means within an experiment followed by the same letter do not differ significantly (Fisher's LSD, *P*=0.05). AA (acetic acid).

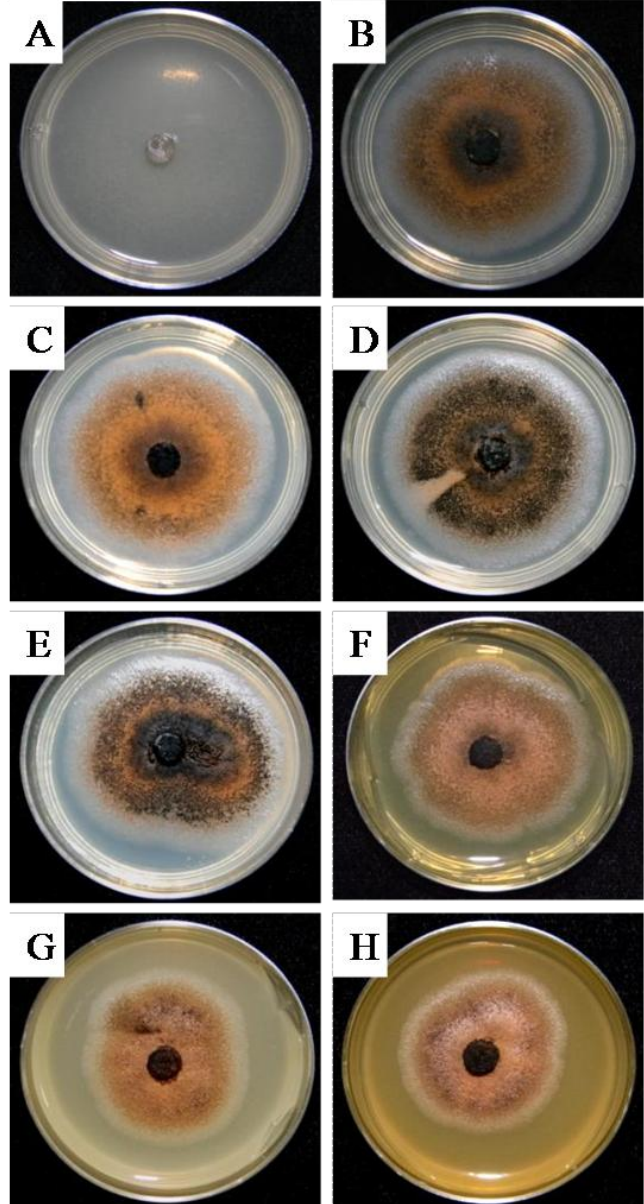


Figure 5.17. Colony phenotypes of *Colletotrichum orbiculare* on media amended with chitosan. A) Positive control (Quadris® 50 WG), B) non-amended media, C) acetic acid, D) chitosan 96, E) chitosan 86 acid, F) chitosan 1 G) chitosan #2 and H) chitosan #3.

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APPENDIX 1

Table 1. Morphological characterization of *Colletotrichum orbiculare* grown in potato dextrose agar.

Variable[a]	Mean	Min.	Max
Long conidia	10.2	8.5	12.1
Wide conidia	4.0	2.9	4.6
Long appressorium	13.5	8.8	17.8
Wide appressorium	8.2	6.3	11.0

[a] One hundred conidia and appressorium were randomly selected to be measured.

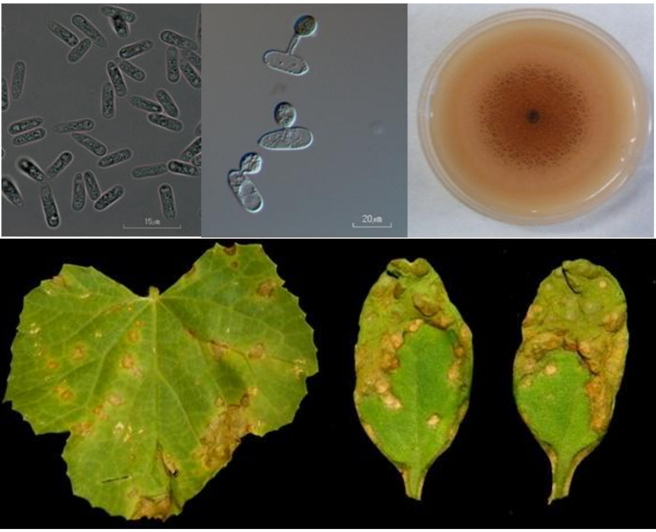

Figure 1. *Colletotrichum orbiculare.* A) Conidia, B) Melanized and non-melanized appressorium, C) *C. orbiculare* grown in green beans agar media and D) Symptoms caused by the fungi in cantaloupe leaves and cotyledons after 5 days post inoculation.

***Colletotrichum orbiculare* ITS4**

```
AATTGGGGTTCCTACCTGATCCGAGGTCACCTGTAAAGAATTTGGGGGTT
TAACGGCAAGAGTCCCTCCGGATCCCAGTGCGAGACGTTAGTTACTACGC
AAAGGAGGCTCCGGGAGGGTCCGCCACTACCTTTAAGGGCCCACGTCGGC
CGTGGGGCCCCAAAACCAAGCGGTGCTTGAGGGTTGAAATGACGCTCGAA
CAGGCATGCTCGCCAGAATGCTGGCGAGCGCAATGTGCGTTCAAAGATTC
GATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCG
TTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTAAAAGTTTTAATTA
TTTGCTTGTGCCACTCAGAAGAGACGTCGTGTAAATAGAGTTTGGTTTCC
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CCCGCGAGAGGCTTCCCCTGCCCGCCGAAGCAACGGTTAGGTACGTTCAC
AGGTGTTATATAGCGGCAAA
```

APPENDIX 2

University of Kentucky

Field Experiment 2010 'Paragon' Tomato

```
Trial ID: Merari2010               Protocol ID: Merari2010
Location: South Farm           Study Director: Merari Feliciano
                                 Investigator: Kenny Seebold
```

Block	1	2	3	4	5	6	7	8	9	10	11
	10 101	1 102	3 103	7 104	6 105	8 106	11 107	9 108	4 109	5 110	2 111
	2 201	6 202	8 203	4 204	1 205	10 206	9 207	11 208	7 209	3 210	5 211
	11 301	9 302	3 303	7 304	10 305	5 306	2 307	1 308	8 309	6 310	4 311
	4 401	5 402	2 403	6 404	11 405	10 406	7 407	8 408	1 409	9 410	3 411

Figure 1. Treatments arrangement in a Randomized Complete Block Design with four replicates. The same arrangement was used for all trials.

University of Kentucky

Field Experiment 2010 'Paragon' Tomato

Trial ID: Merari2010 | Protocol ID: Merari2010
Location: South Farm | Study Director: Merari Feliciano
Investigator: Kenny Seebold

Reps: 4 | Plots: 10 by 20 feet
Spray vol: 40 gal/ac | Mix size: 3 liters (min 2.7808)

Trt No.	Type	Treatment Name	Form Conc	Form Unit	Form Type	Rate	Rate Unit	Appl Code	Appl Description	Amt Product to Measure	Plot No. By Rep 1	2	3	4
01	FUNG	Sonata	1.38	%	SC	4	qt/a	ABCDEFGH	7 day schedule	74.99 ml/mx	102	205	308	409
02	FUNG	Kocide 3000	46	%	DF	1	lb/a	ABCDEFGH	7 day schedule	8.987 g/mx	111	201	307	403
03	FUNG	Trilogy	5.46	LB/GAL	SL	2.0	% v/v	ABCDEFGH	7 day schedule	59.99 ml/mx	103	210	303	411
04	FUNG	Serenade	14.6	%	WP	3	lb/a	ABCDEFGH	7 day schedule	26.96 g/mx	109	204	311	401
05	FUNG	Bordeaux	7	%	WP	4	oz wt/gal	ABCDEFGH	7 day schedule	89.87 g/mx	110	211	306	402
06	FUNG	Chitosan	85	%	WP	2	% w/v	ABCDEFGH	7 day schedule	59.99 g/mx	105	202	310	404
07	FUNG	Regalia	5	%	SC	2	qt/a	ABCDEFGH	7 day schedule	37.5 ml/mx	104	209	304	407
08	FUNG	Quadris	50	%	WG	3.2	oz/a	ABCDEFGH	7 day schedule	1.797 g/mx	106	203	309	408
09	FUNG	Chitosan SG	85	%	WG	2	% w/v	ABCDEFGH	7 day schedule	59.99 g/mx	108	207	302	410
10	FUNG	Water									101	206	305	406
11	FUNG	Lime sulfur				3.25	fl oz/gal	ABCDEFGH	7 day schedule	Unknown Fm Ds	107	208	301	405

Sort Order: Treatment

Product quantities required for listed treatments and applications in one trial:

Amount*	Unit	Treatment Name	Form Conc	Form Type	Lot Code
749.919	ml	Sonata	1.38	SC	
89.869	g	Kocide 3000	46	DF	
599.935	ml	Trilogy	5.46	SL	
269.608	g	Serenade	14.6	WP	
898.698	g	Bordeaux	7	WP	
599.935	g	Chitosan	85	WP	
374.959	ml	Regalia	5	SC	
17.974	g	Quadris	50	WG	
599.935	g	Chitosan SG	85	WG	

* 'Per area' calculations based on spray volume= 40 gal/ac, mix size= 3 liters (mix size basis).
* Product amount calculations increased 25 % for overage adjustment.
* 'Per volume' calculations use spray volume= 40 gal/ac, mix size= 3 liters.
* Adjusted for multiple applications in treatment list.

Trial Comments

Table 1. Spray plan and the amount required for each application.

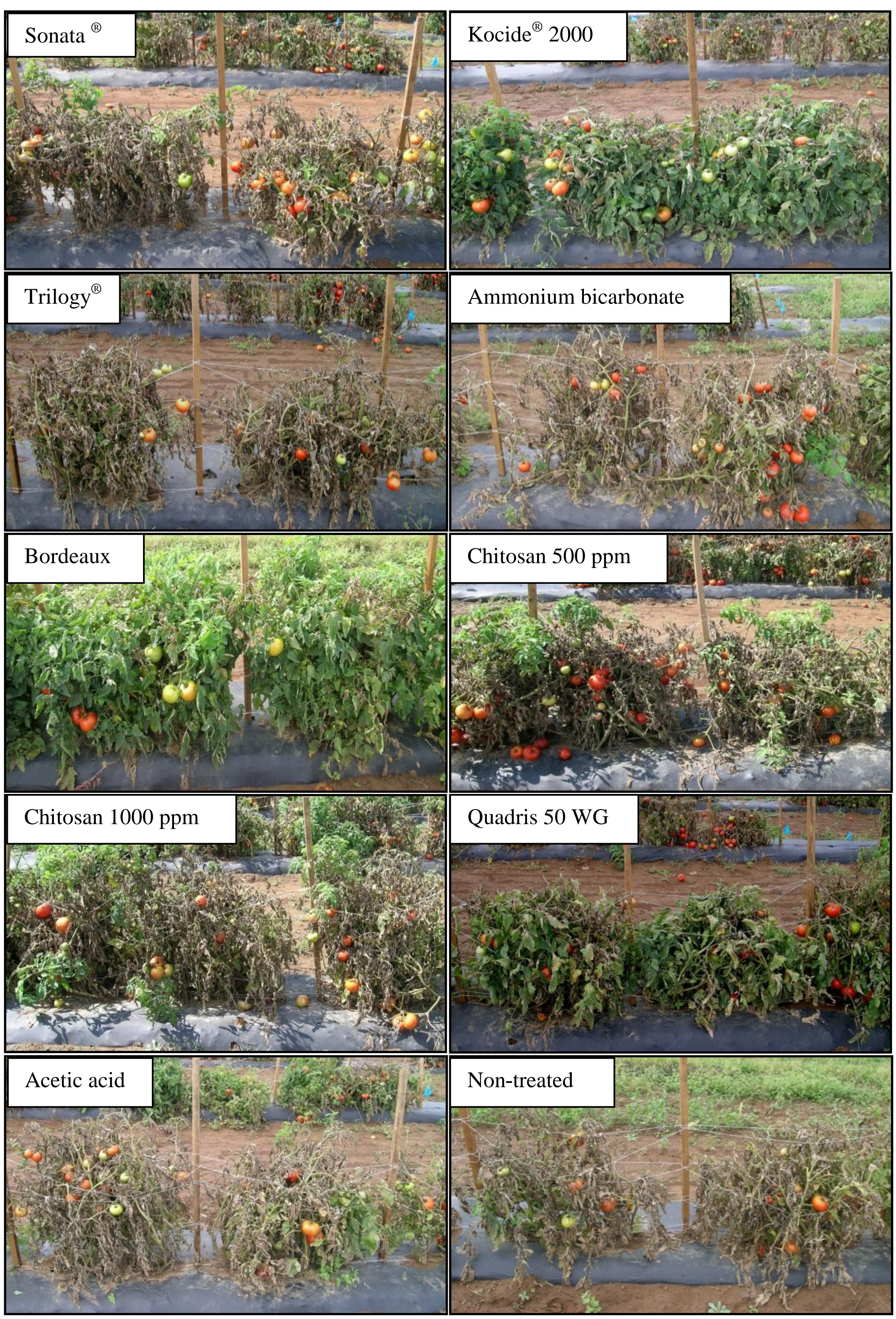

Figure 2. Representative picture of treatments plot from experiment I in 2009.

Figure 3. Representative picture of treatments plot from experiment II in 2010.

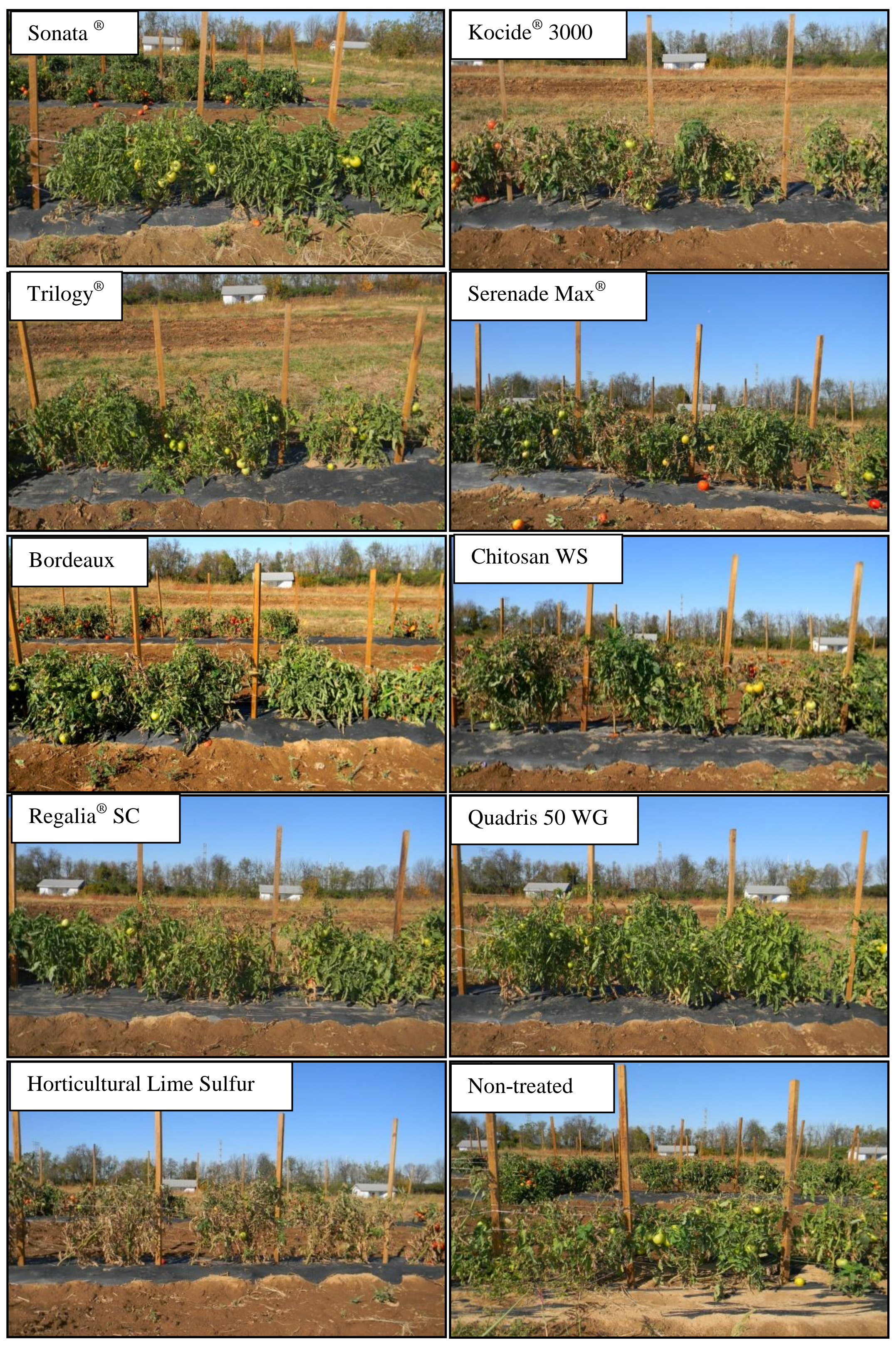

Figure 4. Representative picture of treatments plot from experiment III in 2010.

Variable	N	R^2	R^2 Aj	CV	
AUDPC stand	40	0.75	0.64	27.81	
Analysis of Variance (SC type III)					
F.V.	**SC**	**gl**	**CM**	**F**	**p-valor**
Model	2589.39	12	215.78	6.69	<0.0001
Treatments	2283.99	9	253.78	7.87	<0.0001
Block	305.4	3	101.8	3.16	0.0409
Error	870.51	27	32.24		
Total	3459.9	39			

Test:LSD Fisher Alfa=0.05 DMS=8.23820					
Error: 32.2413 gl: 27					
Treatments	Means	n			
2	8.85	4	A		
8	9.19	4	A		
5	11.11	4	A		
7	20.49	4		B	
3	20.58	4		B	
6	23.98	4		B	C
10	25.07	4		B	C
1	26.88	4		B	C
9	28.4	4		B	C
4	29.62	4			C

Test:LSD Fisher Alfa=0.05 DMS=5.21030				
Error: 32.2413 gl: 27				
Block	Means	n		
3	16.12	10	A	
2	20.29	10	A	B
4	21.57	10		B
1	23.69	10		B

Figure 5. Analysis of variance of sAUDPC for field experiment I.

Variable	**N**	**R²**	**R² Aj**	**CV**		
AUDPC stand	40	0.53	0.31	41.17		
Analysis de la Variance (SC type III)						
F.V.	SC	gl	CM	F	p-valor	
Model	9178.5	12	764.88	2.49	0.0237	
Treatments	7521.91	9	835.77	2.73	0.0211	
Blocks	1656.59	3	552.2	1.8	0.1709	
Error	8279.99	27	306.67			
Total	17458.5	39				

Test:LSD Fisher Alfa=0.05DMS=25.40736						
Error: 306.6663 gl: 27						
Treatments	Medias	n				
2	22.2	4	A			
5	24.28	4	A	B		
8	26.2	4	A	B	C	
7	40.68	4	A	B	C	D
3	43.65	4	A	B	C	D
1	46.23	4	A	B	C	D
6	47.8	4		B	C	D
11	50.93	4			C	D
10	58.9	4				D
4	64.5	4				D

Test:LSD Fisher Alfa=0.05DMS=16.06902						
Error: 306.6663 gl: 27						
Blocks	Medias	n				
2	32.12	10	A			
1	42.9	10	A	B		
4	45.75	10	A	B		
3	49.37	10		B		

Figure 6. Analysis of variance of sAUDPC for field experiment II

Variable	**N**	**R^2**	**R^2 Aj**	**CV**	
AUDPC STAND	40	0.72	0.59	47.07	
Analysis de la Variance (SC type III)					
F.V.	SC	gl	CM	F	p-valor
Model	7685.95	12	640.5	5.7	0.0001
Treatments	7631.81	9	847.98	7.55	<0.0001
Blocks	54.14	3	18.05	0.16	0.9219
Error	3033.92	27	112.37		
Total	10719.9	39			

Test:LSD Fisher Alfa=0.05 DMS=15.37965					
Error: 112.3673 gl: 27					
Treatments	Means	n			
8	7.94	4	A		
5	13.89	4	A	B	
2	14.44	4	A	B	
7	17.33	4	A	B	
1	18.71	4	A	B	
3	19.14	4	A	B	
10	22.37	4	A	B	
4	22.73	4	A	B	
6	27.7	4		B	
11	60.94	4			C

Test:LSD Fisher Alfa=0.05 DMS=9.72694					
Error: 112.3673 gl: 27					
Blocks	Means	n			
III	20.83	10	A		
IV	22.22	10	A		
II	23.04	10	A		
I	24	10	A		

Figure 7. Analysis of variance of sAUDPC for field experiment III.

APPENDIX 3

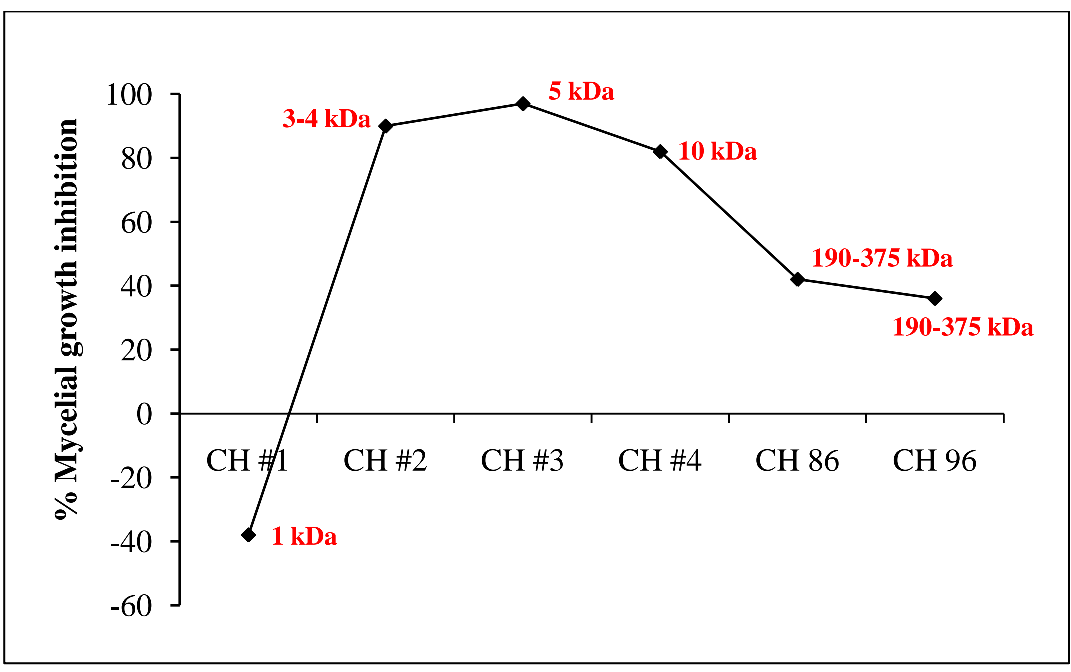

Figure 1. Comparison of the antifungal activity of chitosan with different molecular weight. All chitosan compared at 200 µg/

LITERATURE CITED

Abd-El-Karem, F., El-Mougy, N. S., El-Gamal, N. G. and Fotouh, Y. O. 2006. Use of chitin and chitosan against tomato root rot disease under greenhouse conditions. Journal of Agriculture and Biological Sciences 2(4): 147-152.

Agrawal, G. K., Rakwal, R., Tamogami,S., Yonekura, M., Kubo, A. and Saji, H. 2002. Chitosan activates defense/stress responses in the leaves of *Oryza sativa* seedlings. Plant Physiol. Biochem. 40:1061-1069.

Agrios, G. N. 2005. Plant Pathology. 5ta ed. Elsevier Academic Press Publication. San Diego, California. 922 pp.

Ait, B. E., Eullaffroy, P., Clément, C. and Vernet, G. 2004. Chitosan improves development and protects *Vitis vinifera* L. against *Botrytis cinerea*. Plant Cell Rep 22:608-614.

Anonymous. 1996. RPD No. 920-Anthracnose of cucumber, muskmelon, watermelon, and other cucurbits. Integrated Pest Management (IPM). Reports on Plant Diseases. University of Illinois at Urbana-Champaign, Extension. 2pp.

Aziz, A., Trotel-Aziz, P., Dhuicq, L., Jeandet, P., Couderchet, M., and Vernet, G. 2006. Chitosan oligomers and copper sulfate induce grapevine defense reactions and resistance to gray mold and downy mildew. Phytopathology 96:1188-1194.

Baird, S., Scoles, M., Bellows, B. and Ferry, E. 2005. Souther Organic Resource Guide: A Reference Handbook of Organic Resources in the South Arkansas, Kentucky, Louisiana, Mississippi, and Tennessee. *Independent Organic Inspectors Association (IOIA) and National Center for Appropriate Technology (NCAT).*138 pp.

Baley, J. A., Nash, C., Morgan, L. W., and O'Connell, R. J. 1996. Molecular taxonomy of *Colletotrichum* species causing anthracnose on the Malvaceae. Phytopathology 86:1076-1083.

Bautista, B. S., Hernández, L. A. N., Velázquez, V. M. G., Hernández, L. M. Ait, B. E., Bosques, M. E. and Wilson, C. L. 2006. Chitosan as a potential natural compound to control pre and postharvest diseases of horticultural commodities. Crop Protection 25:108-118.

Bautista, B. S., Hernández, L. M., Bosques, E. and Wilson, C. L. 2003. Effect of chitosan and plant extracts on growth of *Colletotrichum gloesporioides*, anthracnose levels and quality of papaya. Crop Protection 22:1087-1092.

Baysal, F., McSpadden-Gardener, B., Cardina, J., Kleinhenz, M. and Miller, S. 2009. Effect of field management practices on disease development, soil chemistry and yield in organic tomatoes. Acta Hort. (ISHS) 808:113-116.

Bell, A. A., Hubbard, J. C., Liu, L., Davis, R. M. and Subbarao, K. V. 1998. Effect of chitin and chitosan on the incidence and severity of Fusarium yellows of celery. Plant Dis. 82:322-328.

Benhamou, N., Kloepper, J. W. and Tuzum, S. 1998. Induction of resistance against *Fusarium* wilt of tomato by combination of chitosan with an endophytic bacterial strain: ultrastructure and cytochemistry of the host response. Planta 204:153-168.

Ben-Shalom, N., Ardi, R., Pinto, R., Aki, C. and Fallik, E. 2003. Controlling gray mold caused by *Botrytis cinerea* in cucumber plants by means of chitosan. Crop Protection 22:285-290.

Bouchra, C., Mohamed, A., Mina, I. H. and, Hmamouchi, M. 2003. Antifungal activity of essential oils from several medicinal plants against four postharvest citrus pathogens. Phytopathol. Mediterr., 42:251-256.

Brammall, R. A. 1993. Effect of foliar fungicides treatment on early blight and yield of fresh market tomato in Ontario. Plant Dis. 77:484-488.

Burpee, L. and Latin, R. 2008. Reassessment of fungicide synergism for control of dollar spot. Plant Dis. 92:601-606.

Ceylan, E. and Fung, D. Y. C. 2004. Antimicrobial activity of spices. Journal of rapid methods and automation in microbiology 12:1-55.

Chaerani, R. and Voorrips, R. E. 2006. Tomato early blight (*Alternaria solani*): the pathogen, genetics, and breeding for resistance. Journal of General Plant Pathology 72(6):335-347.

Clarete, C. E., Aparecida, B. E., Florentino, P. S. and Cia, P. 2007. Avaliacao de quitosana, aplicada em pos-colheita, na protecao de uva Italia contra *Botrytis cinérea*. Summa Phytopathol. 33(3):215-221.

Coakley, S. M., Scherm, H., Chakraborty, S. 1999. Climate change and plant disease management. Annu. Rev. Phytopathol. 37:399-426.

Cohen, Y. and Gisi, U. 1993. Uptake, translocation and degradation of ^{14}C-cymoxanil in tomato plants as affected by mancozeb and oxadixyl. Crop Prot. 12:284-292.

Coolong, T., Masabni, J., Strang, J., Jones, T. Bessin, R. and Seebold, K. 2008. An IPM Scouting Guide for Common Pests of Solanaceous Crops in Kentucky. University of Kentucky, Kentucky Cooperative Extension: ID-172. 28 pp.

Coolong, T., Pfeiffer, J., Slone, D., and Poston, A. L. 2009. Fresh Market Tomato Variety Performance in 2009. University of Kentucky, Lexington, KY 40546. 4 pp.

Couch, H. B. and Smith, B. D. 1991. Synergistic and antagonistic interactions of fungicides against *Pythium aphanidermatum* on perennial ryegrass. Crop Protection 10(5):386-390.

Cuero, R. G. Osuji, G. and Washington, A. 1991. N-carboxymethyl chitosan inhibition of aflatoxin production: role of zinc. Biotechnol. Lett. 13:41-44.

Daeschel, M. A. 1992. Procedures to detect antimicrobial activities of microorganisms. Food Biopreservatives of Microbial Origin. B. Ray and M. A. Daeschel, eds. CRC Press, Boca Raton, FL. Pages 57-80.

Daferera, D. J., Ziogas, B. N. and, Polossiou, M. G. 2000. GC-MS analysis of essential oils from some greek aromatic plants and their fungitoxicity on *Penicillium digitatum*. J. Agric. Food Chem. 48:2576-2581.

De Waard, M. A. 1996. Synergism and antagonism in fungicide mixtures containing sterol demethylation inhibitors. Phytopathology 86:1280-1283.

Delate, K. 2003. Fundamentals of Organic Agriculture. Iowa State University, PM 1880. http://www.extension.iastate.edu/Publications/PM1880.pdf

Delespaul, Q., De Billerbeck, V. G., Roques, C. G., Michel, G., Marquier-Vinuales, C. and, Bessier, J. M. 2000. The antifungal activity of essential oils as determined by different screening methods. Journal of Essential Oil Research 12:256-266.

Dimitri, C. and Oberholtzer, L. 2009. Marketing U.S. organic foods: recent trends from farm to consumers. Economic Information Bulletin 58.U. S. Dept. of Agriculture, Economic Research Service.

Diver, S., Kuepper, G. and Born, H. 1999. Organic tomato production. ATTRA 800-346-9140.

Doares, S. H., Syrovetst, T., Weilert, E. W. and, Ryan, C. A.1995. Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. Proc. Natl. Acad. Sci. 92:4095-4098.

Dutta, P. K., Dutta, J. and Tripathi, V. S. 2004. Chitin and chitosan: Chemistry, properties and applications. Journal of Scientific & Industrial Research 63:20-31.

Edelson, J. V. and Magaro, J. J. 1988. Development of onion thrips, *Thrips tabaci* Lindeman, as a function of temperature. *Southwest Entomol*. 13:171-176.

El Ghaouth A, Arul J, Benhamou N, Asselin A, Be´langer RR. 1994. Effect of chitosan on cucumber plants: suppression of *Pythium aphanidermatum* and induction of defense reactions. *Phytopathology* 84:313–320.

El Ghaouth, A, Arul J, Grenier, J. and, Asselin, A. 1992. Antifungal activity of chitosan on two postharvest pathogens of strawberry fruits. Phytopathology 82:398-402.

El Ghaouth, A., Arul J., Wilson, C. and, Benhamou, N. 1994. Ultrastructure and cytochemical aspects of the effect of chitosan on decay of bell pepper fruit. Physiological and Molecular Plant Pathology 44:417-432.

El-Ghaouth, A., Smilanick, J. L., Brown, G. E., Ippolito, A., Wisniewski, M., and Wilson, C. L. 2000. Application of *Candida saitoana* and glycolchitosan for the control of postharvest diseases of apple and citrus fruit under semi-commercial conditions. Plant Dis. 84:243-248.

EPA. (2002). 2000-2001 pesticide market estimates: Historical data. Retrieved from: http://www.epa.gov/oppbead1/pestsales/01pestsales/historical_data2001_3.htm

EPA. 2006. Re-registration eligibility decision (RED) for coppers. EPA 738-R-06-020.

EPA.2011. Chitosan; Poly-D-glucosamine (128930) Fact Sheet. http://www.epa.gov/opp00001/biopesticides/ingredients/factsheets/factsheet_128930.htm

Fandohan, P., Gbenou, J. D., Gnonlonfin, B., Hell, K., Marasas, W. F. O., and Wingfield, M. J. 2004. Effect of essential oils on the growth of *Fusarium verticillioides* and fumonisin contamination in corn. J. Agric. Food Chem 52:6824-6829.

Fang, L. X., Qiang, F. X., Yang, S., Pu, W. T. and Xing, S. Z. 2008. Effects of molecular weight and concentration of chitosan on antifungal activity against *Aspergillus niger*. Iranian Polymer Journal 17(11):843-852.

FAOSTAT. 2009. http://faostat.fao.org/

Farr, D. F., Bills, G. F., Chamuris, G. P., and Rossman, A. Y. 1989. Fungi on plants and plant products in the United States. St Paul: APS Press.

Farag, R. S., Daw, Z. Y., Hewedi, F. M. and, EL-Baroty, G. S. A. 1989. Antimicrobial activity of some Egyptian spice essential oils. J. Food Prot. 52:665-667.

Feng, W. and Zheng, X. 2005. Essential oils to control *Alternaria alternata in vitro* and *in vivo*. Food Control 18:1126-1130.

FRAC. 2009. FRAC Code List©: Fungicides sorted by mode of action (including FRAC Code numbering). http://www.frac.info/frac/index.htm

Gisi, U. 1996. Synergistic interaction of fungicides mixtures. Phytopathology 86(11):1273-1279.

Gleason, M. L., Sisson, A. J., Massman, J. M. and Mueller, D. S. 2004. Control of anthracnose of muskmelon with fungicides in MELCAST warning system. F&N Test Vol 60:V076.

Gold, M. V. 2007. Alternative farming systems information center. USDA. Online publication. http://www.nal.usda.gov/afsic/pubs/ofp/ofp.shtml

Gul, S. A. and Reddy, A. S. N. 2000. Inhibition of fungal and bacterial plant pathogens by synthetic peptides: *In Vitro* growth inhibition, interaction between peptides and inhibition of disease progression. MPMI 13(8):847–859.

Hadwiger, L. A., and McBride, P. O. 2006. Low-level copper plus chitosan applications provide protection against late blight of potato. Online. Plant Health Progress doi:10.1094/PHP-2006-0406-01-RS.

Hawthorne, S. B., Rickkola, M.-L., Screnius, K., Holm, Y., Hiltunen, R. and Hartonen, K. 1993. Comparison of hydrodistillation and supercritical fluid extraction for the determination of essential oils in aromatic plants. J. Chromatogr 634:297-308.

Herath, H. and Abeywickrama, K.. 2008. *In vitro* application of selected essential oils and their major components in controlling fungal pathogens of crown rot in Embul banana (*Musa acuminata*– AAB). International Journal of Food Science and Technology 43 (3):440–447.

Hervieux, V., Yaganza, E. S., Arul, J., and Tweddell, R. J. 2002. Effect of organic and inorganic salts on the development of *Helminthosporium solani*, the causal agent of potato silver scurf. Plant Dis. 86:1014-1018.

Hirano, A. and Nagao, N. 1989. Effects of chitosan, pectic acid, lysozyme, and chitinase on the growth of several phytopathogens. Agri. Biol. Chem. 11: 3065-3066.

Homma, Y., Arimoto, Y., and Misato, T. 1981. Studies on the control of plant diseases by sodium bicarbonate formulation. 2. Effect of sodium bicarbonate on each growth stage of cucumber powdery mildew fungus (*Sphaerotheca fuliginea*) in its life cycle. J. Pestic. Sci. 6:201-209.

Horsfall, J. G. and Heuberger, J. W. 1942. Causes, effects and control of defoliation on tomatoes. Conn. Agric. Exp. Stn. Bull. 456:184-223.

Horst, R. K., Kawamoto, S. O., and Porter, L. L. 1992. Effect of sodium bicarbonate and oils on the control of powdery mildew and black spot of roses. Plant Dis. 76:247-251.

Ingram, D. T. and Millner, P. D. 2007. Factors affecting compost tea as a potential source of *Escherichia coli* and *Salmonella* on fresh produce. Journal of Food Protection 70(4):828-834.

Iriti, M. and Faoro, F. 2008. Abscisic acid is involved in chitosan-induced resistance to tobacco necrosis virus (TNV). Plant Physiology and Biochemistry 46:1106-1111.

Isman, M. B. 2000. Plant essential oils for pest and disease management. Crop Protection 19:603-608.

Jayaraj, J., Rahman, M., Wan, A. and, Punja, Z. K. 2009. Enhanced resistance to foliar fungal pathogens in carrot by application of elicitors. Annals of Applied Biology 1-10.

Jeun, Y. C. and Park, E. W. 2003. Ultrastructures of the leaves of cucumber plants treated with DL-3-Aminobutyric acid at the vascular bundle and the penetration sites after inoculation with *Colletotrichum orbiculare*. Plant Pathol. J. 19(2):85-91.

Jones, J. B., Jones, J. P., Stall, R. E. and Zitter, T. A. 1991. Compendium of tomato diseases. American, Phytopathological Society, St. Paul, MN. 73 pp.

Joslin, K. R. M. and Taber, H. G. 2003. Control of the foliar disease, *Septoria lycopersici*, in organic tomato production. Department of Horticulture, Iowa State University, Ames, IA 50011.

Kasaai, R. M. 2009. Various methods for determination of the degree of N-Acetylation of chitin and chitosan: A Review. J. Agric. Food Chem. 57:1667-1676.

Keinath, A. P. 2000. Effect of protectant fungicide application schedules on gummy stem blight epidemics and marketable yield of watermelon. Plant Dis. 84:254-260.

Kimura, A., Takano, Y., Furasawa, I., and Okuno, T. 2001. Peroxisomal metabolic function is required for appressorium-mediated plant infection by *Colletotrichum lagenarium.* The Plant Cell 13:1945-1957.

Kokkini, S. K., R.; Dardiotis, A.; Krigas, N. and Lanaras, T. 1997. Autumn essential oils of greek oregano. Phytochemistry 44:883-886.

Kolyadina, O. A., Murinov, K. Yu., Kuramshina, A. R., Kabal'nova, N. N. and Murinov, Yu. I. 2006. Russian Journal of Physical Chemistry 80(12):2002-2006.

Konsler, T.R., and D.B. Shoemaker (ed). 1980. Growing Trellised Tomatoes In Western North Carolina. AG-60. North Carolina Agricultural Extension Service. Greensboro, NC. 44 p.

Kubo, Y., K. Suzuki, I. Furusawa, and M. Yamamoto. 1985. Melanin biosynthesis as a prerequisite for penetration by appressoria of *Colletotrichum lagenarium*: site inhibition by melanin-inhibiting fungicides and their action on appressoria. Pestic. Biochem. Physiol. 23:47–55.

Kwack, M. S., Kim, E. N., Lee, H., Kim, J-W., Chun, Se-C. and Kim, K. D. 2005. Digital image analysis to mesasure lesion area of cucumber anthracnose by *Colletotrichum orbiculare*. J. Gen. Plant Pathol 71:418-421.

Leben, C., and Daft, G. C. 1968. Cucumber anthracnose: Influence of nightly wetting of leaves on number of lesions. Phytopathology 58:264-265.

Lewis, M. L., Mera, J .R. and Miller, S. A. 2004. Evaluation of fungicides and bactericides for the control of foliar and fruits diseases of processing tomatoes, 2004. F&N 60:V110.

Little, M. T. and Hills, J. F. 1978. Agricultural Experimentation, Design and Analysis. Wiley and Sons, Inc. USA. 350pp.

Liu, H., Du, Y., Wang, X. and Sun, L. (2004) Chitosan kills bacteria through cell membrane damage. Int J Food Microbiol, 95:145–155.

Lizama-Uc, G. Estrada, M. I. A., Caamal-Chan, M. G., Souza, P. R. Oropeza, S. C., Islas, F. I. and Zuñiga, A. J. J. 2007. Chitosan activates a MAP-kinase pathway and modifies abundance of defense-related transcripts in calli of *Cocos nucifera* L. Physiological and Molecular Plant Pathology 70:130-141.

Madden, L. V., Hughes, G., and Van den Bosh, F. 2007. The study of plant disease epidemics. APS PRESS, St. Paul, Minnesota. 421 pp.

Madden, L., Pennypacker, S. P. and MacNab, A. A. 1978. FAST, a forecast system for *Alternaria solani* in tomato. Phytopathology 68:1354-1358.

Manjunatha, G., Niranjan-Raj, S., Prashanth, G. N., Deepak, S., Amruthesh, K. N., and Shetty, H. S. 2009. Nitric oxide is involved in chitosan-induced systemic resistance in pearl millet against downy mildew disease. Pest Manag. Sci. 65(7):737-43.

McGrath, M. T. and Moyer, D. D. 2003. Evaluation of compost tea and the biofungicide Sonata for foliar disease in organically produced tomatoes, 2003. F&N 59:V053.

McGrath, M.T. 2007. Evaluation of treatments for managing foliar diseases in organically-produced tomato. Acta Hort. (ISHS) 808:137-142.

Monroe, J. S., Santini, J. B., and Latin, R. 1997. A model defining the relationship between temperature and leaf wetness duration, and infection of watermelon by *Colletotrichum orbiculare*. Plant Dis. 81:739-742.

Moretti, M. D. L., Sanna-Passino, G., Demontis, S. and Bazzoni, E. 2002. Essential oil formulations useful as a new tool for insect pest control. AAPS PharmSciTech. 3(2): 64–74.

Moyer, C. and Peres, N. A. 2008. Evaluation of biofungicides for control of powdery mildews of gerbera daisy. Proc. Fla. State Hort. Soc. 121:389-394.

Müller-Riebau, F., Berger, B., and Yegen, O. 1995. Chemical composition and fungitoxic properties to phytopathogenic fungi of essential oils of selected aromatic plants growing wild in turkey. J. Agric. Food Chem. 43:2262-2266.

Muñoz, Z., Moret, A. and Garcés, S. 2009. Assessment of chitosan for foliar inhibition of *Colletotrichum* sp. on tomatoes and grapes. Crop Protection 28:36-40.

Nash, A. F., and Gardner, R. G. 1988. Tomato early blight resistance in a breeding line derived from *Lycopersicon hirsutum* PI 126445. Plant Dis. 72:206-209.

Obradovic, A., Mavridis, A., Rudolph, K., Janse, J. D., Arsenijevic, M., Jones, J. B., Minsavage, G. V., and Wang, J.-F. 2004. Characterization and PCR-based typing of *Xanthomonas campestris* pv. *vesicatoria* from pepper and tomatoes in Serbia. Eur. J. Plant Pathol. 110:285-292.

Olivier, C., MacNeil, C. R., and Loria, R. 1999. Application of organic and inorganic salts to field-grown potato tubers can suppress silver scurf during potato storage. Plant Dis. 83:814-818.

OMRI. 2010. OMRI Products List Exceeds 2000 Products. http://www.omri.org/news/94498/omri-products-list-exceeds-2000-products

OMRI. 2011. The OMRI Products List data. http://www.omri.org

OMRI. 2011. Chitosan. http://www.omri.org/simple-gml-search/results/CHITOSAN

Ordoñez, V. C., Ferrera, C. R. and Hernández, C. L. V. 2009. In vitro antifungal effects of potassium bicarbonate on *Trichoderma* sp. and *Sclerotinia sclerotiorum*. Mycoscience 50:380-387.

Palma,G. J., Huang, I. C., Jansson, H.B., Salinas, J., López, L. L.V., Read, N.D. 2009. Chitosan permeabilizes the plasma membrane and kills cells of *Neurospora crassa* in an energy dependent manner. Fungal Genetics and Biology 46:585–594.

Palmer, C. L., Horst, R. K., and Langhans, R. W. 1997. Use of bicarbonates to inhibit in vitro colony growth of *Botrytis cinerea.* Plant Dis. 81:1432-1438.

Park, R.D., Jo, K.J., Jo, Y.Y., Jin, Y.L., Kim, K.Y., Shim, J.H. and Kim, Y.W. 2002 Variation of antifungal activities of chitosan on plant pathogens. J Microbiol Biotechnol. 12:84–88.

Parker, S. K., Gleason, M. L., and Nutter, F. W., Jr. 1995. Influence of rain events on spatial distribution of Septoria leaf spot of tomato. Plant Dis. 79:140-152.

Pernezny, K., Momol, T., Peres, N. Raid, R., and Roberts. P. 2007. 2007 Florida Plant Disease Management Guide: Chemical Control Guide for Diseases of Vegetables. Revision No.181. University of Florida, IFAS Extension. PPP6. 81pp.

Prabuseenivasan, S., Jayakumar, M. and, Ignacimuthu, S. 2006. *In vitro* antibacterial activity of some plant essential oils. BMC Complementary and Alternative Medicine 6:39-47.

Prusky, D., Stanley, F. and, Dickman, M. B. 2000. *Colletotrichum*, host specificity, pathology, and host interaction. St. Paul, Minn.: APS Press. 393 pp.

Qin, C., Li, Huirong, Xiao, Q., Liu, Y., Zhu, J. and Du, Y. 2006. Water-solubility of chitosan and its antimicrobial activity. Carbohydrate Polymers 63:367-374.

Rabae, E. I. Badawy, M. E. T., Stevens, C. V., Smagghe, G. and Steurbaut, W. 2003. Chitosan as antimicrobial agent: applications and mode of action. Biomacromolecules 4(6):1457-1465.

Reganold, J. P., Elliot, L. F. and Unger, Y. L. 1987. Logn-term effects of organic and conventional farming on soil erosion. Nature 330(26):370-372.

Rhodes, J. and Rastal, B. 2008. Chitosan as an antimicrobial agent. Ingredients and additives. http://www.fpiinternational.com/articles/ingredients_additives/032_FTI007.pdf

Rotem, J. (ed.). 1994. The Genus Alternaria: Biology, Epidemiology and Pathogenicity. St Paul, Minnesota, USA, American Phytopathological Society. 326 pp.

Rowell, B., Bessin, R., Masabni, J., Strang, J., Jones, T. and, Seebold, K. 2006-07. Vegetable production guide for commercial growers. University of Kentucky, Kentucky Cooperative Extension: ID-36. 140 pp.

Russo, M. G., G.; Bocchini, P.; Carnacini, A. 1998. Essential oil chemical composition of wild populations of italian oregano spice (*Origanum vulgare* ssp. *hirtum* (Link) Ietswaart): A preliminary evaluation of their use in chemotaxonomy by cluster analysis. J. Agric. Food Chem. 1998, 46, 3741-3746.

Sakaguchi, A., Miyaji, T., Tsuji, G. and Kubo, Y. 2010. A kelch repeat protein, cokel1p, associates with microtubules and is involved in appressorium development in *Colletotrichum orbiculare*. MPMI 23(1): 103-111.

Samoucha, Y. and Cohen, Y. 1984. Synergy between metalaxyl and mancozeb in controlling dawny mildew in cucumbers. Phytopathology 74:1434-1437.

Samoucha, Y. and Cohen, Y. 1988. Synergistic interactions of cymoxanil mixtures in the control of metalaxyl –resistance *Phytophthora infestans* of potato. Phytopathology 74:1434-1437.

Sbragia, J. R. 1975. Chemicals control of plant disease: An exciting future. Annu. Rev. *Phytopathol*. 13:257-269.

Schumann, L. G. and D' Arcy, J. C. 2006. Essential Plant Pathology. The American Phytopathological Society. 338 pp.

Seaman, A. Dillards, H., Cobb, A. and Porter, S. 2004. Tomato foliar disease control using OMRI-aproved materials. Organic Farming Research Foundation Project Report: 03-s-29.

Shad, A. G. and Reddy, A. S. N. 2000. Inhibition of fungal and bacterial plant pathogens by synthetic peptides: In vitro growth inhibition, interaction between peptides and inhibition of disease progression. MPMI 13(8):847-859.

Shalom, N. B., Ardi, R., Pinto, R., Aki, C. and Fallik, E. 2003. Controlling gray mold caused by *Botrytis cinerea* in cucumber plants by means of chitosan. Crop Protection 22:285-290.

Shovan, L.R., Bhuiyan, M. K. A., Begum, J. A. and Pervez, Z. 2008. *In vitro* control of *Colletotrichum dematium* causing anthracnose of soybean by fungicides, plant extracts and *Trichoderma harzianum*. Int. J. Sustain. Crop Prod. 3(3):10-17

Smilanick, J. L., Margosan, D. A., Mlikota, F., Usall, J., and Michael, I. F. 1999. Control of citrus green mold by carbonate and bicarbonate salts and the influence of commercial postharvest practices on their efficacy. Plant Dis. 83:139-145.

Soliman, K. M., and Badeaa, R. I. 2002. Effect of oil extracted from some medicinal plants on different mycotoxigenic fungi. Food and Chemical Toxicology 40:1669-1675.

Soylu, E. M., Soylu, S., and Kurt, S. 2006. Antimicrobial activities of the essential oils of various plants against tomato late blight disease agent *Phytophthora infestans*. Mycopathologia 161:119-128.

Stössel, P. and Leuba, J. L. 1984. Effect of chitosan, chitin and some aminosugars on growth of various soilborne phytopathogenic fungi. Journal of Phytopathology 111(1):82-90.

Streit, B. 1984. Effect of high copper concentrations on soil invertabrates (earthworms and oribatid mites). Oecologia, 64:381-388.

Takano, Y. Kikuchi, T. Kubo, Y. Hamer, J. E. Mise, K. and Furusawa, I.2000. The *Colletotrichum lagenarium* MAP kinase gene CMK1 regulates diverse aspects of fungal pathogenesis. MPMI 13 (4):374–383.

Tarantilis, P. and. Polissiou, M. G. 1997. Isolation and identification of the aroma components from saffron (*Crocus sativus*). J. Agric. Food Chem. 45:459-462.

Terreaux, R. Domard, M., Viton, C. and Domard, A. 2006. Interactions study between the copper II ion and constitutive elements of chitosan structure by DFT calculations. Biomacromolecules 7:31-37.

Thompson, D. C., and Jenkins, S. F. 1985. Influence of cultivar resistance, initial disease, environment, and fungicide concentration and timing on anthracnose development and yield loss in pickling cucumber. Phytopathology 75:1422-1427. .

Tikhonov, V. E., Stepnova, E. A., Babak, V. G., Yamskov, I. A., Palma, G. J., Jansson H., Lopez, L. L. V., Salinas J., Gerasimenko, D. V., Avdienko, I. D., and Varlamov, V. P. 2006. Bactericidal and antifungal activities of a low molecular weight chitosan and its N-/2(3)-(dodec-2-enyl) succinoyl/-derivatives. Carbohydrate Polymers 64:66–72.

Tsuji, G., Fujii, S., Tsuge, S., Shiraishi, T. and Kubo, Y. 2003. The Colletotrichum lagenarium Ste 12-like gene CST is essential for appressorium penetration. MPMI, 16(4):315-325.

USDA-GOV.2004. *Report of the Compost Tea Task Force*.
http://www.ams.usda.gov/nop

USDA, 2011. National Organic Program. What is organic?
http://www.ams.usda.gov/AMSv1.0/nop

Uthairatanakij, A., Teixeira, J. A. and, Obsuwan, K. 2007. Chitosan for improving orchid production and quality. Orchid science and Biotechnology 1(1):1-5.

Vallad, G. E. 2009. Evaluation of fungicides for early blight and target spot control on tomato, Fall 2009. University of Florida, Gulf Coast Research & Education Center. 5 pp.

van Bruggen, A. H. C. 1995. Plant disease severity in high-input compared to reduced-input and organic farming systems. Plant Disease 79(10):976-984.

Van Zwieten, L., Rust, J., Kingston, T., Merrington, G., and Morris, S. 2004. Influence of copper fungicide residues on occurrence of earthworms in avocado orchard soils. Science of the Total Environment 329:29-41.

Vesentini, D., Steward, D., Singh, A. P., Ball, R., Daniel, G. and, Franich, R. 2007. Chitosan-mediated changes in cell wall composition, morphology and ultrastructure in two wood-inhabiting fungi. Mycological Research III 875-890.

Vidhyasekaran, P. 2004. Concise encyclopedia of plant pathology. The Haworth Press, Inc., NY. 619 pp.

Walker-Simmons, M. K. and Ryan, C. A. 1984. Proteinase inhibitor synthesis in tomato leaves. Plant Physiol. 76:787-790.

White, T. J., T. Bruns, S. Lee, and J. W. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Academic Press, Inc., New York. 315-322 pp.

Wilson, C. L., Solar, J. M., El Ghaouth, A., and Wisniewski, M. E. 1997. Rapid evaluation of plant extracts and essential oils for antifungal activity against *Botrytis cinerea*. Plant Dis. 81: 204-210.

Wszelaki, A. L and Miller, S. A. 2005. Determining the efficacy of disease management products in organically-produced tomatoes. Online. Plant Health Progress, doi:10.1094/PHP

Wszelaki, A. L., Butler, T. J. and, Steiner, C. P. 2003. Evaluation of approved materials for the control of foliar and fruits diseases of organic fresh-market tomatoes, 2003. B&C, 19:PT013.

Wszelaki, A. L., Walker, S. D., Steiner, C. P. and, Miller, S. A. 2002. Evaluation of alternative for the control of foliar and fruit diseases of organic processing tomatoes, 2002. B&C Test 18:PT008.

Yeop, L. J., Sik, M. S. and Kook, H. B. 2005. Isolation and antifungal activity of kakuol, a propiophenone derivative from *Asarum sieboldi* rhizome. Pest Manag Sci 61:821-825.

Zehnder, G., Gurr, G. M. Kühne, S., Wade, M. R., Wratten, S. D. and Wyss, E.. 2007. Arthropod Pest Management in Organic Crops. Annu. Rev. Entomol., 52:57-80.

Zitter, A. T., Hopkins, L. D. and Thomas, E. C. 1996. "Compendium of cucurbit diseases." American Phytopathological Society, APS Press, MN. 87 pp.

Zitter, T. A. 2010. Listing by Category of 2010 OMRI Product List, Web edition: http://vegetablemdonline.ppath.cornell.edu/NewsArticles/OMRI%20Product%20List.pdf

Zitter, T. A. and Drennan, J. L. 2005. Biofungicides and organically-approved products compared with conventional fungicides for potato production. F&N, 61:V027.

Zitter, T. A., Drennan, J. L., Mutschler, M. A. and Kim, M-J. 2005. Control of early blight of tomato with genetic resistance and conventional and biological sprays. *Acta Hort* 695: 181-190.

Zitter, T.A., Hopkins, D.L. and, Thomas, C. E. 1996. Compendium of Cucurbit Diseases. St. Paul, Minn.: APS Press. 87 pp.

Ziv, O., and Zitter, T. A. 1992. Effects of bicarbonate and film-forming polymers on cucurbit foliar diseases. Plant Dis. 76:513-517

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PUBLICATIONS

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- **Feliciano, M.** and Vincelli, P. 2009. Effect of pH, concentration and dialysis on antifungal activity and phytotoxicity of β 1-4 linked polymer of glucosamine (chitosan). *Phytopathology* 99:S33 (abstract).

- **Feliciano, M**., Cabrera-Asencio, I. and, Rivera-Vargas. 2008. Effect of thrips (Thysanoptera: Thripidae) damage in the severity of purple blotch disease of onion caused by *Alternaria* sp. under tropical conditions. *Phytopathology* 99:S53 (abstract).

- **Feliciano, M**., Cabrera-Asencio, I. and, Rivera-Vargas, L. 2008. *Frankliniella occidentalis, F. schultzei* and *F. fusca* (Thysanoptera: Tripidae) in Puerto Rico. *The Journal of Agriculture of the University of Puerto Rico*, 92(1-2):107-110.

- **Feliciano, M**. and L. Rivera Vargas. 2006. First report of powdery mildew of onion (*Allium cepa* L.) caused by *Leveillula taurica* (Lev.) Arn. in Puerto Rico. Proceedings of the Caribbean Food Crops Society, XLII: (2): 188-192.