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FINDING SYNERGY: IMPROVING BIOPESTICIDE EFFICACY FOR DISEASE CONTROL
THROUGH CO-APPLICATION WITH THE NATURAL PRODUCT CHITOSAN

BY

LIZA DEGENRING

Bachelor of Science, Boston College, 2016

Master of Science, University of New Hampshire, 2019

DISSERTATION

Submitted to the University of New Hampshire

In Partial Fulfillment of

The Requirements for the Degree of

Doctor of Philosophy

In

Agricultural Sciences

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Liza DeGenring

This dissertation was examined and approved in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Agricultural Science by:

Dissertation Director, Anissa Poleatewich, Assistant Professor, Agriculture,
Nutrition and Food Systems

Rebecca Sideman, Full Extension Professor, Agriculture, Nutrition and Food
Systems

Cheryl Smith, Extension Professor Emerita, Agriculture, Nutrition and Food
Systems

Kari Peter, Associate Research Professor, Department of Plant Pathology and
Environmental Microbiology, Penn State University

Marta Lima, Research Assistant Professor, School of Plant and
Environmental Sciences, Virginia Tech

On June 27, 2023

Approval signatures are on file with the University of New Hampshire Graduate School.

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standard (GS), grower standard + chitosan (GS+C), and grower standard + biopesticide + chitosan (GS+B+C). Postharvest treatments include water control, Tidal Grow high molecular weight (TG HMW) at 1.0 %, Tidal Grow low molecular weight (TG LMW) at 1.0 %, and ARMOUR-Zen 15 (AZ) at 1.0 %. 139

Figure A-8. Representative McIntosh apples from pre- and postharvest treatments 14 days post infestation (dpi) with *Colletotrichum fioriniae* in experiment 6. Pre-harvest sprays included grower standard (GS), grower standard + chitosan (GS+C), and grower standard + biopesticide + chitosan (GS+B+C). Postharvest treatments include water control, Tidal Grow high molecular weight (TG HMW) at 1.0 %, Tidal Grow low molecular weight (TG LMW) at 1.0 %, and ARMOUR-Zen 15 (AZ) at 1.0 %. 140

ABSTRACT**FINDING SYNERGY: IMPROVING BIOPESTICIDE EFFICACY FOR DISEASE CONTROL
THROUGH CO-APPLICATION WITH THE NATURAL PRODUCT CHITOSAN**

by

Liza DeGenring

University of New Hampshire

Annual crop losses caused by plant diseases are estimated worldwide at 220 billion US dollars. Conventional fungicides are the primary means to control these diseases, however there are growing concerns over human health, effects on non-target species, and environmental contamination. Furthermore, many plant pathogens have developed fungicide resistance due to overexposure to chemicals with single modes of action. There is a worldwide trend to explore alternative tools to reduce the use of synthetic fungicides while continuing to reduce plant disease. Among these control strategies are the use of antagonistic microorganisms (biopesticides) and naturally occurring compounds that have fungicidal activity. Chitosan is a promising natural compound documented to have antifungal and disease suppressive effects. Chitosan has been used successfully as a postharvest application to prevent storage rot and extend shelf life of perishable fruits and vegetables. There is limited research on preharvest application of chitosan for reducing pre-harvest and post-harvest diseases. The overall goal of this research was to investigate biopesticide-chitosan synergisms in two cropping systems to improve biological control of pre- and post-harvest plant diseases. These systems were chosen to represent two plant types (woody and herbaceous) and two production industries (perennial field production and greenhouse production). The herbaceous cropping system focused on the investigation of chitosan's ability to suppress gray mold on petunia leaves caused by *Botrytis*

cinerea using in vitro and in planta approaches. In this research I found that chitosan products, both reagent grade and commercial, reduced *B. cinerea* growth in vitro and lesion size in planta. For the woody cropping system, I focused on the investigation of chitosan's efficacy and the potential synergisms of chitosan and a biopesticide to suppress fungal above-ground apple diseases. Results indicate that pre-harvest chitosan applications can reduce foliar diseases on apple but was most effective when overlaid onto a grower standard treatment. Additionally, the addition of chitosan to a biopesticide spray did not enhance the biopesticide's efficacy under the research conditions. Postharvest chitosan treatments greatly reduced postharvest incidence and severity of rots caused by *Penicillium expansum* and *Colletotrichum fioriniae*. Results from this research demonstrate that chitosan can suppress foliar and fruit fungal diseases and has the potential to play a role in a disease management program for reduction of above-ground fungal pathogens under various cropping systems.

CHAPTER 1

Introduction

1.1. Shifts in disease management

Agricultural systems are facing new production challenges in the 21st century. Global food demand is forecasted to increase 100-110% from 2005 to 2050 (Tilman et al. 2011) and yet environmental stressors (drought, topsoil/nutrient losses, high salinity, flooding, etc.) are causing significant losses in yield (Porter et al. 2014). Losses due to insect pests, weeds, and pathogens have been estimated at 27-42% for major field crops (Oerke 2006). The Food and Agriculture Organization of the United Nations (FAO) estimates that annual crop losses caused by plant disease worldwide are at US \$220 billion (FAO 2019). Traditionally, synthetic fungicides are the primary means to control plant diseases (Morton and Staub 2008). However, there are growing concerns over risks to human, non-pest species, and environmental health associated with pesticide usage, as well as the development of fungicide-resistant pathogens (Pimentel 2005; Ekström and Ekbohm 2011; Xia et al. 2006; Lamichhane et al. 2016; Gomiero et al. 2011; Wilson and Tisdell 2001; Janisiewicz and Korsten 2002). Overexposure to chemicals with single modes of action can drive the development of fungicide-resistant strains of pathogens, making disease management more difficult and could ultimately lead to devastating crop losses (Wilson 1997; Janisiewicz and Korsten 2002; Ma and Michailides 2005). Concerns over synthetic fungicide use have led to the development of alternative approaches to disease management. Among these control strategies are the use of antagonistic microorganisms and naturally occurring compounds that have fungicidal activity as part of an integrated pest management (IPM) strategy.

1.2. Biocontrol agents

In biological control, antagonist microorganisms (also known as biocontrol agents (BCAs)) are utilized to suppress the activity of plant pathogens through one or more modes of action (Whipps 2001; van Lenteren 2000; Kumar et al. 2021). BCAs can utilize direct or indirect antagonism of the plant pathogen, leading to suppression of the pathogen's activity and reduce disease symptoms (Pal and McSpadden Gardener 2006; Baker 1986; Whipps 2001; Raymaekers et al. 2020). Direct antagonism occurs when the BCA produces antibiotics that kill (or interfere with) the pathogen or through parasitism and predation of the pathogen (Pal and McSpadden Gardener 2006; Belanger et al. 2012). Microorganisms can also be indirectly antagonistic to pathogens through competition for nutrients and space (Lugtenberg and Kamilova 2009; Raymaekers et al. 2020) and activation of induced systemic resistance (ISR) in the plant host (Kloepper et al. 2004; van Loon et al. 1998). ISR occurs when the plant's defense mechanisms are triggered by the beneficial microorganism, allowing the plant to be protected from a future attack (Pieterse et al. 2014; Compant et al. 2005). Because BCAs often suppress disease via multiple modes of action, there is low risk for the development of resistance and thus biopesticides are considered more durable than conventional fungicides. Additionally, the modes of actions of BCAs are unique from synthetic fungicides and can be used in rotation with fungicides to reduce overall risk of the development of fungicide resistance (Ons et al. 2020).

Several biocontrol agents have been vigorously tested, including species in the genera *Bacillus*, *Pseudomonas*, *Trichoderma*, *Streptomyces*, and antagonistic yeasts (Paulitz et al. 2001; Syed Ab Rahman et al. 2018). Several of these biocontrol agents have been commercialized and sold as microbial biopesticides for use in agriculture to reduce pre- and post-harvest diseases (Glare et al. 2012; Raymaekers et al. 2020; Pandit et al. 2022; Droby et al. 2009). After more

than forty years of research aimed at developing effective biopesticides (Cook and Baker 1983; Baker 1986), their adoption as pre- and post-harvest treatments has not been widespread (Glare et al. 2012; Fravel 2005; Nicot et al. 2011). This is largely due to variable performance of biopesticides under commercial conditions (Fravel 2005; Wilson 1997; Chandler et al. 2011; Marian and Shimizu 2019). Microbial biopesticides often fail to grow and maintain high enough populations in the rhizosphere or phyllosphere or do not produce antifungal compounds at levels necessary to suppress pathogen activity (Glare et al. 2012; Chandler et al. 2011; Dastogeer et al. 2020). Recently, several natural compounds have been found to have a synergistic effect on biopesticide efficacy in reducing plant diseases. This synergism may be due to the direct activity of the natural product or indirect effects on biopesticide persistence (such as providing a food source or protection from harmful environmental factors) and/or stimulation of a host defense response.

1.3. The natural product: chitosan

Natural compounds, such as cellulose, chitin, and chitosan, have documented disease suppressive effects through direct inhibition of plant pathogens and stimulation of the plants' immune system (Davis et al. 1992; Kokalis-Burelle et al. 1992; Orzali et al. 2017; Pichyangkura and Chadchawan 2015). Chitin is a component of fungal cell walls and the exoskeletons of insects and the shells of crustaceans, making it the second most abundant polysaccharide found in nature after cellulose (Kaur and Dhillon 2014; Sharif et al. 2018). Chitosan is a natural β -(1,4)-glucosamine polymer that is formed through the deacetylation of chitin (Kaur and Dhillon 2014; Pichyangkura and Chadchawan 2015; Raafat and Sahl 2009). Chitosan is more soluble in solution compared to chitin and thus chitosan is predominately found in commercial formulations (Tripathi and Dubey 2004; Sharif et al. 2018). Chitosan is used in many industries,

such as pharmacology, biotechnology, medicine, and agriculture (Bautista-Baños et al. 2006; Orzali et al. 2017). It is non-toxic to humans and has a low environmental impact (Rinaudo 2006). Additionally, chitosan is EPA-approved as a biopesticide and FDA-approved as a GRAS (generally recognized as safe) substance (Romanazzi and Feliziani 2016). Research has shown that chitosan has strong potential as a crop protection tool for use in agriculture (Figure 1-1). For example, chitosan induces host defense responses (Jia et al. 2018), has fungistatic properties against several fungi, such as *Botrytis cinerea* (Pers.:Fr.), *Rhizopus stolonifer* (Ehrenb.:Fr.) Vuill, *Penicillium expansum* link, *Fusarium oxysporum* f. sp. *radicis-lycopersici* Jarvis and Showmaker (Benhamou and Theriault 1992; El-Ghaouth et al. 2000b; Hernández-Lauzardo et al. 2008), and has antibacterial properties, especially against gram-positive bacteria (Figure 1-1) (No et al. 2002).

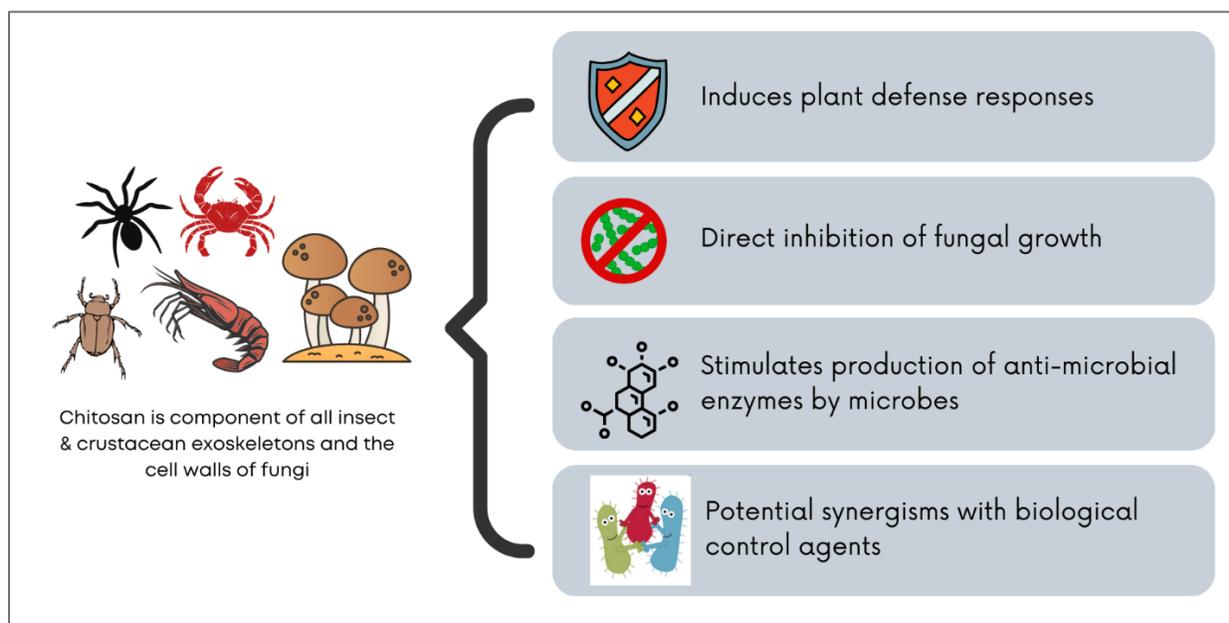


Figure 1-1. Chitosan modes of action leading to disease suppression.

1.4. Postharvest applications of chitosan

Chitosan has been used successfully in postharvest applications to prevent storage rot and extend shelf life of perishable fruits (apple, citrus, pear, strawberry, grape, and tomato) and

vegetables (cucumber and bell pepper) (Li et al. 2015; Zhang et al. 2011; Romanazzi and Feliziani 2016; Liu et al. 2007; Bautista-Baños et al. 2006; Sharif et al. 2018). There appears to be more than one mode of action by which chitosan preserves fruits and vegetables and reduces disease. Chitosan forms a film on the fruit, slowing the ripening process (El Ghaouth et al. 1991a) and providing a moisture barrier, thus preventing weight loss and reducing respiration rate (Chien et al. 2007; El-Ghaouth et al. 1991b; Li et al. 2015). A chitosan post-harvest dip can improve firmness, total soluble solids (TSS) content, titratable acidity, and ascorbic acid (all of which correlate to the quality of the fruit) (Chien et al. 2007). The chitosan barrier on the fruit prevents the outward flux of nutrients, interfering with the establishment of a nutritional relationship between the host and pathogen (El-Ghaouth et al. 1994b). The lack of nutrients from the fruit results in aged and nutrient-deprived fungal cells (El-Ghaouth et al. 1994b). Studies have shown that chitosan can cause cellular disorganization of fungal pathogens through cell wall loosening, cytoplasm disintegration, and excessive branching and swelling of the cell wall (Ait Barka et al. 2004; El-Ghaouth et al. 1994b; Benhamou et al. 1998). El Ghaouth et al. (1994b) found that when a wounded area was treated with a chitosan solution, the host's primary cell walls below the wounded site showed no signs of alteration even when large numbers of fungal cells were in the wounded area. This suggests that chitosan may impair the pathogen's ability to produce macerating enzymes (El-Ghaouth et al. 1994b). In addition to the damage caused to invading pathogens, chitosan can stimulate a defense response in the host tissue, such as cell wall thickening, formation of hemispherical protuberances along host cell walls, and occlusion of many intercellular spaces with a fibrillar material (El-Ghaouth et al. 1994b, 1997). When applied to harvested fruits, chitosan may up-regulate the production of defense-related compounds such as polyphenoloxidase (PPO) and peroxidase (POD) (Li et al. 2015; Liu et al.

2007), and antioxidants and enzymatic activity of anti-fungal enzymes, such as β -1,3-glucanase (Wang & Gao, 2013). Chitosan may also cause an increase in the production of compounds associated with carbohydrate catabolism and energy production, aconitase, NADH dehydrogenase, and malate dehydrogenase, which can provide energy for resisting a pathogen (Li et al. 2015).

1.5. The role of chitosan molecular weight and concentration on disease suppression

Several grades of chitosan are available that vary in biological source, purity, degree of deacetylation, viscosity, chitosan concentration (% chitosan of a solution), and molecular weight (MW) (Raafat and Sahl 2009). In published literature, there are varying, and at times conflicting, results on the effect of MW and chitosan concentration on plant disease suppression and plant growth promotion. Hernández-Lauzardo et al. (2008), found that low (1.74×10^4 Da), medium (2.38×10^4 Da), and high (3.07×10^4 Da) MWs of chitosan amended media decreased mycelial growth, however low molecular weight had the greatest inhibition. Chien et al. (2007) similarly found that post-harvest application of lower MW chitosan was able to reduce fungal diseases and improve fruit quality better than high MW chitosan treatments. In contrast, Hernández-Lauzardo et al. (2008) reported that high MW chitosan reduced *in vitro* sporulation of *R. stolonifer* more than low or medium molecular weight, but sporulation may be more associated with isolate type than chitosan treatment (Bautista-Baños et al. 2005). Chitosan has also been tested against bacterial plant pathogens. It appears that MW greatly affects the suppression of gram-negative bacteria whereas a wide range of MW are effective against gram-positive bacteria (No et al. 2002; Younes et al. 2014). MW can also affect a plant's response to abiotic stress. Krupa-Małkiewicz and Fornal (2018) found that higher MW chitosan was able to decrease the adverse effects of salinity, but this effect was not seen at lower MW. In addition to MW, chitosan

concentration is an important consideration. A higher concentration (1-2.5 mg·ml⁻¹) of chitosan tend to correlate with greater inhibition of fungal growth (Hernández-Lauzardo et al. 2008; Yu et al. 2012; El-Ghaouth et al. 2000b; Muñoz and Moret 2010; El-Ghaouth et al. 1997; Benhamou and Theriault 1992); however, a higher concentration can potentially cause phytotoxicity and have negative effects on plant growth (Ait Barka et al. 2004). El Ghaouth et al. (1994a) found no phytotoxicity when cucumber plants were growing in a nutrient solution supplemented with chitosan but there was a change in the root morphology (secondary shoots were shorter and thicker). Due to the conflicting data on MW and chitosan concentration, it is crucial for researchers to investigate these variables to identify the most effective treatment for a given crop species.

1.6. Preharvest application of chitosan

Compared to postharvest application, there are limited documented examples of preharvest application of chitosan to reduce plant disease (Bautista-Baños et al. 2006). Grape plantlets grown on 1.75% (v/v) chitogel (a derivative of chitosan) exhibited improved growth and increased gas exchange compared to control plants (Ait Barka et al. 2004). Additionally, chitogel reduced the growth of *B. cinerea* in both the gel form as a growing media and as a spray onto grape plantlets (Ait Barka et al. 2004). In another study, 1% chitosan (w/v) applied as a soil drench to tomato plants significantly reduced wilt incidence caused by *Ralstonia solanacearum* (Smith) and promoted plant growth (Algam et al. 2010). A nutrient solution supplemented with chitosan was effective in controlling the incidence of root rot on cucumber caused by *Pythium aphanidermatum* (Edson) Fitzp. (El-Ghaouth et al. 1994a). Some research has focused on pre-harvest chitosan sprays to reduce quality loss and decay of fruit post-harvest (Bhaskara Reddy et al. 2000). Similar to results achieved with post-harvest applications, strawberry fruit treated pre-

harvest with chitosan were firmer, ripened at a slower rate, and had decreased decay caused by *B. cinerea* (Bhaskara Reddy et al. 2000).

Research has indicated that chitosan can activate plant defense systems (Benhamou 1996) and this may play a role in its disease suppressive effects. Chitosan elicits a host response characterized by increased enzymatic activity of chitinase and β -1,3-glucanases, and enhanced production of callose cell wall appositions in the host's epidermis and outer cortex (Algam et al. 2010; El-Ghaouth et al. 1994a; Benhamou et al. 1998). After inoculation with *P. aphanidermatum*, chitosan-treated cucumber plants exhibited several defense reactions in the root tissue: oversized papilla deposition in the host cell walls and plugging of intercellular spaces in the cortical and endodermis tissues which create physical barriers against the pathogen (El-Ghaouth et al. 1994a). Benhamou and Theriault (1992) reported similar results with chitosan treated tomato plants infected with *F. oxysporum* f. sp. *radicis-lycopersici*. Interestingly, the induction of physical barriers such as wall appositions, xylem vessel occlusion, and increase in papilla production, were seen primarily on tomato plants that were infected but not on the uninfected chitosan-treated plants, suggesting that the chitosan may only sensitize the plant to respond rapidly when under attack (Benhamou and Theriault 1992). Furthermore, a reduction in tomato root lesions caused by *Fusarium* spp. was reported on tomato plants treated with both a chitosan root treatment and a leaf spray, suggesting that chitosan-induced resistance is systemic (Benhamou and Theriault 1992).

1.7. Potential synergisms with biopesticides

A few studies have suggested that natural compounds, such as chitin and chitosan, may have agriculturally useful synergisms when applied with biopesticides (Yu et al. 2012; Benhamou et al. 1998), but this potential combination is understudied. A few researchers have

studied the effects of chitin/chitosan combined with plant-beneficial yeasts and bacteria and identified some interesting interactions. There are several hypotheses regarding the mechanisms behind these synergisms including (1) alteration of the host physiology and defense capacity (2) enhanced biocontrol agent population growth and (3) increased biocontrol agent chitinase production (Figure 1-2) (Lu et al. 2014; Kokalis-Burelle et al. 1992; Yu et al. 2008).

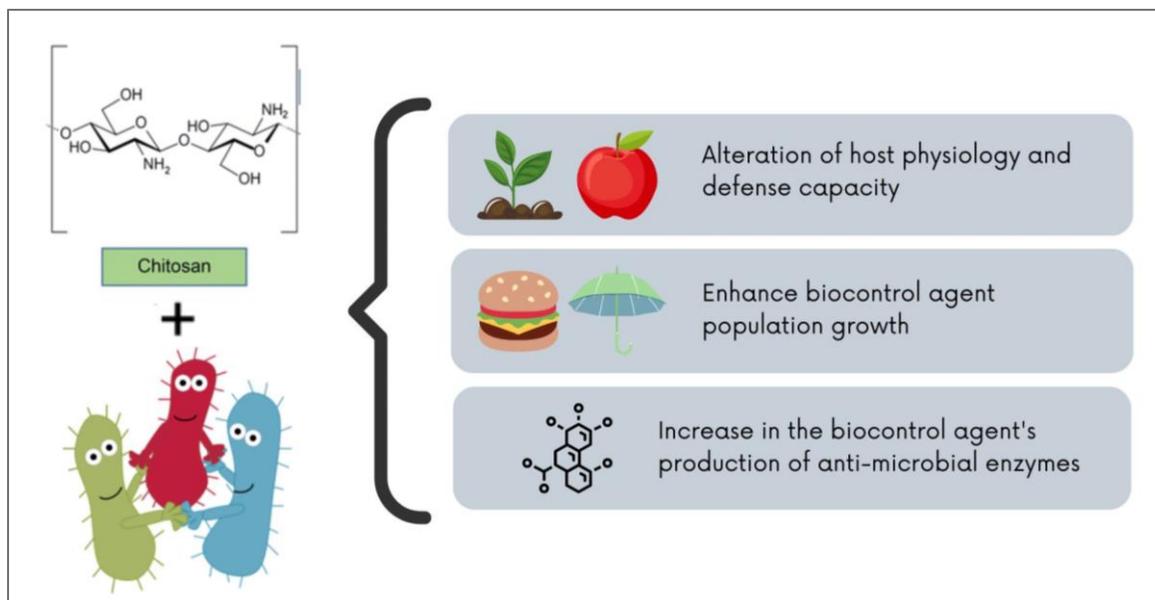


Figure 1-2. Mechanisms behind the potential synergism of chitosan and biocontrol agents for suppressing plant diseases.

In relation to host physiology, chitosan reduced respiration and ripening, enhancing biopesticide efficacy as the protective effect of biopesticides in post-harvest application is reduced with an increase in tissue ripeness (Wilson et al. 1996). An application of chitosan creates a film that reduces the rate of tissue ripening which may aid the biopesticide's ability to reduce disease (El-Ghaouth et al. 2000b). Another possible synergism of a biopesticide-chitosan application is a heightened plant defense response. A *Bacillus pumilus* Meyer and Gottheil (PGPR strain PE 34) and chitosan treatment resulted in an amplified defense response on tomatoes infected with *F. oxysporum* f. sp. *radicislycopersici* (Benhamou et al. 1998).

Chitosan is also thought to have a direct effect on biocontrol agents (BCAs). For example, yeasts grown on chitin-amended media have been shown to have higher antagonistic activity against post-harvest diseases compared to yeasts harvested from media without chitin (Lu et al. 2014; Yu et al. 2008; Ge et al. 2010). A combined application of a biocontrol yeast, *Cryptococcus laurentii* (Kufferath) Skinner, and 0.5% (w/v) chitosan was able to inhibit *P. expansum* (the causal agent of blue mold) growth on pears 5 days post inoculation (Yu et al. 2012). Biocontrol yeast populations harvested from chitin amended media grew more rapidly in apple and pear wounds than compared to yeast harvested from non-chitin amended media (Lu et al. 2014; Yu et al. 2008).

There is some research that suggests that chitin/chitosan may act as a food source for the BCA, specifically actinomycetal communities. Cretoiu et al. (2013) reported that a chitin amendment raised suppressiveness of the soil, particularly toward *Verticillium dahliae* Kleb., and this was hypothesized to be due to shifts in the growth and survival of certain microbial communities, specifically actinobacteria and *Oxalobacteraceae*. A combination application of chitosan and the biopesticide, *Streptomyces melanosporofaciens* strain EF-76 (which has exhibited chitosanolytic activities), decreased the disease severity and incidence of common potato scab (caused by *Streptomyces scabies* Lambert & Loria) compared to the control and the individual treatments, although this additive effect was not always observed (Beauséjour et al. 2003). Kokalis-burelle et al. (1992) reported a 60% reduction in early leafspot of peanut when plants were treated with *Bacillus cereus* and chitin compared to the non-treated control and a 1.3 log increase in *B. cereus* population levels compared to the non-chitin amended leaves. It was hypothesized that the chitin stimulated production of anti-fungal enzymes and helped the beneficial microbe persist long enough to compete with the pathogen by providing protection

from harmful environmental variables and by providing a nutrient source (Kokalis-Burelle et al. 1992). These results are consistent with the effects of other food source amendments, such as cellulose (Davis et al. 1992). The addition of cellulose (0.5% w/v) to an application of *Chaetomium globosum* enhanced the survival and growth of the biopesticide and subsequently resulted in greater disease control of flyspeck (caused by *Zygophiala jamaicensis* E. Mason) and sooty blotch (caused by *Gelodes pomigena* (Shchwein) on apple (Davis et al. 1992).

In summary, there appears to be a synergistic effect of chitosan and biopesticides on plant disease suppression but there are large gaps in our knowledge regarding the modes of actions driving these effects. Chitosan's ability to reduce disease, directly and indirectly, and its potential synergism with biopesticides, make it a valuable tool for use in Integrated Pest Management (IPM) programs. *Furthering our understanding of how chitosan reduces disease pre-harvest and post-harvest and what synergisms occur between biopesticides and chitosan will allow us to develop best practices for disease management.* This research will provide growers with innovations that improve environmental stewardship, profitability, and aid in creating a sustainable production system.

1.8. Research Objectives

The overall goal of this research was to investigate biopesticide-chitosan synergisms leading to improved biological control of pre- and post-harvest above-ground plant diseases. Two model systems, greenhouse ornamentals and tree fruit, were used in this research. These systems were chosen to represent two plant types (woody and herbaceous) and two production industries (perennial field production and greenhouse production). Within this dissertation, each chapter had multiple objectives summarized here:

Chapter 2. Inhibition of *Botrytis cinerea* growth and suppression of gray mold on petunia leaves using chitosan. The objectives of this chapter were to (1) evaluate antimicrobial activity of chitosan *in vitro*, (2) evaluate the effect of chitosan MW and concentration on suppression of *B. cinerea* in planta, and (3) compare commercial and reagent grade chitosan formulations for disease suppression under greenhouse conditions.

Chapter 3. Integration of chitosan and biopesticides to suppress pre-harvest diseases of apple. The objectives of this chapter were to evaluate effects of chitosan to (1) suppress fungal pathogens of apple when applied alone or in combination with a commercial biopesticide on a research orchard, (2) suppress fungal pathogens of apples when applied as part of conventional fungicide program on a commercial orchard, and (3) reduce the quantity of overwintering spores of *V. inaequalis* in orchard leaf litter.

Chapter 4. Postharvest chitosan sprays reduce bitter rot and blue mold on apple fruit. The objectives of this chapter were to (1) identify non-phytotoxic concentrations of chitosan on apple fruit, (2) evaluate commercial chitosan products for reduction of postharvest disease severity on inoculated fruit, (3) evaluate the effect of pre-harvest chitosan applications on suppression of latent infections, postharvest rots, and fruit quality, and (4) evaluate the effect of pre-harvest plus postharvest chitosan applications on suppression of *Penicillium expansum* and *Colletotrichum fioriniae* on inoculated fruit.

CHAPTER 2

Inhibition of *Botrytis cinerea* growth and suppression of gray mold on petunia leaves using chitosan

Liza DeGering, Ryan Dickson, and Anissa Poleatewich

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Abstract

Exogenous application of chitosan has been shown to reduce plant disease severity in food crops; however, less is known about the potential use of chitosan in floriculture. The objective of this study was to investigate the effectiveness of chitosan to suppress gray mold on petunia leaves caused by *Botrytis cinerea* using *in vitro* and *in planta* approaches. We also aimed to determine if chitosan molecular weight influences efficacy. Medium and high molecular weight reagent grade chitosan reduced growth of *B. cinerea* *in vitro* at chitosan concentrations ranging from 1.25% to 2.0% (v/v), while low molecular weight reagent grade chitosan only reduced growth at 2.0% (v/v). In detached leaf assays, all reagent grade chitosan treatments reduced *Botrytis* lesion size on petunia leaves up to 65% compared to the water control. The commercial product Tidal Grow reduced *in vitro* growth of *Botrytis*, starting at 0.5%, and reduced disease severity at 0.75% on petunia leaves. The commercial product ARMOUR-Zen 15 reduced *Botrytis* growth *in vitro* at 3.75% and higher and reduced disease severity at 0.3% and 1.0% on petunia leaves. Under greenhouse conditions, low, medium, and high molecular weight reagent grade chitosan and ARMOUR-Zen 15 at 0.4% chitosan reduced *Botrytis* lesion size on petunia leaves up to 60% compared to the water control. Suppression *in vitro* suggests that chitosan may have direct phytotoxic effects on fungal growth, however our *in planta* and

greenhouse trials suggest that additional modes of action may also play a role in the observed suppressive effects.

Keywords: *Botrytis cinerea*, chitosan, petunia, floriculture, natural products

2.1. Introduction

Botrytis blight or gray mold, caused by *Botrytis cinerea* Pers.:Fr, (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) is an important plant pathogen in greenhouse floriculture, causing losses during production, shipping, and in retail (Daughtrey et al. 2000). Infection of bedding plants can result in latent infections that appear in postharvest storage and shipping (Bennett et al. 2020). *B. cinerea* is classified in the genus *Botryotinia* within the Ascomycota phylum and is considered an imperfect fungus due to the rarity of a sexual phase. Symptoms of *B. cinerea* range by host but often infected leaves and petals have gray to brown spots that develop the characteristic gray, fuzzy sporulation of gray mold (Schumann and D’Arcy 2010). Wounded or senescent tissues are especially susceptible to invasion, thus leaves in the lower canopy and flowers are very susceptible in floriculture crops (Daughtrey et al. 2000). *B. cinerea* can overwinter in or on plant and soil debris in the greenhouse as mycelium and sclerotia (Schumann and D’Arcy 2010). Conidia development from mycelium requires humid conditions and can easily spread vast quantities of conidia spores when plant material is disturbed, mist is present, or humidity rises rapidly, resulting in rapid dispersion and infection under greenhouse conditions (Daughtrey et al. 2000).

While cultural practices, such as reducing leaf wetness and relative humidity, can decrease the rate of infection, foliar application of fungicides is the most common way of mitigating this disease (Hausbeck and Moorman 1996). Concern over fungicide resistance is increasing. For example, *B. cinerea* resistance to common fungicides in floriculture production,

such as benzimidazole and dicarboximide, is widespread throughout North American and European greenhouse floriculture operations (Moorman and Lease 1992; Yourman and Jeffers 1999; Samarakoon et al. 2017). Concerns over chemical residues and the development of fungicide resistance have led to increased interest in the development of alternative tools, such as the use of natural products, for an integrated approach to managing *B. cinerea* (Lamichhane et al. 2016; Xia et al. 2006).

Natural products, such as chitosan, have been gaining interest with growers as a tool to promote plant growth and reduce disease without the risk of fungicide resistance. Chitosan, a derivative of chitin, is a natural β -(1,4)-glucosamine polymer that is a component of insect and crustacean exoskeletons and fungal cell walls (Sharif et al. 2018; Kaur and Dhillon 2014). Chitosan has potential for use in agriculture due to its ability to help plants tolerate biotic and abiotic stress (Hidangmayum et al. 2019). Specifically, chitosan has been shown to have fungistatic properties against several microorganisms, including the plant pathogenic fungi *B. cinerea*, *Rhizopus stolonifer* (Ehrenb.:Fr.) Vuill, *Penicillium expansum* (Link), *Fusarium oxysporum* f. sp. *radicis-lycopersici* Jarvis and Shoemaker (Benhamou and Theriault 1992; El-Ghaouth et al. 2000b; Hernández-Lauzardo et al. 2008). Chitosan has bactericidal properties, particularly against gram-positive bacteria (No et al. 2002). Chitosan and its derivatives have also been shown to elicit plant defenses, leading to reduced disease (Jia et al. 2018).

Information about disease suppressive properties of chitosan has predominately come from postharvest research where chitosan has been shown to prevent storage rot and extend shelf life of perishable fruits and vegetables (El-Ghaouth et al. 2000a; Li et al. 2015; Zhang et al. 2011; El-Ghaouth et al. 2000b; Romanazzi and Feliziani 2016; Liu et al. 2007). There are limited examples of preharvest application of chitosan to reduce plant disease (Bautista-Baños et al.

2006). Grape plantlets grown on 1.75% (v/v) chitogel (a derivative of chitosan) exhibited improved growth and reduced disease caused by *B. cinerea* compared to control plants (Ait Barka et al. 2004). Ben-Shalom and colleagues (2003) found that chitosan significantly reduced *B. cinerea* incidence on cucumber plants when applied 24 hours prior to pathogen challenge (Ben-Shalom et al. 2003). Another study reported that chitosan was effective at controlling powdery mildew (*Podosphaera pannosa* (syn. *Sphaerotheca pannosa* var. *rosae*)) on roses (Wojdyla 2001). While research has shown potential for disease suppression on food crops, there is little known about ornamental crops, though studies have shown that chitosan can have a biostimulant effect on ornamentals. For example, chitosan can increase plant root and shoot growth, flower number per plant, flower head fresh and dry weight, and overall chlorophyll content in several ornamental plants (Abdul-Hafeez and Ibrahim 2021; Salachna and Zawadzińska 2014; Ohta et al. 1999, 2004). Further, chitosan can enhance the production of essential oils in German chamomile plants (Abdul-Hafeez and Ibrahim 2021) and decrease the number of days to flowering for freesia and prairie gentian plants (Salachna and Zawadzińska 2014; Ohta et al. 1999, 2004).

Several grades of chitosan are available commercially that vary in biological source, purity, degree of deacetylation, viscosity, and molecular weight (Raafat and Sahl 2009). The molecular weight (MW) of most commercial chitosans ranges from 50-2,000 kDa (Gonçalves et al. 2021). Research has shown that MW and concentration of chitosan can influence antimicrobial activity (No et al. 2002; Liu et al. 2006). However, there are varying, and at times conflicting, results on the effect of MW and chitosan concentration on plant disease suppression and plant growth promotion. Several studies have reported that low MW chitosan is more effective against fungal diseases compared to medium or high MW chitosan (Hernández-

Lauzardo et al. 2008; Chien et al. 2007), but high MW chitosan is more effective against abiotic stress (Krupa-Malkiewicz and Fornal 2018). Higher concentrations (1.0-2.5 mg·ml⁻¹) of chitosan tend to correlate with greater inhibition of fungal growth compared to lower concentrations (0.5-0.75 mg·ml⁻¹) (Hernández-Lauzardo et al. 2008; Yu et al. 2012; El-Ghaouth et al. 2000b; Muñoz and Moret 2010; El-Ghaouth et al. 1997; Benhamou and Theriault 1992); however, higher concentrations can cause phytotoxicity (Ait Barka et al. 2004). Phytotoxicity may be partly related to the fact that chitosan is not water-soluble and must be dissolved in acid, resulting in solutions with pH ranging from 3-5. Overall, research has shown that phytotoxicity and effective dose are crop species dependent. Therefore, it is crucial for researchers to investigate the most effective chitosan use strategy for a given crop species.

Although there is increasing interest in chitosan as an integrated pest management (IPM) tool, there are still large gaps in our knowledge regarding the potential for use in greenhouse horticulture. Research is needed to identify benefits and best-use practices to provide growers with science-based recommendations. The objectives of this study were to (1) evaluate antimicrobial activity of chitosan *in vitro*, (2) evaluate effect of chitosan molecular weight and concentration on suppression of *B. cinerea in planta*, and (3) compare commercial and reagent grade chitosan formulations for disease suppression under greenhouse conditions.

2.2. Materials and Methods

2.2.1. Plant material.

Petunia (*Petunia × atkinsiana* D. Don) was selected as a model due to its importance as a floriculture crop and susceptibility to Botrytis blight. Cuttings of petunia cv. Supertunia® Black Cherry were obtained from Pleasant View Gardens, Inc. (Loudon, NH). Cuttings were dipped in the rooting hormone Hormodin 1 (OHP, Inc., Morrisville, NC) and stuck in square 0.28-liter pots

filled with pre-moistened sphagnum peat mix (ProMix BX General, Premier Tech Horticulture, Quakertown, PA). For growth room experiments, plants were grown in a walk-in growth room set to 22°C and 70% relative humidity. A 16-hour photoperiod was provided using 56-watt fluorescent grow lights (Lithonia Lighting, Conyers, GA). Plants were fertilized with 20-3-19 NPK commercial water-soluble fertilizer (Jack's Professional LX, JR Peters Inc., Allentown, PA). Over the cropping period, the fertilizer was increased from 100 mg·L⁻¹ to 200 mg·L⁻¹ N as the plants grew. For greenhouse experiments, cuttings of Petunia cv. Supertunia® Black Cherry were rooted in square 6.35 cm pots filled with pre-moistened sphagnum peat mix (ProMix BX General, Premier Tech Horticulture, Quakertown, PA). Plants were grown on open mesh benches under a 16-hour photoperiod using 400-watt high-pressure sodium (HPS) lamps (PL Light Systems Inc., Beamsville, Ontario) and fertilized through stackable 4-way driplines (Netafim Irrigation Inc., Fresno, CA) with 100mg·L⁻¹ of 20-3-19 NPK commercial water-soluble fertilizer (Jack's Professional LX, JR Peters Inc., Allentown, PA) for two weeks post-rooting. The plants were then fertilized with 150 mg·L⁻¹ of the same fertilizer for one more week and then the fertilizer was increased to 200 mg·L⁻¹ for the remainder of the cropping period. Plants were watered at 36.5 mL per minute 1-3 times per day depending on plant growth.

2.2.2. *Pathogen isolate and inoculum preparation.*

Botrytis cinerea (Pers.:Fr.) isolated from infected petunia was maintained on Difco potato dextrose agar (PDA) plates. To prepare inoculum, *B. cinerea* PDA plates were incubated for 5 days at room temperature in the dark. Cultures were then exposed to 14 h darkness/10 h light for 7 days to induce sporulation. A spore suspension was obtained by flooding the cultures with 10 mL of sterile Sabouraud Maltose Broth (SMB) containing 0.1% (v/v) Tween 80 (VWR, Radnor, PA). Conidia were dislodged using a sterile FisherBrand cell spreader (Fisher Scientific,

Hampton, NH). The resulting suspension was filtered through 4 layers of sterile cheesecloth. Conidial concentration was determined with a hemocytometer (Hausser Scientific, Horsham, PA) and adjusted to 5.0×10^5 spores·mL⁻¹ (Ait Barka et al. 2004; El-Ghaouth et al. 1992).

2.2.3. *Chitosan products and preparation of purified chitosan.*

Low (50-190 kDa), medium (200-300 kDa), and high (310-375k Da) MW chitosan powders (>75% deacetylated) were obtained from Sigma Aldrich (St. Louis, MO). To prepare stock solutions, 5 g of chitosan were dissolved in 200 mL of sterile MilliQ water with 5 mL of glacial acetic acid and stirred for 24 h at room temperature (Meng and Tian 2009). The volume was then raised to 450 mL with sterile MilliQ water, and the stock solution pH was adjusted to 5.0 by adding 1 M sodium hydroxide (NaOH). For use in experiments, the stock solution of chitosan was adjusted to the desired concentration. Experiments included a 1% (v/v) glacial acetic acid control prepared at pH 5.0. The nonionic surfactant CapSil (4oz/100gal) was added to all solutions for *in planta* experiments to improve coverage and reduce phytotoxicity.

Commercial chitosan products Tidal Grow (high MW at 1% and 2% and low MW at 4%) was obtained from Tidal Vision Inc. (Bellingham, WA) (exact MWs are proprietary but are within the range of the reagent-grade chitosan MW), and ARMOUR-Zen 15 (15% chitosan) was obtained from Botry-Zen Ltd (Dunedin, New Zealand).

2.2.4. *Antimicrobial activity of chitosan against B. cinerea at different concentrations in vitro.*

To determine if reagent grade and commercial chitosan formulations have direct antimicrobial activity against *B. cinerea*, an *in vitro* assay was performed. This study consisted of seven chitosan treatments (four commercial product treatments and three reagent grade materials) at nine concentrations (Table 2-1), with each chitosan treatment tested in a separate experiment. The reagent grade and Tidal Grow treatments were tested at 0.0, 0.25, 0.5, 0.75, 1.0,

1.25, 1.5, 1.75, and 2.0% (v/v) chitosan. Due to the low pH of Tidal Grow, additional treatments were added in which the pH was adjusted to 5.0 using 1 M NaOH (Table 2-1). ARMOUR-Zen 15 was prepared at the undiluted rate (15%) and then the dose was cut in half four times to achieve 0.4, 1.5, 3.75, 7.5% (v/v) chitosan, in addition to a 0.0% control. In a preliminary trial, acetic acid spread onto ¼ strength PDA (9.75 g/L) plates had no effect on *B. cinerea* mycelial growth (data not shown), suggesting that the addition of acetic acid to the reagent grade chitosan solution did not result in disease suppression. Treatments were applied by spreading 300 µl of each of the prepared chitosan formulations onto ¼ strength PDA and then leaving them to dry for 24 hours. Next, plates were inoculated with a 5 mm plug of *B. cinerea* and incubated at 90% relative humidity and 22°C. Mycelial growth of *B. cinerea* was assessed by measuring the lesion diameter vertically and horizontally across the lesion (D1 and D2) after 0, 48, 72 and 96 hours using a digital caliper. Each chitosan treatment was applied to four replicate plates and the experiment was conducted twice. Treatments were arranged in a completely randomized design.

The area of the mycelial growth at each time point was calculated using the following formula:

$$\text{Area} = \left[\frac{1}{2} (D1) * \frac{1}{2} (D2) \right] * \pi$$

To compare growth over time, the area under the growth curve (AUGC) was calculated for each plate using the area under the disease progress curve (AUDPC) formula (Shaner and Finney 1977).

Table 2-1. Reagent grade and commercial chitosan treatments, and final solution pH, tested for antimicrobial activity against *B. cinerea* in vitro. Treatments were applied at 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0% chitosan (v/v). Each treatment was tested in a separate experiment.

Treatment ^x	pH
Reagent grade low molecular weight	5.3
Reagent grade medium molecular weight	5.3
Reagent grade high molecular weight	5.3
Tidal Grow 2%	3.6
Tidal Grow 4%	3.8
Tidal Grow 2% - pH adjusted ^y	5.0
Tidal Grow 4% - pH adjusted	5.0

^x 300 ul of each treatment were spread onto a ¼ PDA plate 24 hours prior to pathogen challenge

^y pH was adjusted with 1M NaOH

2.2.5. *Effect of molecular weight and concentration of reagent grade chitosan on B. cinerea disease suppression in planta.*

This experiment consisted of four treatments (high, medium, and low MW chitosan and acetic acid) applied at 6 application rates [0.0, 0.1, 0.2, 0.3, 0.4, or 0.5% (v/v) concentration] in which the 0.0% consisted of water. Each of the 24 treatments were applied to six replicate plants. Plants were grown in a walk-in growth room and treatments were arranged in a randomized complete block design. This experiment was conducted twice. Four weeks after rooting, petunia plants were sprayed to glistening with the treatments. Treatments were evaluated using both detached leaf and whole plant assessments.

Twenty-four hours after the chitosan application, six leaves per treatment (one from each plant) were collected and placed in glass Petri dishes with moistened sterile filter paper. The leaves were inoculated with a 10 µL drop of a 5.09×10^5 spores·mL⁻¹ suspension of *B. cinerea*. Three additional leaves per treatment served as non-inoculated controls and received 10 µL of SMB. After inoculation, the Petri dishes were incubated in the dark for 12 h and then placed under light. Lesion diameters were measured 48 hours post inoculation (hpi) using a digital caliper. Lesion area was calculated using the formula described for the *in vitro* tests.

For the whole plant assay, plants were inoculated twenty-four hours post chitosan application. Four plants per treatment were sprayed with a 5.09×10^5 spores·mL⁻¹ suspension of *B. cinerea* to run-off. The two remaining plants per treatment were sprayed with SMB to run-off. After pathogen inoculation, relative humidity was maintained at 95-99%. Four days post inoculation (dpi), the plants were evaluated for disease severity on a 0-100% infected tissue scale.

2.2.6. *Effect of commercial chitosan products on B. cinerea disease suppression in planta.*

This experiment consisted of four treatments: Tidal Grow 2%, ARMOUR-Zen 15, water control, and a CapSil control. Tidal Grow was applied at 0.25, 0.4, 0.5, and 0.75% concentrations (v/v) and ARMOUR-Zen 15 was applied at 0.3, 1.0, and 2.0% (v/v) concentrations.

Concentrations of each product were selected based on manufacturer recommended rates.

Treatments were applied to eight replicate petunia plants. Plants were grown in a walk-in growth room, treatments were arranged in a randomized complete block design, and the experiment was conducted twice. Four weeks after rooting, plants were sprayed to glistening with their assigned treatments. Twenty-four hours post chitosan application, 12 leaves were collected randomly per treatment to assess disease using a detached leaf disease assay, as described for the reagent grade *in planta* tests. Whole plant assays were not conducted because the reagent grade chitosan *in planta* assays demonstrated that detached leaf assays were more consistent and therefore were best for observing differences among treatments.

2.2.7. *Greenhouse evaluation of chitosan for disease suppression.*

Greenhouse experiments were conducted to compare the efficacy of commercial and reagent grade chitosan products under greenhouse conditions. Four weeks after rooting, petunia plants were sprayed to glistening with each of the nine treatments: high, medium, and low MW

chitosan, Tidal Grow 1%, Tidal Grow 2%, and ARMOUR Zen 15%, and acetic acid applied at 0.4% (v/v). These treatments were compared to a CapSil control and a water control. Each treatment was applied to 12 replicate plants in the greenhouse. Treatments were arranged in a randomized complete block design, and the experiment was conducted twice. Detached leaf assays were used to assess treatment effects on disease severity.

2.2.8. Data analysis

Data analysis was performed using RStudio version 2021.09.0 "Ghost Orchid" (RStudio Team 2020). To establish homoscedasticity, all detached leaf lesion area data were transformed by adding 0.5 and taking the square root prior to analysis. Data were analyzed with an analysis of variance (ANOVA) using partial (type II) sums of squares ('car' package). Post hoc Dunnett and Tukey means separation tests were conducted using least squared adjusted treatment means obtained via the 'emmeans' package in Rstudio. A Dunnett's test was used for the *in vitro* and two *in planta* experiments to determine the minimum chitosan concentration needed to reduce disease compared to the control. A Tukey's test was used to identify differences among treatments and thus infer the most effective chitosan product under greenhouse conditions. To investigate the relationship between chitosan concentration and disease suppression, a regression analysis was performed in the 'emmeans' package using the "poly" method. Graphs were created in Rstudio using the package 'ggplot2'.

2.3. Results

2.3.1. Reagent grade chitosan products suppress *B. cinerea* growth *in vitro*.

For low MW chitosan, plates treated with 2.0% chitosan concentration had significantly lower AUGC of *B. cinerea* compared to the control ($P < 0.001$). A regression analysis showed that there is a significant negative linear ($P < 0.001$) and quadratic ($P = 0.004$) relationship

between AUGC and low MW chitosan concentration ($R^2 = 0.12$) (Figure 2-1) in which increasing concentration results in reduced fungal growth.

For medium MW chitosan, plates treated with 1.25% to 2.0% chitosan had significantly lower AUGC compared to the control ($P < 0.001$). Further, a regression analysis showed that there is a significant negative linear ($P < 0.001$) relationship between AUGC and medium MW chitosan concentration ($R^2 = 0.45$) (Figure 2-1) in which increasing concentration results in reduced fungal growth.

For high MW chitosan, plates treated with 1.25% to 2.0% chitosan concentrations had significantly lower AUGC compared to the control ($P \leq 0.001$). Plates treated with 1.5% and 2.0% chitosan reduced fungal growth up to 54% and 51%, respectively, compared to the water control. A regression analysis showed that there is a significant negative linear ($P < 0.001$) relationship between AUGC and high MW chitosan concentration ($R^2 = 0.51$) (Figure 2-1) in which increasing concentration results in reduced fungal growth.

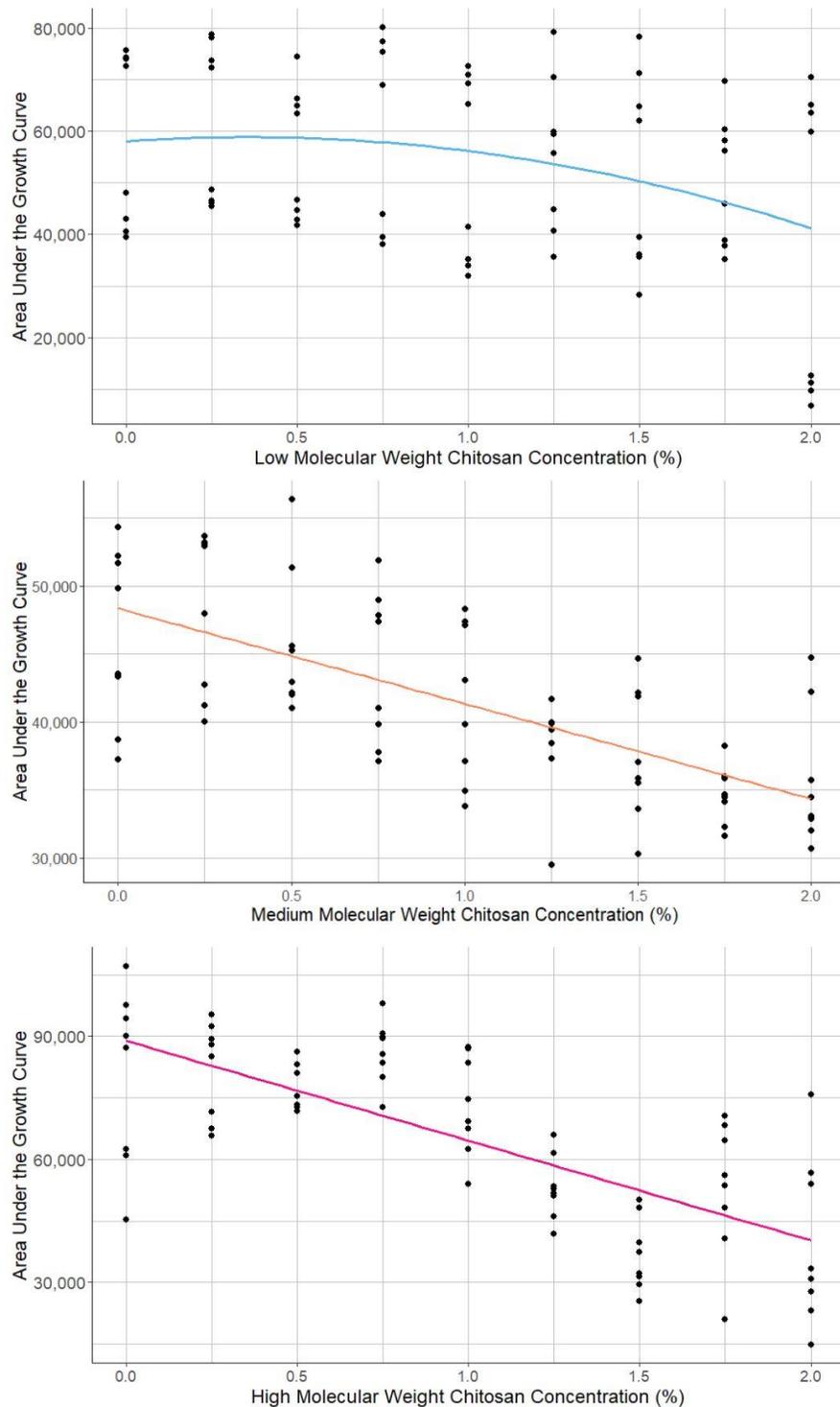


Figure 2-1. Scatter plots representing the effect of reagent grade chitosan products at three molecular weights (MW) and nine chitosan concentrations (v/v) on area under the growth curve (AUGC) of *B. cinerea* mycelium in vitro. For low MW chitosan, a quadratic regression line was the best fit ($R^2 = 0.12$). For medium and high MW chitosan data, a linear regression line was the best fit ($R^2 = 0.45$; $R^2 = 0.51$ respectively).

2.3.2. Commercial chitosan products suppress *B. cinerea* growth in vitro.

For Tidal Grow 2% (high MW), plates treated with 0.25% to 2.0% chitosan concentration had significantly lower AUGC of *B. cinerea* compared to the control ($P \leq 0.008$). Plates treated with 1.5% to 2.0% chitosan showed fungal growth reduced by 81% to 96% compared to the water control. A regression analysis showed that there is a significant negative linear ($P < 0.001$) relationship between AUGC and Tidal Grow 2% chitosan concentration ($R^2 = 0.89$) (Figure 2-2) in which increasing concentration results in reduced fungal growth. When the pH of the Tidal Grow 2% was adjusted from an original pH of 3.6 to the new pH of 5.0, only the plates treated with 1.25% to 1.5% chitosan concentrations had significantly lower AUGC compared to the control ($P \leq 0.025$). Further, a regression analysis showed that there is a significant linear ($P < 0.001$) and quadratic ($P = 0.009$) relationship between AUGC and Tidal Grow 2% chitosan concentration ($R^2 = 0.24$) (Figure 2-2) in which increasing concentration results in reduced fungal growth.

For Tidal Grow 4% (low MW), plates treated with 0.5% to 2.0% chitosan concentrations had significantly lower AUGC of *B. cinerea* compared to the control ($P \leq 0.001$). Plates treated with 1.5% to 2.0% chitosan had the lowest AUGC and showed fungal growth reduced by 56% to 71% compared to the water control. A regression analysis showed that there is a significant linear ($P < 0.001$) and quadratic ($P = 0.004$) relationship between AUGC and Tidal Grow 4% chitosan concentration ($R^2 = 0.73$) (Figure 2-2) in which increasing concentration results in reduced fungal growth. When the pH of the Tidal Grow 4% was adjusted from an original pH of 3.8 to the new pH of 5.0, the plates treated with 1.0% to 2.0% chitosan had significantly lower AUGC compared to the control ($P \leq 0.021$). The greatest suppression was observed on plates treated with 1.5% to 2.0% chitosan; these had the lowest AUGC ($P \leq 0.001$). A regression

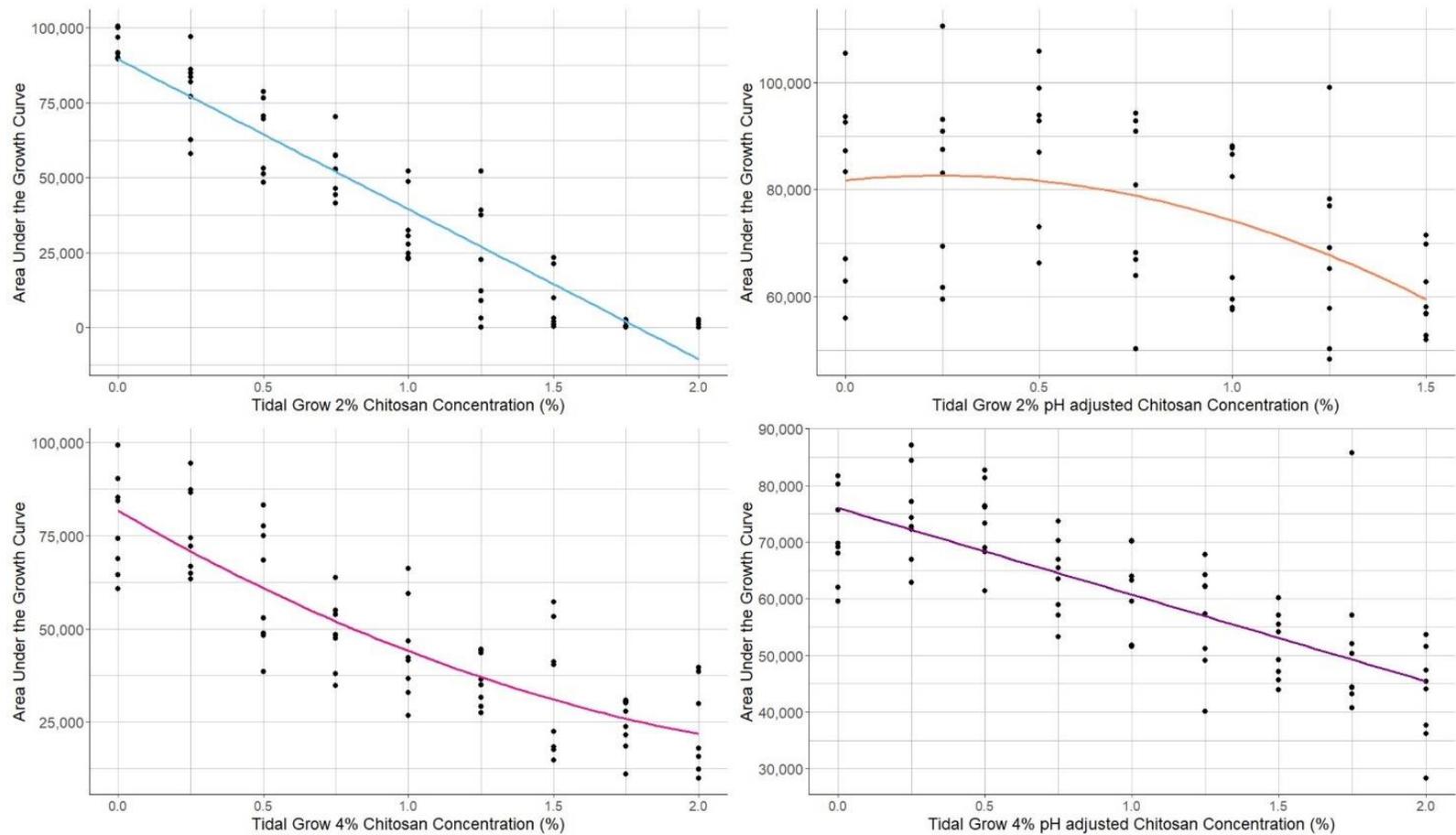


Figure 2-2. Scatter plots representing the effect of commercial chitosan products, Tidal Grow 2% and 4%, at nine chitosan concentrations (v/v) on area under the growth curve of *B. cinerea* mycelium in vitro. For Tidal Grow 2% pH adjusted data and Tidal Grow 4%, a quadratic regression line was the best fit ($R^2 = 0.24$; $R^2 = 0.73$ respectively). Tidal Grow 2% pH adjusted was only evaluated at chitosan concentrations of 0.0% to 1.5% due to the necessity of diluting the original product to raise the pH to 5.0. For Tidal Grow 2% and Tidal Grow 4% pH adjusted data, a linear regression line was the best fit ($R^2 = 0.89$; $R^2 = 0.56$ respectively).

analysis showed that there is a significant linear ($P < 0.001$) relationship between AUGC and Tidal Grow 4% chitosan concentration ($R^2 = 0.56$) (Figure 2-2) in which increasing concentration results in reduced fungal growth.

Plates treated with ARMOUR-Zen at 3.75%, 7.5%, and 15.0% chitosan concentrations had significantly lower AUGC of *B. cinerea* compared to the control ($P \leq 0.017$) (Table 2-2). Plates treated with 7.5% to 15% chitosan showed fungal growth reduced up to 70% and 94%, respectively, compared to the water control. A regression analysis showed that there is a significant linear ($P < 0.001$) and quadratic ($P < 0.001$) relationship between AUGC and ARMOUR-Zen 15% chitosan concentration ($R^2 = 0.82$) in which increasing concentration results in reduced fungal growth.

Table 2-2. Effect of commercial chitosan product, ARMOUR-Zen 15%, at five chitosan concentrations (v/v) on area under the growth curve of *B. cinerea* mycelium in vitro. Data represent least-square means of eight replicates with each value $\times 10^4$. For each reagent grade product, means followed by the same letter are not significantly different ($\alpha=0.05$) as determined by the Tukey HSD Post-hoc test.

ARMOUR-Zen 15% chitosan (v/v) tested	Mean AUGC	std error
0.0	10.0 a	0.40
0.4	9.6 a	0.24
1.5	10.3 ab	0.92
3.75	6.7 b	0.71
7.5	1.6 c	0.42
15	0.1 c	0.07

2.3.3. Low, medium, and high molecular weight reagent grade chitosan reduce *B. cinerea* on detached petunia leaves.

The reagent grade chitosan products were analyzed separately to determine the effect of MW at different chitosan concentrations. Plants treated with 0.3% and 0.4% low MW chitosan had significantly smaller lesions compared to the control ($P \leq 0.015$) (Figure 2-3). When applied at 0.4%, low MW reduced lesion size on leaves up to 65% compared to the water control. A regression analysis showed that there is a significant negative linear ($P = 0.003$) and positive

quadratic ($P = 0.01$) relationship between *B. cinerea* lesion size and low MW chitosan concentration ($R^2 = 0.17$) (Figure 2-4) in which increasing concentration results in reduced disease. Plants treated with 0.5% chitosan exhibited larger average lesion size compared to 0.4% for low MW.

Plants treated with 0.4% and 0.5% medium MW chitosan had significantly smaller lesions compared to the control ($P \leq 0.031$) (Figure 2-3). A regression analysis showed that there is a significant negative linear ($P = 0.002$) relationship between *B. cinerea* lesion size and medium MW chitosan concentration ($R^2 = 0.13$) (Figure 2-4) in which increasing concentration results in reduced disease.

Plants treated with any concentration of the high MW chitosan ($P \leq 0.009$), aside from 0.1% ($P = 0.236$), had significantly smaller lesions compared to the control (Figure 2-3). When applied at 0.5%, high MW reduced lesion size on leaves up to 63% compared to the water control. A regression analysis showed that there is a significant negative linear ($P < 0.001$) relationship between *B. cinerea* lesion size and high MW chitosan concentration ($R^2 = 0.31$) (Figure 2-4) in which increasing concentration results in reduced disease.

There was no significant difference between plants treated with any concentration of acetic acid and the control ($P \geq 0.898$) (Figure 2-3). Based on these data, we determined that a chitosan of 0.4% was most effective at reducing disease without causing phytotoxicity and advanced that rate for reagent grade chitosan treatments for the next set of experiments.

Whole plant disease severity was low across treatments for replicate experiment 1 and 2 (the average for the disease control plants was 30%). Plants treated with 0.1% and 0.4% low MW chitosan had significantly less *B. cinerea* disease severity compared to the control ($P \leq$

0.034). Plants treated with high MW chitosan concentrations of 0.2% and 0.3% significantly reduced disease severity compared to the control ($P \leq 0.038$). Medium MW chitosan (regardless

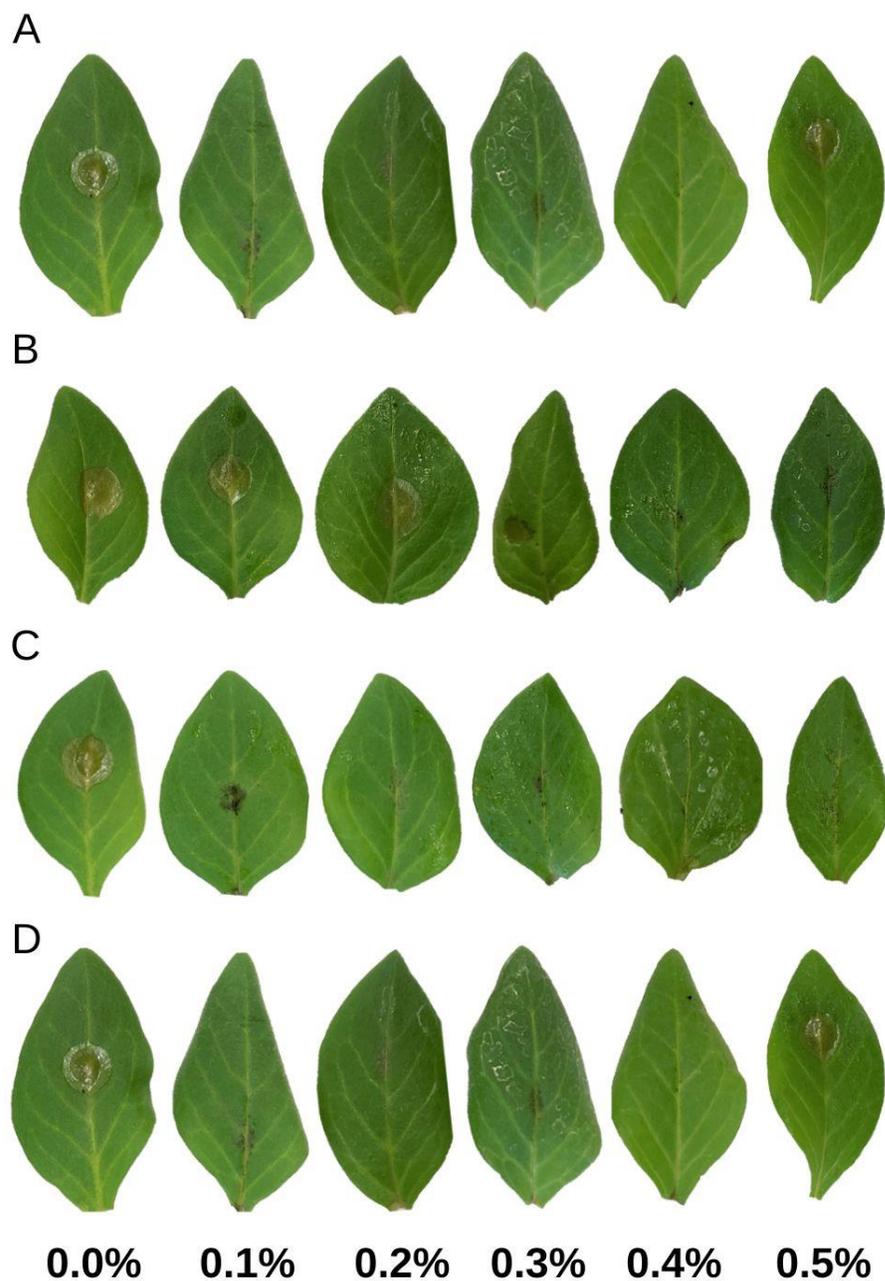


Figure 2-3. Representative detached leaves treated with (A) low molecular weight reagent grade chitosan (B) medium molecular weight chitosan (C) high molecular weight chitosan, and (D) acetic acid at 0%, 0.1%, 0.2%, 0.3%, 0.4%, and 0.5% and challenged with *B. cinerea* 24 hours post chitosan application in a detached leaf assay.

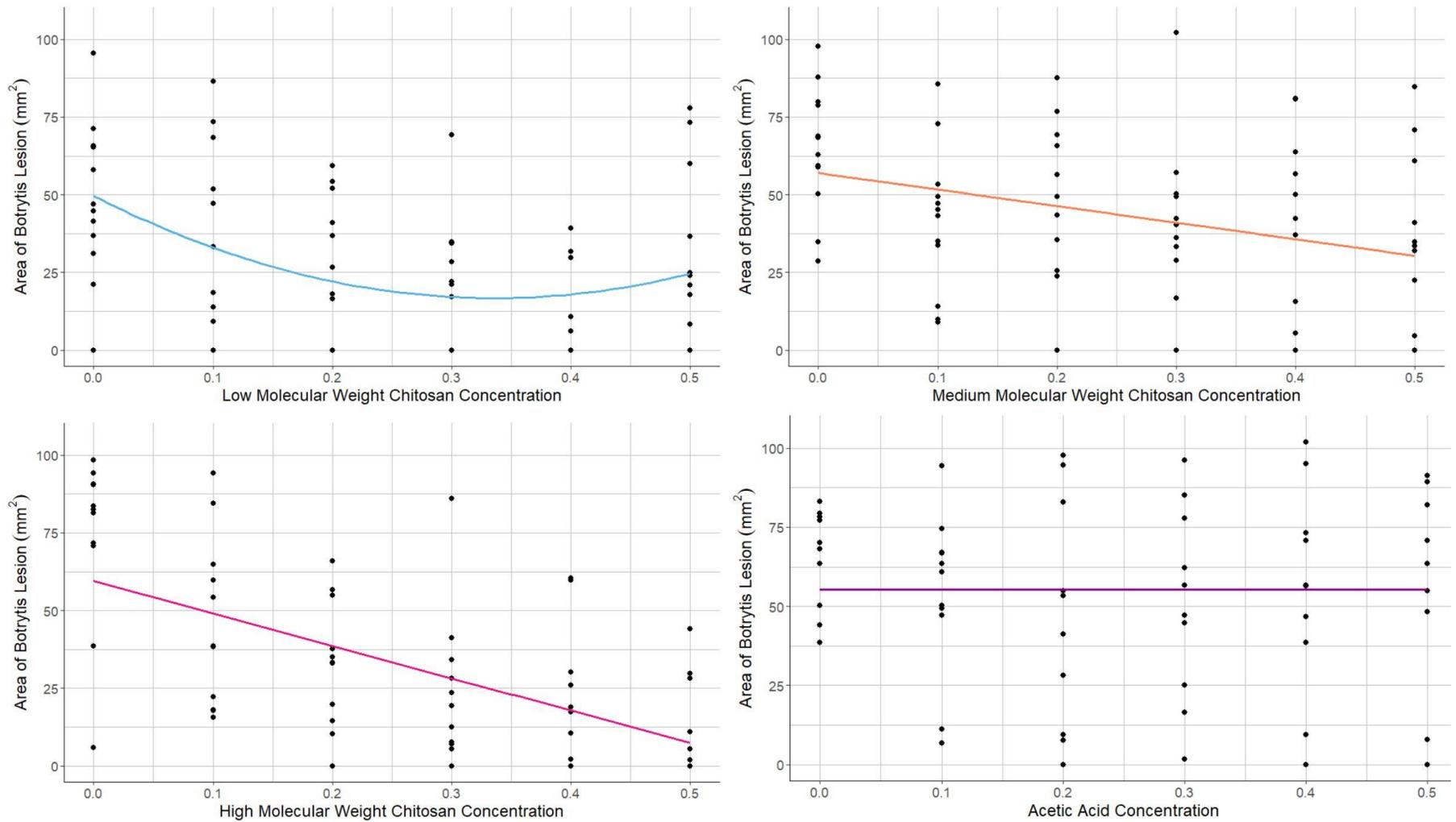


Figure 2-4. Scatter plots representing the effect of reagent grade chitosan products at low, medium, and high molecular weights, and acetic acid at five chitosan concentrations (0.1, 0.2, 0.3, 0.4, and 0.5%) with a control (0.0%). For low molecular weight data, a quadratic regression line was the best fit ($R^2 = 0.17$). For medium molecular weight, high molecular weight, and acetic acid data, a linear regression line was the best fit ($R^2 = 0.13$; $R^2 = 0.31$; $R^2 = 0.0007$ respectively).

of concentration) had no effect on *B. cinerea* disease severity at the 0.05 confidence interval but trended toward significance ($P = 0.057$). Acetic acid concentrations had no effect on *B. cinerea* disease compared to the control.

2.3.4. *ARMOUR-Zen and Tidal Grow reduce B. cinerea on detached petunia leaves.*

Plants treated with 0.75% Tidal Grow had significantly smaller *B. cinerea* lesions compared to the control ($P = 0.05$) (Figure 2-5) representing a 31% reduction in disease severity. Tidal Grow treatments at 0.25%, 0.4%, and 0.5% did not significantly reduce lesion size, however plants treated with 0.5% Tidal Grow did trend towards significance ($P = 0.073$). Regression analysis indicated that *B. cinerea* lesion size decreased linearly with increasing Tidal Grow chitosan concentration ($P = 0.019$). Plants treated with ARMOUR-Zen 15 at all concentrations (0.3, 1.0, and 2.0%) had significantly smaller lesions compared to the control ($P \leq 0.025$) (Figure 2-5). However, ARMOUR-Zen 15 applied at 1.0% resulted in the greatest suppression in which lesions on treated leaves were 75% smaller than the control. Regression analysis showed a significant quadratic ($P = 0.025$) but not linear ($P = 0.181$) relationship between concentration of ARMOUR-Zen and lesion size in which increasing ARMOUR-Zen 15 chitosan concentration results in increased disease suppression up to a certain point. There was no significant difference in lesion size between the water + CapSil and the water control ($P = 0.994$), suggesting that CapSil played no role in suppressing *B. cinerea* growth on the leaves.

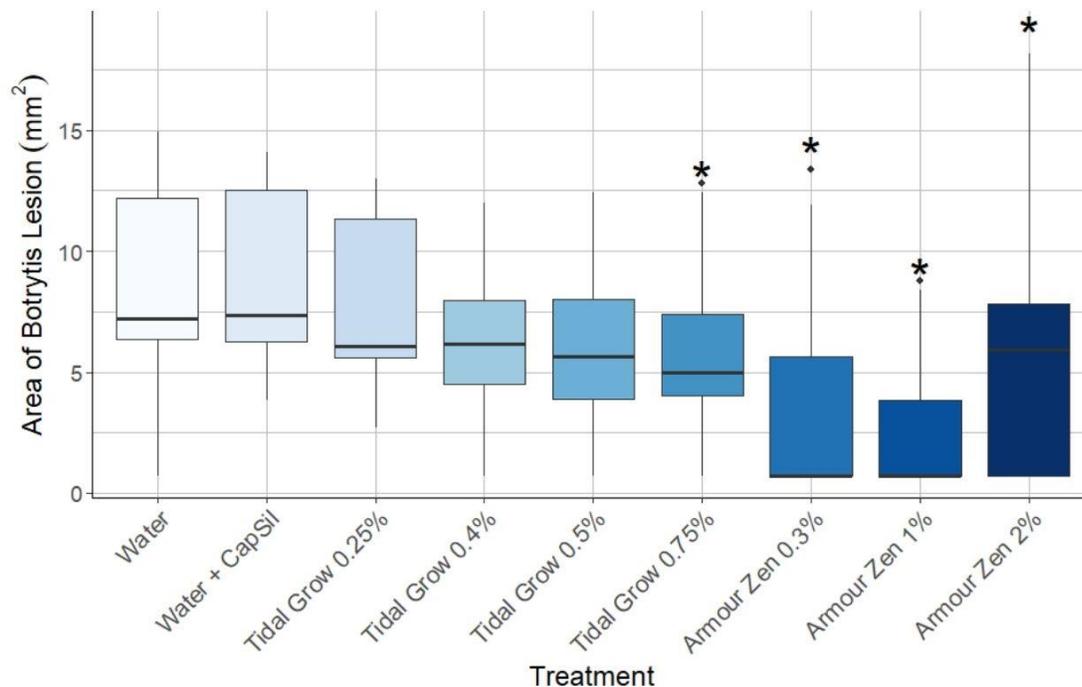


Figure 2-4. Effect of commercial chitosan products on *B. cinerea* lesion size 48 hours post inoculation on petunia leaves. Treatment means followed by an asterisk (*) differ statistically from the water control according to Dunnett's test ($n = 24$) at $\alpha=0.05$. The lines in the boxplot represent median, while the box represents the Interquartile Range (IQR). The start and end of the lines represent the minimum value in the data ($Q_3 - 1.5 \times \text{IQR}$), while any outliers are represented as dots.

2.3.5. Reagent grade chitosan and ARMOUR-Zen reduce *B. cinerea* on petunia grown under greenhouse conditions.

Plants treated with low, medium, high MW chitosan, or ARMOUR-Zen at 0.4% had 47% to 60% smaller *B. cinerea* lesions compared to the control ($P \leq 0.001$) (Figure 2-6). Tidal Grow did not reduce *B. cinerea* lesion size ($P = 1.00$). There was no significant difference in lesion size between the water + CapSil, the acetic acid at 0.4% ($P \geq 0.351$) and the water control, suggesting that CapSil and the acetic acid are not playing a role in suppressing *B. cinerea* growth on the leaves. There was no significant difference in lesion size between plants treated with low, medium, high MW, and Armour-Zen 15 at 0.4% chitosan ($P \geq 0.981$).

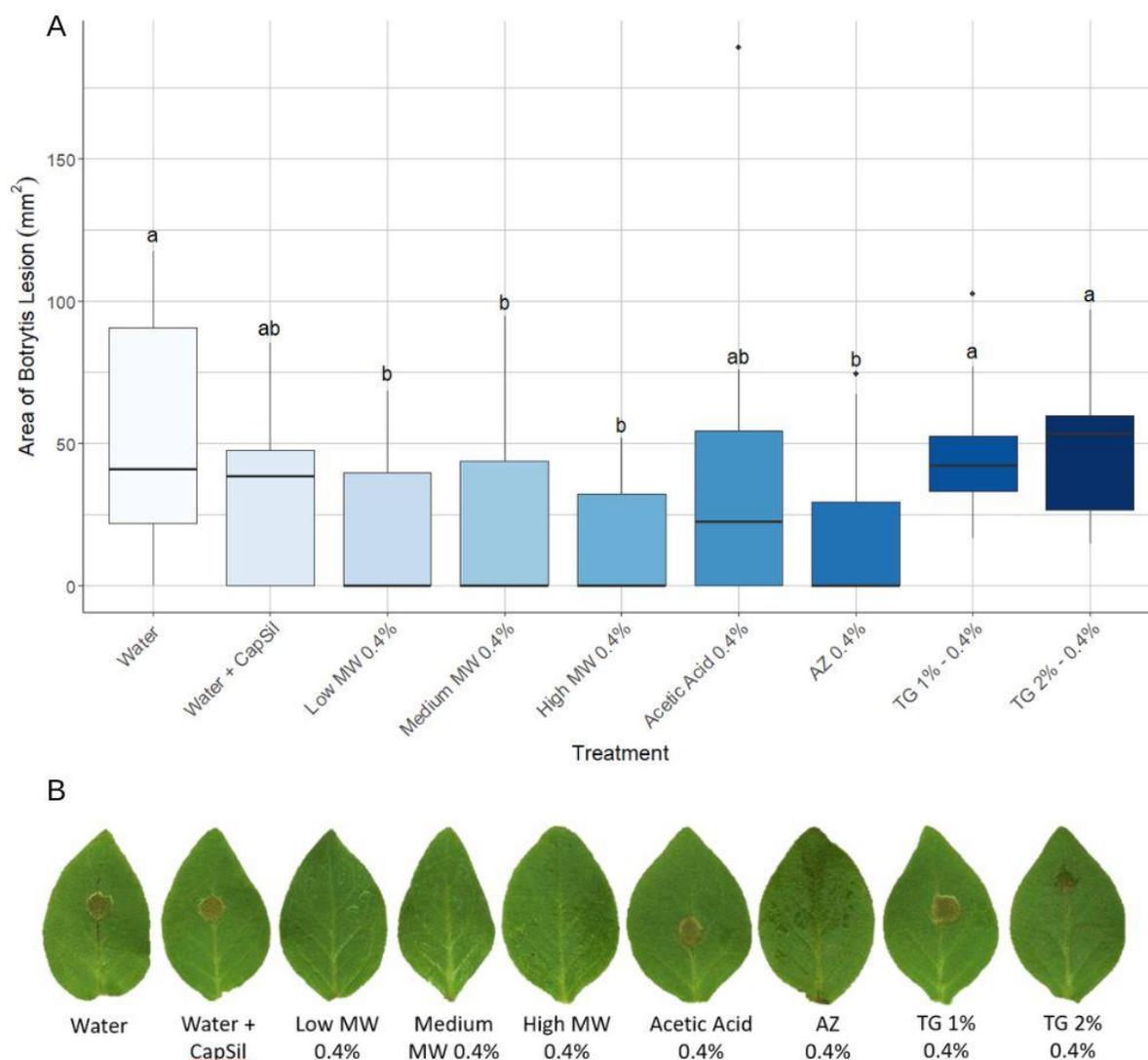


Figure 2-5. (A) Effect of reagent grade chitosan and commercial chitosan products on *B. cinerea* lesion size 48 hours post inoculation on petunia leaves collected from greenhouse grown petunia plants. All treatments, except for the water control, were mixed with the nonionic surfactant CapSil (4oz/100gal). Treatment means followed by the same letter are not significantly different ($\alpha=0.05$) as determined by the Tukey HSD Post-hoc test ($n = 21$). The lines in the boxplot represent median, while the box represents the Interquartile Range (IQR). The start and end of the lines represent the minimum value in the data ($Q_3 - 1.5 \times IQR$), while any outliers are represented as dots. (B) Representative detached leaves of each treatment challenged with *B. cinerea* 24 hours post chitosan application in a detached leaf assay.

2.4. Discussion

We found that foliar applications of reagent grade chitosan can suppress gray mold disease severity on petunia leaves up to 65% and commercial products suppress disease up to

75% compared to the water control. Results of this study demonstrate that chitosan can inhibit gray mold caused by *B. cinerea* on petunia, indicating that chitosan has potential to be used by growers as part of an IPM program to manage gray mold in greenhouse crop production. Use of chitosan as part of an integrated strategy may also help growers mitigate the risk of *B. cinerea* resistance to chemical fungicides (Muñoz et al. 2019; Samarakoon et al. 2017) by providing growers with a rotational product that has a mode of action distinct from synthetic fungicides. Additional research is needed to evaluate efficacy compared to fungicides and to investigate compatibility with other grower practices. While chitosan has been shown to suppress gray mold on food crops (Bhaskara Reddy et al. 2000; Ait Barka et al. 2004; Ben-Shalom et al. 2003) and outdoor floriculture crops (Wojdyla 2001), our study builds on this knowledge, by characterizing suppression of a foliar pathogen on a greenhouse crop. Through conducting this research, we found that efficacy differed among chitosan products and concentration. We also found that efficacy *in vitro* did not always translate to efficacy *in planta*.

Concentration of chitosan affects efficacy and phytotoxicity

In vitro, reagent grade chitosan products reduced growth of *B. cinerea* at the high range of concentrations we tested and increasing concentration correlated with greater suppression. The relationship between chitosan concentration and antifungal properties *in vitro* has been researched in other studies in which increasing concentrations progressively reduce fungal growth (Yu et al. 2012; Muñoz and Moret 2010; Liu et al. 2007; Hernández-Lauzardo et al. 2008; El-Ghaouth et al. 1997; Benhamou and Theriault 1992). In our study however, disease suppressive effects were not always observed at the high range of concentrations tested *in planta*. In our study, however, foliar disease was more severe when the concentration of low MW chitosan increased from 0.4% to 0.5% and a quadratic regression line best explained the

relationship between concentration and disease severity. We hypothesize that phytotoxicity of chitosan at higher concentrations may explain this breakdown in suppression. In preliminary work, we observed development of necrotic spots on leaves treated with low MW chitosan above 0.25% on non-infested plants (Figure A-1). These phytotoxic effects may promote infection by *B. cinerea*—a necrotrophic pathogen. The R^2 values in some of our regression analyses were low, indicating variability in the data. Some of this variation can be attributed to differences between experiments. Calculation of AUGC values can also magnify the spread of the data resulting in low R^2 values. Overall, these trends indicate a negative correlation between chitosan concentration and disease suppression but variability in chitosan efficacy is something for growers to be aware of.

Our results suggest that there is an optimal concentration at which antifungal activity *in vitro* (1.25% chitosan) and reduced symptom severity *in planta* (0.4% chitosan) are achieved; any increases in concentration provide only incremental improvements. Identifying an optimal concentration (which may vary by pathogen and by plant species) is important for product manufacturers to maximize efficacy at the lowest cost to the grower.

Chitosan type and formulation factors influence chitosan efficacy

While we did not directly compare the reagent grade chitosan products *in vitro*, in general, medium and high MWs were effective at lower concentrations (starting at 1.25%) compared to the low MW (starting at 2.0%). Alternatively, Hernandez-Lauzardo and colleagues (2008) reported that low MW was most effective at reducing growth of *Rhizopus stolonifera in vitro* (Hernández-Lauzardo et al. 2008). In another study, high MW chitosan and a carboxymethyl chitosan derivative had an inhibitory effect on growth of *Fusarium oxysporum f. sp. vasinfectum*, *Alternaria solani* and *Valsa mali* (Guo et al. 2006). While there are conflicting

reports on which MW is most effective, it is possible that fungal species differ in their sensitivity to chitosan *in vitro* (Hernández-Lauzardo et al. 2008). Additionally, the degree of acetylation, structure of derivatives, and preparation can affect the physiochemical properties of chitosan and thus suppression of fungal growth (Younes et al. 2014; Hernández-Lauzardo et al. 2008).

The pH of chitosan solutions has been shown to influence disease suppression by chitosan. (Nassr and Barakat 2013) reported that low pH of the media solution can inhibit *B. cinerea* conidia germination and, thus, there may be a similar effect on mycelial growth. In our study suppression was greater for the Tidal Grow products at pH 3.6 to 3.8 compared to the Tidal Grow products adjusted to 5.0, suggesting that the low pH was playing an inhibitory role. However, even at pH 5.0, the Tidal Grow products at chitosan concentrations > 1.25% reduced *B. cinerea* growth from 15% to 40% compared to the water control.

Commercially available products show promise for greenhouse use

Both commercial products (Tidal Grow and ARMOUR-Zen 15) exhibited direct antifungal properties in the *in vitro* assays, with Tidal Grow 2% and 4% being effective at lower chitosan concentrations ($\geq 0.25\%$ and $\geq 0.5\%$ respectively) than ARMOUR-Zen 15 ($\geq 3.75\%$). In another study testing ARMOUR-Zen, Reglinski and colleagues (2010) reported inhibition of *B. cinerea* in liquid media containing ARMOUR-Zen at 0.016, 0.03, 0.125, and 0.25 g L⁻¹ chitosan (representing 0.0016%, 0.003%, 0.0125%, and 0.025% chitosan). Growth inhibition was accompanied by retraction of cytoplasm and shrinkage of the mycelium (Reglinski et al. 2010). Differences in study design may explain discrepancies between studies. Reglinski et al. evaluated chitosan in solution, whereas we evaluated a chitosan coating to mimic chitosan applied as a foliar spray.

In planta, both commercial products reduced severity of gray mold symptoms by 31% to 75%, although ARMOUR-Zen 15 was effective at lower concentrations (starting at 0.3%) than Tidal Grow (0.75%). The reduction in lesion area by ARMOUR-Zen 15 (~75%) was comparable to that seen with the reagent grade chitosan products (up to 65%). While ARMOUR-Zen has been tested against *B. cinerea* in grape production (Calvo-Garrido et al. 2013; Reglinski et al. 2010; Feliziani et al. 2013) and against *Alternaria solani* and *Xanthomonas vesicatoria* in tomato production (Ramkissoon et al. 2016), this research is the first to evaluate Tidal Grow and ARMOUR-Zen for disease suppression under greenhouse conditions. Results from this study provide evidence that these chitosan products may be effective tools for greenhouse growers. Additional studies are needed to compare efficacy of chitosan products to fungicides and to determine compatibility with other pest and disease management tools (beneficial insects, biopesticides, and chemical pesticides).

Efficacy differed between in vitro and in planta assays

We observed differences in the effect of chitosan concentration on *B. cinerea* growth *in vitro* and disease severity *in planta*. *In vitro* assays are useful for examining anti-microbial activity of products but are not always accurate predictors of efficacy *in planta* (Andrews 1992). For example, we found that concentrations >1.25% reduced *B. cinerea* growth *in vitro*, while disease suppression was observed at only 0.4% to 0.5% *in planta*.

Research has shown that chitosan can activate plant defense systems and this may play a role in its suppressive effects (Benhamou 1996) and explain differences between our *in vitro* and *in planta* assays. Chitosan elicits a host response characterized by increased enzymatic activity of chitinase and β -1,3-glucanases, and enhanced production of callose cell wall appositions in the host's epidermis and outer cortex (Algam et al. 2010; El-Ghaouth et al. 1994a; Benhamou et al.

1998). Other studies have shown that chitosan can have a direct antimicrobial effect on fungal pathogens by causing cellular disorganization such as cell wall loosening, cytoplasm disintegration, and excessive branching and swelling of the cell wall (Ait Barka et al. 2004; El-Ghaouth et al. 1994b; Benhamou et al. 1998). We hypothesize that the different chitosan products we tested vary in their modes of action. Tidal Grow products appear to be more effective at reducing *B. cinerea* through direct antagonistic properties, while the mode of action of ARMOUR-Zen may be to activate the plant's defense response. This research suggests that there is still much to learn about chitosan and its modes of action.

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CHAPTER 3

Integration of chitosan and biopesticides to suppress pre-harvest diseases of apple

Liza DeGenring, Kari Peter, and Anissa Poleatewich

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Abstract

The natural product chitosan has been shown to reduce plant disease severity and enhance the efficacy of microbial biocontrol agents in several crops. However, little is known about the potential synergisms between chitosan and biopesticides and best use practices in apple production. The objectives of this study were to evaluate the effect of pre-harvest application of chitosan alone and in combination with a commercial biopesticide to suppress fungal diseases of apple and to investigate the potential for chitosan to reduce the quantity of overwintering *Venturia inaequalis* spores in orchard leaf litter. Chitosan products, Tidal Grow and ARMOUR-Zen 15, and a commercial biopesticide, Serenade ASO, were tested in a research orchard in Pennsylvania and commercial orchards in New Hampshire. Chitosan applications reduced apple scab incidence and severity up to 55% on fruit compared to the water control. Chitosan also reduced sooty blotch, flyspeck, and rust incidence on fruit. Furthermore, a chitosan + biopesticide treatment overlaid onto a grower standard spray program reduced disease more effectively than the grower standard alone. However, this efficacy was dependent on cultivar and pathogen. Chitosan did not reduce overwintering *V. inaequalis* ascospores. This research provides evidence that pre-harvest chitosan applications have the potential for disease management in apple production.

Keywords: chitosan, biopesticides, natural products, apple scab, powdery mildew, *Bacillus subtilis*

3.1. Introduction

The development and adoption of alternative products for managing plant diseases have been driven by growing concerns over the use of conventional fungicides (Lamichhane et al. 2016; Pimentel 2005). Research has found that synthetic pesticides can leave chemical residues on produce, have detrimental impacts on human health, non-pest species, and the environment, and can lead to the development of fungicide resistance (Ekström and Ekbohm 2011; Wilson and Tisdell 2001; Gomiero et al. 2011). Extensive research has focused on the use of microbial biocontrol agents (BCAs) for the management of preharvest and postharvest diseases over the last 30 years (Raymaekers et al. 2020; Pandit et al. 2022; Droby et al. 2009; Janisiewicz and Korsten 2002), resulting in the commercialization of several biopesticide products. Barriers to widespread commercial use of BCAs include high cost of production, limited shelf-life, and variable performance under the environmental conditions found in commercial farms (Fravel 2005; Marian and Shimizu 2019; Chandler et al. 2011). BCAs often fail to grow and maintain high enough populations in the rhizosphere and phyllosphere or do not produce antifungal compounds at levels necessary to suppress pathogen activity (Glare et al. 2012). Finding products that enhance the efficacy of BCAs could improve disease management. Several natural compounds, such as cellulose, chitin, and chitosan, have shown a synergistic effect on biopesticide efficacy for reducing plant diseases (Kokalis-Burelle et al. 1992; Davis et al. 1992; Beauséjour et al. 2003; Yu et al. 2012).

Chitosan has been gaining interest as an alternative tool to promote plant growth and reduce disease. Chitosan is a natural β -(1,4)-glucosamine polymer, derived from chitin and found

in insect and crustacean exoskeletons and fungal cell walls (Sharif et al. 2018; Kaur and Dhillon 2014). Chitosan has been shown to induce a host response (Jia et al. 2018) and have fungistatic properties against several pathogenic fungi, such as *B. cinerea* (Pers.:Fr), *Rhizopus stolonifer* (Ehrenb.:Fr.) Vuill, *Penicillium expansum* (Link), *Fusarium oxysporum* f. sp. *radicis-lycopersici* Jarvis and Shoemaker (Hernández-Lauzardo et al. 2008; Benhamou and Theriault 1992; El-Ghaouth et al. 1992; DeGenring et al. 2023).

Much of the research evaluating chitosan as a disease management tool has focused on post-harvest application to reduce storage rots and extend the shelf-life of fruits and vegetables (Romanazzi and Feliziani 2016; Li et al. 2015; Zhang et al. 2011; El-Ghaouth et al. 1991b; Liu et al. 2007). There is limited research on the pre-harvest application of chitosan to reduce plant diseases (DeGenring et al. 2023; Bautista-Baños et al. 2006; Poleatewich 2010). Chitosan dips for tomato seeds significantly reduced disease symptoms of *Fusarium oxysporum* f. sp. *radicis-lycopersici* and *Pythium aphanidermatum* (Edson) Fitzp. (Benhamou and Theriault 1992; Lafontaine and Benhamou 1996; Benhamou et al. 1994). A combination of chitosan seed treatment and foliar spray on pearl millet reduced downy mildew disease caused by *Sclerospora graminicola* (Sacc.) (Sharathchandra et al. 2004). Chitosan sprays have reduced disease caused by *B. cinerea* on petunia (DeGenring et al. 2023) and cucumber plants (Ben-Shalom et al. 2003). While these studies show promise for the pre-harvest application of chitosan, there are still gaps in knowledge pertaining to how efficacy is affected by molecular weight, concentration of chitosan, solution pH, and number of applications (DeGenring et al. 2023; Bautista-Baños et al. 2006; Bhaskara Reddy et al. 2000).

A limited number of studies have suggested that natural compounds, such as chitin and chitosan, may have agriculturally useful synergisms when applied with a BCA (Yu et al. 2012;

Benhamou et al. 1998). There are several hypotheses regarding the mechanisms behind these synergisms including a heightened plant defense response, enhanced BCA population growth, and increased chitinase production by BCAs (Lu et al. 2014; Kokalis-Burelle et al. 1992; Yu et al. 2008). For example, *Bacillus pumilus* (PGPR strain SE 34) combined with chitosan resulted in an amplified defense response on tomatoes infected with *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Benhamou et al. 1998).

Additionally, both chitin and chitosan have shown to directly enhance BCAs', specifically yeasts, antagonistic activity and population growth in postharvest applications (Ge et al. 2010; Lu et al. 2014; Yu et al. 2008, 2012). There is some research to suggest that chitin may act as a food source for BCAs and thereby enhance biocontrol activity. Kokalis-burelle et al. reported a 60% reduction in early leafspot of peanut when plants were treated with *Bacillus cereus* strain 304 and chitin compared to the non-treated control and a 1.3 log increase in *B. cereus* population levels compared to the non-chitin amended leaves. It was hypothesized that the chitin stimulated production of anti-fungal enzymes and helped the BCA persist long enough to compete with the pathogen by providing protection from harmful environmental variables and by providing a nutrient source (Kokalis-Burelle et al. 1992). These results are consistent with the effects of other food source amendments, such as cellulose (Davis et al. 1992).

Further research is needed to evaluate the efficacy of pre-harvest commercial chitosan applications and the potential synergisms of a combined application of chitosan and a BCA. Apple fruit production is an excellent model to address these knowledge gaps due to the multiple fungal pathogens present throughout the growing season.

Apple scab, caused by the fungus *Venturia inaequalis* (Cke.) Wint., is one of the most destructive diseases of apple (*Malus domestica* Borkh.) in the Northeast, where the moist and

warm conditions during the growing season favor disease (MacHardy 1996). *V. inaequalis* is an ascomycete fungus that has both sexual and asexual forms (Gauthier et al. 2018). *V. inaequalis* overwinters in infected fallen leaf and fruit debris as pseudothecial initials. In spring following sexual reproduction, the pseudothecia release ascospores from within sacs (asci) during wet conditions, at approximately the same time as apple trees break dormancy. As a polycyclic pathogen, ascospores serve as primary inoculum to initiate primary infections. Once primary infection is complete, asexual reproduction occurs on infected leaves and buds resulting in the production of secondary inoculum, the conidia (Gauthier et al. 2018). Conidia spread by wind and rain resulting in secondary infections of which there can be multiple cycles over a growing season. The severity of an apple scab epidemic is determined largely by weather conditions, inoculum pressure, cultivar susceptibility, and primary lesions produced by primary infection (MacHardy 1996). Thus, growers can reduce inoculum pressure by reducing overwintering of *V. inaequalis* in orchard leaf litter (Sutton et al. 2000) and reducing infections during primary infection (Holb 2006). Growers rely predominately on fungicides for reducing both primary and secondary infections in their orchards. However, the development of fungicide resistance by *V. inaequalis* is of large concern, and several classes of fungicides, such as benzimidazole, strobilurin, and demethylation inhibitors, have already lost their effectiveness (Ma and Michailides 2005; Holb 2009; Turecek and Köller 2004). *Bacillus* based biopesticides have been effective at reducing apple scab compared to a non-treated control (Poleatewich et al. 2012; Travis et al. 2005) but typically do not achieve control comparable to conventional fungicides (Yoder et al. 2006; Cromwell et al. 2011).

In addition to apple scab, the prevalence of powdery mildew (*Podosphaera leucotricha* (Ellis & Everh.) E.S. Salmon), cedar-apple rust (*Gymnosporangium juniperi-virginianae*

Schwein.), and sooty blotch and flyspeck (Díaz Arias et al. 2010) are common diseases affecting apple production in the northeastern United States (Holb et al. 2017). *P. leucotricha* is an ascomycete fungus in the Erysiphaceae family. The fungus overwinters in terminal and lateral shoot and blossom buds. Primary infection occurs with the dispersion of asexual conidia (Marine et al. 2010). Infection is high during periods of hot and humid weather but does not require the presence of leaf wetness. During the growing season, the pathogen continuously produces conidia and multiple infection cycles can occur (Marine et al. 2010). To reduce primary inoculum, it is critical that growers either prune infected shoots or apply chemical sprays prior to green tip as the fungus is already overwintering in the buds (Holb et al. 2017).

G. juniperi-virginianae is a basidiomycete fungus in the Pucciniaceae family (Snover-Clift and Jensen 2015). This rust needs two hosts to complete its life cycle: juniper and apple. The rust overwinters on their juniper host as stem galls and can potentially survive multiple years in this structure (Snover-Clift and Jensen 2015). The galls produce teliospores that spread by wind to infect the apple host (Snover-Clift and Jensen 2015). Growers must focus on this primary infection to reduce symptoms on apples, however the presence of juniper outside of the orchard can reduce a farmer's ability to minimize inoculum.

Sooty blotch and flyspeck are a disease caused by a complex of saprophytic fungi that colonize the epicuticular wax layer of apple (Díaz Arias et al. 2010). On going research suggests that multiple species are present in this complex including Dothideomycetes, Capnodiales, and Mycosphaerellaceae (Díaz Arias et al. 2010). It is still unknown which of these species causes the greatest economic threat. Sooty blotch and flyspeck are considered summer fruit diseases and should be managed with sprays in the second half of the summer.

Many growers have adopted Integrated Pest Management (IPM) programs and seek alternative tools to manage disease in their orchards. Our research aims to give growers tools to utilize in replacement of or in rotation with conventional fungicides for managing foliar diseases in apple orchards. This research is a launching point for future experiments to develop recommendations for the use of chitosan products as part of an IPM approach to manage preharvest diseases.

The objectives of this study were to evaluate effects of chitosan to (1) suppress fungal pathogens of apple when applied alone or in combination with a commercial biopesticide on a research orchard, (2) suppress fungal pathogens of apples when applied as part of conventional fungicide program on a commercial orchard, and (3) reduce the quantity of overwintering spores of *V. inaequalis* in orchard leaf litter.

3.2. Materials and Methods

3.2.1. Chitosan Products

The commercial chitosan product ARMOUR-Zen 15 (15% chitosan) was obtained from Botry-Zen Ltd (Dunedin, New Zealand). The commercial chitosan product Tidal Grow (high molecular weight 2%) was obtained from Tidal Vision Inc. (Bellingham, WA) (exact MWs are proprietary but are within the range of the 310-375 kDa). The biopesticide, Serenade ASO, with the active ingredient *Bacillus subtilis* QST 713, was obtained from Bayer AG (Leverkusen, Germany).

3.2.2. Objective 1. Research Orchard Trials

3.2.2.1. Research orchard site

Two experiments were conducted from 2021-2022 in a 0.8-acre research block at the Penn State University (PSU) Fruit Research and Extension Center (FREC) located in Biglerville,

Pennsylvania. The orchard has an average yearly rainfall of 112 cm, average summer temperature ranging from 16 to 28 °C, and Arendtsville gravelly loam soil type (National Cooperative Soil Survey 2003; Turley et al. 2022). The objective of these experiments was to evaluate the efficacy of chitosan alone or in combination with a commercial biopesticide to control pre-harvest diseases of apple. Results from 2021 were used to inform and adjust experiments in 2022. Experiments were conducted on semi-dwarf cultivar ‘Rome’ grafted on M.7 rootstock planted in 2015. Maintenance programs for insect pests and fire blight were applied with an airblast sprayer delivering 100 gallon/acre to the entire orchard following commercial production practices. This research orchard had a history of apple scab and powdery mildew and thus this research relied on natural inoculum. The Network for Environment and Weather Application’s apple scab models were used to collect weather data and to predict infection periods and inoculum load using the weather station located at FREC (NEWA 2023; <https://newa.cornell.edu/>).

3.2.2.2. *Experiment 1. Research orchard trials – 2021*

Five treatments were evaluated for efficacy in reducing pre-harvest diseases of apple: water control, grower standard (GS), chitosan (C), reduced risk (RR), and reduced risk + chitosan (RR+C) (Table 3-1). Reduced risk products are classified as having low impact on the environment, high specificity to target organisms, and low potential for human health risk (Adaskaveg et al. 2005). The chitosan product, Tidal Grow 2%, was applied at 0.025 mg·ml⁻¹ (0.0025% (v/v) chitosan). Each treatment was applied to six replicate trees arranged in a randomized complete block design with a buffer tree in between each treatment tree. Treatments were applied using a boom sprayer at 400 psi, delivering 100 gallon/acre. Treatment applications were made on a 7-15-day interval starting in mid-April for primary *V. inaequalis* infection and

every 10-14 days for secondary infection (Table 3-1). On 8-June, trees were assessed for foliar disease incidence. On 5-October, fruit were harvested and evaluated for disease incidence.

3.2.2.3. *Experiment 2. Research orchard trials – 2022*

Five treatments were evaluated in experiment 2 (Table 3-2). The rate of chitosan was increased from 473 mL/acre (in experiment 1) to 1893 mL/acre (0.1 mg·ml⁻¹ or 0.01% (v/v) chitosan), and the sulfur component of the reduced risk treatment in experiment 1 was removed to focus on synergisms between chitosan and the microbial biopesticide (Table 3-2). Each treatment was applied to five replicate trees arranged in a randomized complete block design with a buffer tree in between each treatment tree. Treatments were applied as described for experiment 1 (Table 3-2). On 14-June, trees were assessed for foliar disease incidence. On 27-September, fruit were harvested and evaluated for disease incidence.

3.2.2.4. *Objective 1. Disease Assessments*

For experiments 1 and 2, trees were evaluated for incidence of five apple diseases: apple scab, powdery mildew, rust, flyspeck, and sooty blotch. In mid-June, trees were assessed for apple scab, powdery mildew, and rust incidence on leaves. For each replicate tree, foliar disease incidence was determined by randomly selecting 10 terminal shoots and evaluating all the leaves on the shoot for apple scab, powdery mildew, and rust incidence. A leaf was counted in the overall incidence of a tree if it had at least one lesion visible with the naked eye. At harvest, in early October of each year, 25 fruit per replicate tree were harvested and immediately evaluated for incidence of apple scab, powdery mildew, rust, sooty blotch, and fly speck. The number of apple scab lesions per fruit were counted. Additionally, 25 fruit per tree were rated for apple scab severity using a 0-6 score as described by Poleatewich et al. (Poleatewich et al. 2012) (Figure 3-

1A). Each of the 25 fruits were also evaluated for powdery mildew severity as a percentage of fruit showing russet symptoms using a rating scale from 0-6 (Figure 3-1B).

Table 3-1. Treatment list, application rate, and applying timing for experiment 1 conducted at Penn State University Fruit Research and Extension Center in 2021. Treatments were applied to ‘Rome’ grafted on M.7 rootstock.

Treatment	Trade Name (Active Ingredient)	Rate (per acre)	Timing¹
Water Control	Water	--	TC-10C
Grower Standard (GS)	Manzate Pro-Stick (<i>Mancozeb</i>)	1361 g (3 lb)	TC-1C
	Captan Gold (<i>Captan</i>)	1134 g (2.5 lb)	TC, 2C-10C
	Luna Sensation (<i>Fluopyram and Trifloxystrobin</i>)	184 mL (5 fl oz)	P, FB
	Inspire Super (<i>Difenoconazole and Cyprodinil</i>)	355 mL (12 fl oz)	PF, 1C
Chitosan (C)	LI 700 (<i>Penetrant</i>)	473 mL (1 pint)	2C-10C
	Tidal Grow (<i>2% Chitosan</i>)	473 mL	TC-10C
Reduced Risk (RR)	Microthiol Disperss (<i>Sulfur 80%</i>)	4536 g (10 lb)	TC-PF
	Serenade ASO (<i>Bacillus subtilis</i> strain QST 713)	3785 mL (4 qt)	1C-10C
Reduced Risk + Chitosan (RR+C)	Microthiol Disperss (<i>Sulfur 80%</i>)	4536 g (10 lb)	TC-PF
	Serenade ASO (<i>Bacillus subtilis</i> strain QST 713)	3785 mL (4 qt)	1C-10C
	Tidal Grow (<i>2% Chitosan</i>)	473 mL	TC-10C

¹Application timings: Tight Cluster (TC, 13 Apr); Pink (P, 27 Apr); Full Bloom (FB, 3 May); Petal Fall (PF, 11 May); 1st Cover (1C, 21 May); 2nd Cover (2C, 4 Jun); 3rd Cover (3C, 18 June); 4th Cover (4C, 30 Jun); 5th Cover (5C, 13 Jul); 6th Cover (6C, 28 Jul); 7th Cover (7C; 13 Aug); 8th Cover (8C; 26 Aug); 9th Cover (9C; 15 Sept); and 10th Cover (10C; 1 Oct).

Table 3-2. Treatment list, application rates, and application timing for experiment 2 conducted at Penn State University Fruit Research and Extension Center in 2022. Treatments were applied to ‘Rome’ grafted on M.7 rootstock.

Treatment	Trade Name (Active Ingredient)	Rate (per acre)	Timing¹
Water Control	Water	--	TC-11C
Grower Standard (GS)	Manzate Pro-Stick (<i>Mancozeb</i>)	1361 g (3 lb)	P-11C
	Captan Gold (<i>Captan</i>)	1134 g (2.5 lb)	P
	Inspire Super (<i>Difenoconazole and Cyprodinil</i>)	355 mL (12 fl oz)	P, 1C
	Miravis (<i>Pydiflumetofen</i>)	101 mL (3.42 fl oz)	B, PF
Chitosan (C)	Tidal Grow (<i>2% Chitosan</i>)	1893 mL	P-11C
Reduced Risk (RR)	Serenade ASO (<i>Bacillus subtilis</i> strain QST 713)	3785 mL (4 qt)	P-11C
Reduced Risk + Chitosan (RR+C)	Serenade ASO (<i>Bacillus subtilis</i> strain QST 713)	3785 mL (4 qt)	P-11C
	Tidal Grow (<i>2% Chitosan</i>)	1893 mL	P-11C

¹Application timings: Tight Cluster (TC, 13 Apr); Pink (P, 21 Apr); Full Bloom (FB, 3 May); Petal Fall (PF, 11 May); 1st Cover (1C, 18 May); 2nd Cover (2C, 26 May); 3rd Cover (3C, 10 June); 4th Cover (4C, 22 Jun); 5th Cover (5C, 8 Jul); 6th Cover (6C, 20 Jul); 7th Cover (7C; 3 Aug); 8th Cover (8C; 17 Aug); 9th Cover (9C; 31 Aug); 10th Cover (10C; 12 Sept); and 11th Cover (11C; 23-Sept).



Figure 3-1. Representation for assessment of (A) apple russet severity and (B) apple scab severity on harvested fruit. Each fruit was assigned a rating (0-6) corresponding to the picture it most clearly resembled. These apples are medium sized (~8 cm in diameter).

3.2.3. Objective 2. On-farm trials

3.2.3.1. Orchard sites

Two experiments were conducted in 2022 on two commercial orchard sites in New Hampshire (NH). These experiments were designed to compare disease suppression of a conventional fungicide spray program alone or in combination with the chitosan product, ARMOUR-Zen 15, applied at $1.5 \text{ mg}\cdot\text{ml}^{-1}$ (0.15% (v/v) chitosan) and the biopesticide, Serenade ASO, applied at $1.5 \text{ mg}\cdot\text{ml}^{-1}$. These farms had a history of apple scab (personal communication with farmer) and thus this research relied on natural inoculum. The NEWA's apple scab models were used to collect weather data and to predict infection periods and inoculum load using the weather station located at each site (NEWA 2023; <https://newa.cornell.edu/>). Maintenance programs for insect pests and fire blight were applied by the growers to both orchards following commercial production practices.

3.2.3.2. Experiment 3. On-Farm Site #1

This trial was conducted on a commercial farm in a 3.0-acre orchard with semi-dwarf cultivars 'McIntosh' and 'Macoun' grafted on Bud.9 or M.9 rootstock that were planted in 2017. This site has an average rainfall of 120 cm, mean temperature of 9°C , and Hollis-Charlton very rocky fine sandy loam soil type (National Cooperative Soil Survey 2016b; NH GRANIT 2020). Three treatments were evaluated: grower standard control (GS), grower standard + chitosan (GS+C), and grower standard + biopesticide + chitosan (GS+B+C) (Table 3-3). Each treatment was applied to seven replicate 'McIntosh' trees and eight replicate 'Macoun' trees. Treatments were applied to trees arranged in a randomized complete block design with a buffer tree in between each treatment tree. The GS treatment was applied using the grower's equipment, a Rears Pul Blast Sprayer (REARS MFG. CO., Coburg, OR), delivering 38-40 gal/A. The C and B+C sprays were applied on a different day as the GS spray using two Dramm backpack BP-4Li

sprayers (Model #BP-4LI and #MS40Li (MUS)) at 150 psi, delivering 23-25 gal/A. Treatment applications were made on ~10-day intervals starting on 5-May for primary *V. inaequalis* infection and ~30-day intervals for secondary infection (Table 3-3). The final treatment application was made on 8-September at harvest. Trees were assessed for foliar disease incidence bi-weekly starting on 7-June at symptom onset. Fruit were harvested on 8-September and put into cold storage at 4°C located at the UNH Woodman Farm until evaluations for disease incidence were initiated.

3.2.3.3. *Experiment 4. On-Farm Site #2*

The second commercial orchard trial took place in a 0.9-acre orchard with semi-dwarf cider cultivars ‘Kingston Black’, ‘Dabinett’, and ‘Wickson’ grafted on M.26 rootstock that were planted in 2018. This site has an average rainfall of 114 cm, average temperature of 6°C, and Gilmanton fine sandy loam soil type (National Cooperative Soil Survey 2016a; NH GRANIT 2020). Three treatments were evaluated: grower standard control (GS), grower standard + chitosan (GS+C), and grower standard + biopesticide + chitosan (GS+B+C) (Table 3-4). Each treatment was applied to four replicate trees arranged in a randomized complete block design. The GS treatment was applied using the grower’s equipment, a Rears powerblast sprayer (REARS MFG. CO., Coburg, OR), delivering 38-40 gal/A. The C and B+C sprays were applied on a different day as the GS spray using two Dramm backpack BP-4Li sprayers (Model #BP-4LI and #MS40Li (MUS)) at 150 psi, delivering 23-25 gal/A. Treatment applications were made on ~10-day intervals starting on 6-May for primary *V. inaequalis* infection and ~30-day intervals for secondary infection (Table 3-4). The final treatment application was made on 4-October at harvest. Maintenance programs for insect pests and fire blight were applied by the grower to the entire orchard following commercial production practices. Trees were assessed for foliar disease

incidence monthly starting on 10-June at symptom onset. Fruit were harvested on 4-October and put into cold storage at 4°C until evaluations for disease incidence were initiated.

Table 3-3. Treatment list, application rates, and application timing for experiment 3 conducted in 2022. Treatments were applied to cultivars ‘Macoun’ and ‘McIntosh’.

Treatment	Trade Name (Active Ingredient)	Rate (per acre)	Timing¹
Grower Standard (GS)	Koverall Fungicide (<i>Mancozeb</i>)	1700 g (3.75 lbs)	GT-P
	Captan Gold (<i>Captan</i>)	2612 mL (0.69 gal)	FB-1C
	Captan Gold (<i>Captan</i>)	3785 mL (1 gal)	1C-5C
	Agro Mos (<i>Copper 4%</i>)	1892 mL (0.5 gal)	3C-5C
Grower Standard + Chitosan (GS+C)	Koverall Fungicide (<i>Mancozeb</i>)	1700 g (3.75 lbs)	GT-P
	Captan Gold (<i>Captan</i>)	2612 mL (0.69 gal)	FB-1C
	Captan Gold (<i>Captan</i>)	3785 mL (1 gal)	1C-5C
	Agro Mos (<i>Copper 4%</i>)	1892 mL (0.5 gal)	3C-5C
	ARMOUR Zen (15% Chitosan)	3785 mL (4 qts)	P-5C
Grower Standard + Biopesticide + Chitosan (GS+B+C)	Koverall Fungicide (<i>Mancozeb</i>)	1700 g (3.75 lbs)	GT-P
	Captan Gold (<i>Captan</i>)	2612 mL (0.69 gal)	FB-1C
	Captan Gold (<i>Captan</i>)	3785 mL (1 gal)	1C-5C
	Agro Mos (<i>Copper 4%</i>)	1892 mL (0.5 gal)	3C-5C
	Serenade ASO (<i>Bacillus subtilis</i> strain QST 713)	3785 mL (4 qts)	P-5C
	ARMOUR Zen (15% Chitosan)	3785 mL (4 qts)	P-5C

¹Application timings based on ‘McIntosh’: Green Tip (GT, 22 April); Pink (P, 5 May); Full Bloom (FB, 16 May); Petal Fall (PF, 26 May); 1st Cover (1C, 7 June); 2nd Cover (2C, 20 June); 3rd Cover (3C, 22 July); 4th Cover (4C, 30 Aug); and 5th Cover (5C; 8 September).

Table 3-4. Treatment list, application rates, and application timing for experiment 4 conducted in 2022. Treatments were applied to cultivars ‘Dabinett’, ‘Wickson’ and ‘Kingston Black’.

Treatment	Trade Name (Active Ingredient)	Rate (per acre)	Timing¹
Grower Standard (GS)	Kocide 3000 (<i>Copper Hydroxide 46.1%</i>)	1814 g (4 lbs)	GT
	Koverall Fungicide (<i>Mancozeb</i>)	1361 g (3 lbs)	P-PF
	Captan Gold (<i>Captan</i>)	3785 mL (4 qts)	FB-3C
	Pristine Fungicide (<i>Pyraclostrobin and Boscalid</i>)	454 g (16 oz)	4C
Grower Standard + Chitosan (GS+C)	Kocide 3000 (<i>Copper Hydroxide 46.1%</i>)	1814 g (4 lbs)	GT
	Koverall Fungicide (<i>Mancozeb</i>)	1361 g (3 lbs)	P-PF
	Captan Gold (<i>Captan</i>)	3785 mL (4 qts)	FB-3C
	Pristine Fungicide (<i>Pyraclostrobin and Boscalid</i>)	454 g (16 oz)	4C
	ARMOUR Zen (15% Chitosan)	3785 mL (4 qts)	P-5C
Grower Standard + Biopesticide + Chitosan (GS+B+C)	Kocide 3000 (<i>Copper Hydroxide 46.1%</i>)	1814 g (4 lbs)	GT
	Koverall Fungicide (<i>Mancozeb</i>)	1361 g (3 lbs)	P-PF
	Captan Gold (<i>Captan</i>)	3785 mL (4 qts)	FB-3C
	Pristine Fungicide (<i>Pyraclostrobin and Boscalid</i>)	454 g (16 oz)	4C
	Serenade ASO (<i>Bacillus subtilis</i> strain QST 713)	3785 mL (4 qts)	P-5C
	ARMOUR Zen (15% Chitosan)	3785 mL (4 qts)	P-5C

¹Application timings based on ‘Dabinett’: Green Tip (GT, 18 April); Pink (P, 6 May); Full Bloom (FB, 17 May); Petal Fall (PF, 26 May); 1st Cover (1C, 10 June); 2nd Cover (2C, 21 June); 3rd Cover (3C, 19 July); 4th Cover (4C, 18 Aug); and 5th Cover (5C, 15 September).

3.2.3.4. *Objective 2. Disease Assessments*

For experiments 3 and 4, trees were evaluated for incidence and severity of five apple diseases: apple scab, powdery mildew, rust, flyspeck, and sooty blotch. Apple scab severity was evaluated by using a rating scale from 0-6 as described by Poleatewich et al. (Poleatewich et al. 2012). Starting in mid-June, treatments were assessed for apple scab, powdery mildew, and rust incidence on leaves as described in Objective 1. Disease assessments were done bi-weekly for experiment 3 and monthly for experiment 4 and continued until harvest (Table 3-4). For each replicate tree, foliar disease incidence was determined by randomly selecting five branches and evaluating five leaves on the branch for disease incidence.

At harvest, 25 fruit per replicate tree were evaluated for incidence of apple scab, powdery mildew, rust, sooty blotch, and fly speck. 25 fruit per tree were rated for apple scab severity using a 0-6 score as described by Poleatewich et al. (Figure 3-1A) (Poleatewich et al. 2012). Each of the 25 fruits were also evaluated for powdery mildew severity as a percentage of fruit showing russet symptoms using a rating scale from 0-6 (Figure 3-1B).

3.2.4. *Objective 3. Evaluation of chitosan to reduce overwintering of *V. inaequalis* in orchard leaf litter.*

The objective of this experiment was to evaluate the efficacy of chitosan to reduce overwintering ascospores of *V. inaequalis* in the orchard. Reducing *V. inaequalis* overwintering and ascospore production has been identified as an important strategy to reduce primary infections of apple scab (MacHardy 1996). Chitosan was compared to urea, a strategy known to reduce overwintering inoculum of *V. inaequalis* (Holb 2009; Sutton et al. 2000). Three treatments were evaluated: 5% urea solution (50 g of agriculture grade urea/L), Tidal Grow 2%, and a water control. In 2020, the application rate of the Tidal Grow 2% was 0.79 mL·L⁻¹ and in

2021, the application rate was $7.9 \text{ mL}\cdot\text{L}^{-1}$. In November 2020 and 2021, apple leaves from different cultivars (i.e. Rome Beauty, Cameo, Gala) infected with *V. inaequalis* were collected from a FREC orchard. The leaves were packed into sachets, which comprised of two 5-inch round window screens with approximately 8 leaves placed between the screens and then stapled shut (Sutton et al. 2000). In 2020, treatments were sprayed until glisten ($\sim 0.7 \text{ mL}$ per leaf) using a hand-held spray bottle onto 9 replicate sachets for a total of 27 sachets (Sutton et al. 2000). In 2021, treatments were sprayed onto 12 replicate sachets for a total of 36 sachets. The sachets were overwintered fixed to the orchard floor of the 5-Cultivar apple research block at FREC.

Ascospore production was evaluated weekly during the ascospore ejection period, from late April to the end of June (or until no more ascospores were observed) (MacHardy 1996). Three sachets per treatment were removed from the orchard each week for about twelve weeks. In the laboratory, the sachets were completely submerged in water and soaked for 1 minute to initiate spore release (MacHardy 1996). The sachets were then placed on top of a vacuum with a slide cover at the bottom to catch spores that were being released (Frey and Keitt 1925). The sachets remained on the vacuum for 30 minutes. Once complete, the slide cover was placed on a slide bottom that contained a drop of Lactophenol blue dye. Ascospores were enumerated under the compound microscope and the number of ascospores per sachet was determined.

3.2.5. Data Analysis

Data analysis was performed using RStudio version 2022.09.6 "Spotted Wakerobin" (RStudio Team 2020). Each experiment was analyzed separately. Additionally, for the NH sites, each cultivar was analyzed separately. Disease incidence and severity ratings on fruit were transformed using an arcsine transformation to achieve homogeneity of variance. For measurements taken over time, the area under the disease progress curve (AUDPC) was

calculated for each experimental unit (DeGenring et al. 2023; Shaner and Finney 1977). Data were analyzed with an analysis of variance (ANOVA) using partial (type II) sums of squares ('car' package). Post hoc Tukey means separation tests were conducted using least squared adjusted treatment means obtained via the 'emmeans' package in Rstudio. Contrasts were also conducted but resulted in the same results as the Tukey tests. Graphs were created in Rstudio using the package 'ggplot2'.

3.3. Results

3.3.1. Objective 1: Experiment 1-2

At FREC, apple scab (*V. inaequalis*), powdery mildew (*P. leucotricha*), cedar-apple rust (*G. juniperi-virginianae*), sooty blotch and flyspeck were observed on leaves and apples. Apple scab was identified by the characteristic olive-green to brown lesions on the leaves and scabby lesions on the fruit (Gauthier et al. 2018). Powdery mildew was identified by the characteristic white powdery growth on the leaves and potential leaf curling (Marine et al. 2010). Additionally, flag shoots were observed in which an entire shoot had a silver-gray appearance because of the infection. On fruit, powdery mildew was identified by russeting and discoloration on the fruit (Marine et al. 2010). Rust was identified by observing yellow to orange lesions on the leaves and fruit and rust projections on the underside of the leaf (Koetter and Grabowski 2019). Sooty blotch and flyspeck are commonly observed together on harvested fruit and are characterized by sooty or cloudy blotches with indefinite borders and defined, black, shiny dots (Peter 2023).

3.3.2. Objective 1: Experiment 1. FREC research trials – 2021

During the 2021 growing season five primary scab infection events were predicted using NEWA modeling (Table A-1). Ascospore discharge was low until 29-April when the ascospore discharge accumulation jumped from 20% to 53%. Ascospore discharge was complete by 10-

May. Three infection events occurred during this two-week period, two of which lasted consecutive days (Figure A-2A).

In June, trees treated with the GS, RR, and RR+C treatments had significantly lower incidence of apple scab on the leaves compared to the water control ($P \leq 0.011$) (Figure 3-2A). Trees treated with C did not have a significant reduction of foliar apple scab incidence compared to the water control ($P = 0.101$). There was no effect of treatment on powdery mildew incidence ($P = 0.206$) or rust incidence ($P = 0.103$) on the leaves (Table A-2).

In October, there was significantly less incidence and severity of apple scab and on fruit harvested from trees treated with the GS, RR, and RR+C treatments compared to the water control fruit ($P \leq 0.001$) (Table 3-5). Fruit from these treatments had 80% to 96% less apple scab incidence and 82% to 97% less apple scab severity compared to the water control fruit. Additionally, all fruit from treated trees had 61% to 99% less apple scab lesions compared to fruit from the water control trees ($P \leq 0.037$) (Table 3-5). Fruit harvested from trees treated with GS, RR, and RR+C treatments had significantly less powdery mildew incidence and russet severity compared to the water control fruit ($P \leq 0.016$) (Table 3-5). For flyspeck incidence, only the trees treated with GS had less disease compared to the water control ($P = 0.017$) (Table 3-5). There was significantly less incidence of sooty blotch on harvested fruit from all treatments compared to the water control ($P \leq 0.033$). Fruit from trees treated with the GS, RR+C, and C treatments had the least amount of sooty blotch (Table 3-5). No fruit evaluated under any treatment showed symptoms of rust.

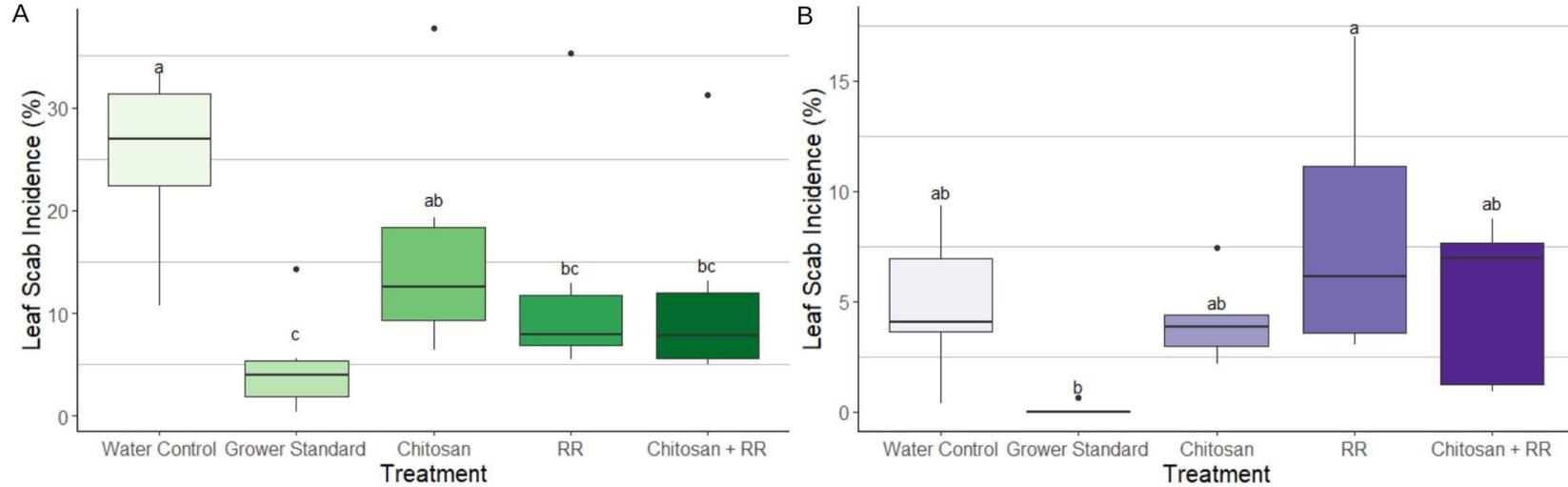


Figure 3-2. Effect of treatments on leaf scab incidence (%) on ‘Rome’ leaves from the (A) 2021 (experiment 1) and (B) 2022 (experiment 2) Penn State University Fruit Research and Extension Center research trials. Treatments included a water control, grower standard, chitosan, reduced risk (RR), and chitosan + reduced risk. Exact chemical treatments for each experiment are listed in Tables 1-2. Treatment means followed by the same letter are not significantly different ($\alpha=0.05$) as determined by the Tukey HSD Post-hoc test. The lines in the boxplot represent median, while the box represents the Interquartile Range (IQR). The start and end of the lines represent the minimum value in the data ($Q_3 - 1.5 \times IQR$), while any outliers are represented as dots.

Table 3-5. Mean disease incidence, mean number of scab lesions, scab severity (score 0-6), or russet severity (score 0-6) \pm standard error on harvested ‘Rome’ fruit from the 2021 research orchard trials (Experiment 1).^{1, 2}

	Water Control	GS	C	RR	RR+C
Scab Incidence (%)	56.2 \pm 6.1 (a)	2.0 \pm 1.4 (b)	39.3 \pm 6.3 (a)	6.7 \pm 1.7 (b)	11.3 \pm 3.3 (b)
Number of Scab Lesions	4.6 \pm 0.7 (a)	0.0 \pm 0.0 (c)	1.8 \pm 0.3 (b)	0.2 \pm 0.1 (c)	0.4 \pm 0.2 (bc)
Scab Severity (0-6)	0.9 \pm 0.1 (a)	0.02 \pm 0.01 (b)	0.5 \pm 0.1 (a)	0.07 \pm 0.02 (b)	0.2 \pm 0.04 (b)
Powdery Mildew Incidence (%)	54.6 \pm 5.6 (a)	26.0 \pm 4.9 (b)	40.0 \pm 4.0 (ab)	28.1 \pm 6.1 (b)	28.0 \pm 4.0 (b)
Russet Severity (0-6)	1.8 \pm 0.2 (a)	1.1 \pm 0.1 (b)	1.3 \pm 0.1 (ab)	0.8 \pm 0.1 (b)	0.9 \pm 0.1 (b)
Sooty Blotch Incidence (%)	57.6 \pm 3.8 (a)	14.7 \pm 2.5 (c)	32.0 \pm 6.3 (bc)	35.4 \pm 5.1 (b)	31.3 \pm 6.8 (bc)
Flyspeck Incidence (%)	24.5 \pm 9.0 (a)	1.3 \pm 1.3 (b)	33.3 \pm 9.6 (a)	19.2 \pm 4.2 (ab)	18.3 \pm 5.2 (a)

¹ Treatments evaluated were water control, grower standard (GS), chitosan (C), reduced risk (RR), and a reduced risk and chitosan mixture (RR+C).

² Within a disease measurement treatment means followed by different letters are significantly different ($\alpha=0.05$) as determined by the Tukey HSD Post-hoc test.

3.3.3. Objective 1 - Experiment 2. FREC research trials – 2022

For the 2022 season, eight primary scab infection events were predicted using NEWA modeling (Table A-1). Similar to 2021, cumulative ascospore discharge jumped from 24% to 61% on 26-April. Ascospore discharge was complete by 20-May. During these 25 days, there were six infection events, four of which lasted multiple days (Figure A-2B).

In June, only trees treated with the GS treatment had significantly lower incidence and severity of foliar apple scab ($P \leq 0.007$) (Figure 3-2B). Additionally, only trees treated with GS treatment had less powdery mildew incidence compared to the water control ($P = 0.030$) (Table 3-6). Trees treated with GS and C had 100% and 70% less rust incidence, respectively, compared to the water control ($P \leq 0.017$) (Table 3-6).

In October, there was significantly less incidence and severity of apple scab on fruit harvested from trees treated with GS compared to the control ($P = 0.001$) (Table 3-6). Apple scab severity on the fruit from trees treated with C was 54% less compared to the water control and was as effective as the GS treatment ($P = 0.071$) (Table 3-6). There was no effect of treatment on powdery mildew incidence ($P = 0.873$) or severity ($P = 0.300$) on harvested apples (Table 3-6). Fruit harvested from trees treated with GS and C had 100% and 92%, respectively, less rust incidence compared to control fruit ($P \leq 0.050$) (Table 3-6). Similarly, fruit from the GS and C treated trees had 92% and 47%, respectively, less sooty blotch incidence and 100% and 68%, respectively, less fly speck compared to the control fruit ($P \leq 0.034$) (Table 3-6).

Table 3-6. Mean disease incidence, mean number of scab lesions, scab severity (score 0-6), or russet severity (0-6) \pm standard error on ‘Rome’ leaves and harvested fruit from the 2022 research orchard trial (Experiment 2).^{1,2}

		Water Control	GS	C	RR	RR+C
Leaves	Scab Severity (0-6)	0.41 \pm 0.07 (ab)	0.01 \pm 0.01 (b)	0.42 \pm 0.09 (ab)	0.66 \pm 0.10 (a)	0.45 \pm 0.09 (ab)
	Powdery Mildew Incidence (%)	39.0 \pm 2.75 (a)	21.1 \pm 2.9 (b)	48.9 \pm 2.0 (a)	52.3 \pm 2.1 (a)	51.2 \pm 2.1 (a)
	Powdery Mildew Shoot Incidence	12.4 \pm 3.9	7.2 \pm 2.4	10.0 \pm 1.6	9.8 \pm 1.7	15.0 \pm 1.2
	Rust Incidence (%)	8.2 \pm 1.3 (a)	0.0 \pm 0.0 (c)	2.4 \pm 1.8 (bc)	5.4 \pm 1.0 (ab)	6.7 \pm 1.5 (ab)
Harvested Fruit	Scab Incidence (%)	29.6 \pm 8.8 (a)	0.8 \pm 0.8 (b)	16 \pm 5.2 (a)	24.8 \pm 4.1 (a)	39.2 \pm 6.0 (a)
	Number of Scab Lesions	2.3 \pm 0.7 (ab)	0.01 \pm 0.01 (b)	2.1 \pm 1.2 (ab)	1.3 \pm 0.4 (ab)	3.6 \pm 0.7 (a)
	Scab Severity (0-6)	0.62 \pm 0.2 (a)	0.01 \pm 0.01 (b)	0.28 \pm 0.1 (ab)	0.36 \pm 0.1 (a)	0.76 \pm 0.2 (a)
	Powdery Mildew Incidence (%)	68.8 \pm 6.5	72.0 \pm 10.0	71.2 \pm 7.1	67.2 \pm 4.6	68.8 \pm 8.2
	Russet Severity (0-6)	1.9 \pm 0.8	1.3 \pm 0.5	0.9 \pm 0.2	1.2 \pm 0.08	1.0 \pm 0.2
	Rust Incidence (%)	9.6 \pm 4.3 (a)	0.0 \pm 0.0 (b)	0.8 \pm 0.8 (b)	11.2 \pm 5.0 (a)	4.8 \pm 3.2 (ab)
	Sooty Blotch Incidence (%)	40.8 \pm 3.9 (a)	3.2 \pm 0.8 (b)	21.6 \pm 6.4 (ab)	33.6 \pm 5.9 (a)	41.6 \pm 4.1 (a)
Flyspeck Incidence (%)	20.0 \pm 7.2 (a)	0.0 \pm 0.0 (b)	6.4 \pm 2.0 (ab)	13.6 \pm 6.5 (a)	21.6 \pm 4.7 (a)	

¹ Treatments evaluated were water control, grower standard (GS), chitosan (C), reduced risk (RR), and a reduced risk and chitosan mixture (RR+C).

² Within a disease measurement, treatment means followed by different letters are significantly different ($\alpha=0.05$) as determined by the Tukey HSD Post-hoc test. Measurements with significant differences are visually represented by the shaded cells

3.3.4. Objective 2 – Experiment 3. NH On-Farm Site #1

For experiment 3 (on-farm site #1) conducted in 2022, six primary scab infection events were predicted using NEWA modeling (Table A-1), however five of these events were multi-day infection periods. Ascospore discharge was steady throughout the spring, although spikes occurred around bloom and petal fall (Figure A-3A). Ascospore discharge was complete by 2-June. At this site, apple scab, powdery mildew, cedar-apple rust, sooty blotch and flyspeck were observed on leaves and apples and were identified as discussed in Section 3.3.1. Additionally, frog eye leaf spot, caused by *Botryosphaeria obtuse* (Schwein.) Shoemaker, was observed and identified by the characteristic small purple flecks on the leaves that enlarge over time resulting in a purple ring around a brown center (Mauch and Rodriguez Salamanca 2022).

Over the season, there was no effect of treatment on AUDPC of scab severity on leaves from cultivars Macoun ($P = 0.789$) or McIntosh ($P = 0.315$) (Table 3-7). However, Macoun trees treated with GS+C had 19-25% lower AUDPC of scab incidence on leaves compared to the GS and GS+B+C treated Macoun trees ($P = 0.038$) (Table 3-7). Additionally, Macoun trees treated with GS+B+C had 50% lower AUDPC of powdery mildew incidence compared to the GS treated Macoun trees ($P = 0.045$) (Table 3-7). There was no effect of treatment on AUDPC of frog eye severity on leaves from cultivars Macoun ($P = 0.400$) or McIntosh ($P = 0.181$) (Table 3-7). However, McIntosh trees treated with GS and GS+B+C had significantly lower AUDPC of frog eye incidence on leaves compared to the GS+C treated trees ($P = 0.050$) (Table 3-7). Rust severity and incidence were not affected by treatment on Macoun trees ($P \geq 0.503$), and McIntosh trees had only a few leaves with rust lesions (Table 3-7).

In September, McIntosh trees treated with GS+B+C had 33-35% lower scab incidence and 64-79% less scab lesions on harvested fruit compared to McIntosh trees treated with GS or

Table 3-7. Effect of grower standard control (GS), grower standard + chitosan (GS+C), and grower standard + biopesticide + chitosan (GS+B+C) on the area under the disease progress curve (AUDPC)¹ for foliar disease incidence or severity ± standard error on cultivars ‘Macoun’ and ‘McIntosh’ from the 2022 New Hampshire on-farm trial #1. Mean disease incidence, mean number of scab lesions, scab severity (score 0-6), or russet severity (score 0-6) ± standard error on harvested apples from experiment 3 conducted in 2022.²

		Macoun			McIntosh		
		GS	GS+C	GS+B+C	GS	GS+C	GS+B+C
Leaves	Scab Incidence	45.5±8.7 (ab)	36.5±9.2 (a)	49.0±7.4 (b)	226.0±26.0	213.7±43.7	236.3±22.4
	Scab Severity	1.42±0.7	1.1±0.4	3.9±0.6	2.8±0.7	3.8±1.0	1.2±0.4
	Powdery Mildew Incidence	54.0±18.5 (a)	37.5±7.9 (ab)	27.5±5.9 (b)	7.1±1.8	6.3±1.5	9.4±5.5
	Rust Incidence	67.0±23.2	74.5±26.3	73.25±27.0	0.0±0.0	0.0±0.0	1.7±0.8
	Rust Severity	1.4±0.5	2.0±0.6	1.6±0.6	0.01±0.01	0.01±0.01	0.05±0.05
	Frog Eye Incidence	148.5±41.1	129.5±32.1	132.0±30.0	42.3±10.4 (b)	104.6±35.2 (a)	48.0±9.9 (b)
	Frog Eye Severity	5.6±2.0	3.8±1.4	3.9±1.3	1.0±0.3	0.8±0.1	3.1±1.5
Harvested Fruit	Scab Incidence (%)	50.4±14.1	46.3±10.1	46.3±15.8	57.5±15.8 (a)	56.0±16.3 (a)	37.6±5.2 (b)
	Number of Scab Lesions	1.4±0.2	1.2±0.2	0.9±0.1	3.9±0.7 (ab)	6.6±1.2 (a)	1.4±0.4 (b)
	Scab Severity (0-6)	0.9±0.1	0.7±0.1	0.6±0.1	1.1±0.1	1.1±0.1	0.5±0.1
	Powdery Mildew Incidence (%)	68±9.2	68.6±4.6	75.5±8.7	90.2±2.4 (a)	59.6±7.5 (b)	78.9±4.1 (ab)
	Russet Score (0-6)	1.1±0.1	1.0±0.1	1.6±0.2	2.0±0.2 (a)	0.7±0.1 (b)	2.0±0.2 (a)
	Sooty Blotch Incidence (%)	1.6±1.6	4.0±1.7	1.0±1.0	1.3±1.3	0.8±0.8	0.8±0.8
	Flyspeck Incidence (%)	19.2±5.4	18.3±4.4	15.0±6.0	8.0±5.4	8.8±4.0	23.9±9.7

¹ The AUDPC was calculated from disease assessments taken bi-weekly starting on 7 June until 15 September.

² Within a disease measurement and within a cultivar (Macoun, McIntosh), treatment means followed by different letters are significantly different ($\alpha=0.05$) as determined by the Tukey HSD Post-hoc test. Measurements with significant differences are visually represented by the shaded cells.

GS+C ($P \leq 0.048$) (Table 3-7). However, there was no effect of treatment on apple scab severity for fruit harvested from cultivars Macoun ($P = 0.634$) or McIntosh ($P = 0.176$) (Table 3-7).

While there was no effect of treatment on powdery mildew incidence or severity on Macoun fruit ($P \geq 0.605$), McIntosh fruit from trees treated with GS+C had 34% less powdery mildew incidence and 65% less powdery mildew severity compared to fruit treated with GS ($P \leq 0.015$) (Table 3-7). There was no effect of treatment on sooty blotch or flyspeck incidence for fruit harvested from cultivars Macoun ($P \geq 0.320$) or McIntosh ($P \geq 0.142$) (Table 3-7). No fruit evaluated under any treatment or cultivar showed symptoms of rust.

3.3.5. Objective 2 - Experiment 4. NH On-Farm Trial #2

For experiment 4 (on-farm site #2), seven primary scab infection events were predicted using NEWA modeling (Table A-1), all of which were multi-day infection periods. Cumulative ascospore discharge jumped from 28% to 50% around bloom. Ascospore discharge was complete by 6-June 6. During these 22 days, there were four infection events (Figure A-3B).

Apple scab was not observed on any trees at NH on-farm site #2. Overall, disease incidence was low at this farm so incidence and severity data for all foliar diseases was combined to evaluate effects of treatments on suppression of all leaf and fruit spots. Over the season, there was no effect of treatment on AUDPC of disease severity on leaves from cultivars Dabinett ($P = 0.355$), Kingston Black ($P = 0.202$), or Wickson ($P = 0.912$) (Table 3-8). However, Kingston Black trees treated with GS+C had 55% lower AUDPC of disease incidence compared to Kingston Black trees treated with GS ($P = 0.049$) (Table 3-8). Additionally, Kingston Black trees treated with GS+B+C had significantly lower AUDPC of powdery mildew incidence compared to Kingston Black trees treated with GS ($P = 0.038$) (Table 3-8). There was no effect of treatment on AUDPC of frog eye lesion severity on leaves from cultivars Dabinett

Table 3-8. Effect of grower standard control (GS), grower standard + chitosan (GS+C), and grower standard + biopesticide + chitosan combination (GS+B+C) on area under the disease progress curve (AUDPC)¹ for disease incidence or severity ± standard error on cultivars ‘Kingston Black’, ‘Dabinett’, and ‘Wickson’ leaves from experiment 4 in 2022.²

	Kingston Black			Dabinett			Wickson		
	GS	GS+C	GS+B+C	GS	GS+C	GS+B+C	GS	GS+C	GS+B+C
Powdery Mildew Incidence	45.5±11 (ab)	61.5±20 (a)	24.0±11 (b)	10.0±2.7	17.5±6.2	27.0±10	13.0±6.0	10.0±5.0	13.0±4.7
Lesion Incidence ³	53.0±12 (a)	24.0±1.6 (b)	31.0±4.4 (ab)	6.0±2.6	11.0±6.0	18.0±4.8	4.0±1.6	9.0±4.1	11.0±3.4
Lesion Severity	4.8±1.7	2.0±0.7	1.5±0.7	0.1±0.1	0.2±0.1	0.3±0.1	0.1±0.0	0.2±0.1	0.2±0.0
Frog Eye Incidence	18.0±6.6 (ab)	9.5±2.4 (b)	37.0±13.3 (a)	7.5±1.9	13.0±2.6	9.0±1.3	6.5±3.9	5.0±2.1	8.0±2.8
Frog Eye Severity	0.3±0.1 (b)	0.1±0.0 (b)	0.9±0.2 (a)	0.1±0.1	0.4±0.1	0.2±0.0	0.1±0.0	0.1±0.0	0.1±0.1

¹The AUDPC was calculated from disease assessments taken monthly starting on 10 June until 4 October.

² Within a disease measurement and within a cultivar (Kingston Black, Dabinett, Wickson), treatment means followed by different letters are significantly different ($\alpha=0.05$) as determined by the Tukey HSD Post-hoc test. Shaded rows represent measurements with significant differences.

³ Lesion incidence represents the observation of any foliar disease on a leaf within a disease assessment.

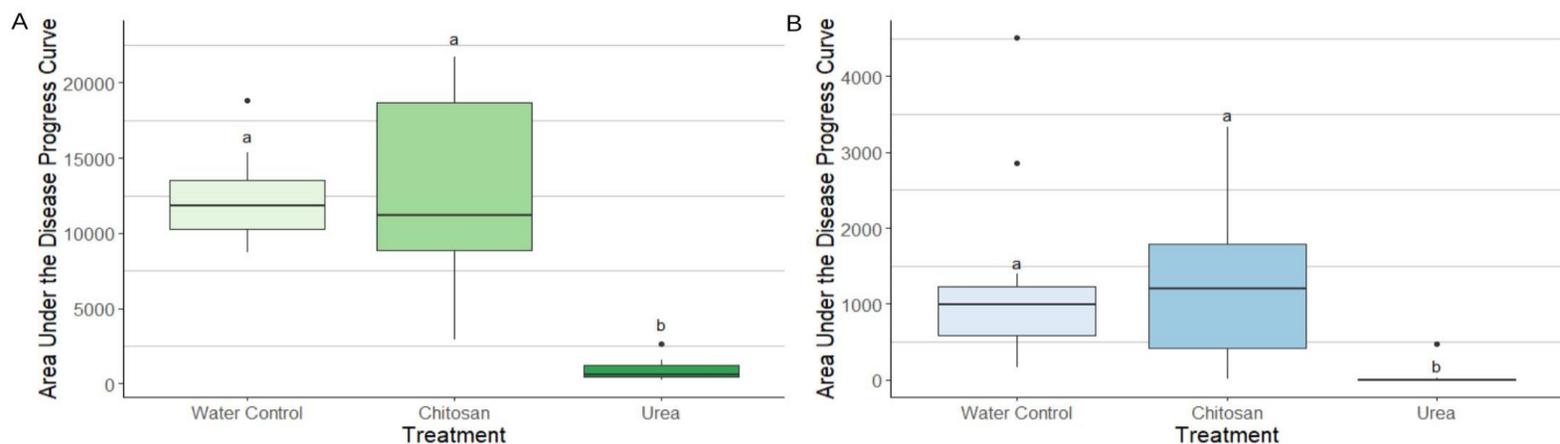


Figure 3-3. Effect of overwintering treatments on area under the disease progress curve (AUDPC) for *Venturia inaequalis* ascospore release starting in March until mid-Summer for (A) 2020-2021 trial and (B) 2021-2022 trial at Penn State University Fruit Research and Extension Center. Treatments were a water control, chitosan (2020/2021: 0.79 mL/L chitosan; 2021/2022: 7.9 mL/L chitosan), and a 5% urea solution. Treatment means followed by the same letter are not significantly different ($\alpha=0.05$) as determined by the Tukey HSD Post-hoc test. The lines in the boxplot represent median, while the box represents the Interquartile Range (IQR). The start and end of the lines represent the minimum value in the data ($Q_3 - 1.5 \times IQR$), while any outliers are represented as dots.

($P = 0.235$) or Wickson ($P = 0.810$) (Table 3-8). However, Kingston Black trees treated with GS or GS+C had 68% and 88%, respectively, lower AUDPC of frog eye severity compared to Kingston Black trees treated with GS+B+C ($P \leq 0.022$) (Table 3-8).

In October, there was no effect of treatment on incidence or severity of powdery mildew or flyspeck on fruit harvested from cultivars Dabinett ($P \geq 0.183$) or Wickson ($P \geq 0.133$) (Table A-3). Cultivar Kingston Black did not produce enough fruit for post-harvest evaluations. No fruit evaluated under any treatment or cultivar showed symptoms of rust or sooty blotch.

3.3.6. Objective 3. Evaluation of chitosan to reduce overwintering of V. inaequalis in orchard leaf litter.

In the 2020-2021 overwintering trials, the 5% urea application significantly reduced AUDPC of ascospore production ($P < 0.001$) compared to the control, but the chitosan application did not reduce AUDPC ($P = 0.996$) (Figure 3-3A). In the 2021-2022 overwintering trials, even with the increase in the percent chitosan, the same results were observed: the chitosan application did not reduce AUDPC of ascospore production ($P = 0.851$), but the 5% urea application significantly reduced AUDPC of ascospore production compared to the control ($P < 0.001$) (Figure 3-3B).

3.4. Discussion

The addition of BCAs and chitosan to an IPM program to manage disease in northeast apple orchards would give growers additional disease control options. This study investigated the use of chitosan in combination with biopesticides, as part of a conventional spray program, and as a tool to reduce primary apple scab infections

Chitosan can reduce disease when applied as part of a conventional fungicide program

Results from this research suggest that chitosan may not be effective as a stand-alone treatment and more research is needed to determine if higher concentrations of chitosan are effective. Over the two-year study at FREC research orchard, two chitosan concentrations were tested. Chitosan concentration was increased in year two (from $0.02 \text{ mg}\cdot\text{ml}^{-1}$ to $0.1 \text{ mg}\cdot\text{ml}^{-1}$) due to low efficacy observed in the first year and previous reports that higher chitosan concentration results in increased disease suppression. Specifically, chitosan concentrations between $1.0\text{-}2.5 \text{ mg}\cdot\text{ml}^{-1}$ tend to correlate with greater fungal growth inhibition compared to lower concentrations ($0.1\text{-}0.75 \text{ mg}\cdot\text{ml}^{-1}$) (Yu et al. 2012; Hernández-Lauzardo et al. 2008; Muñoz and Moret 2010; Benhamou and Theriault 1992; DeGenring et al. 2023). The low incidence of apple scab in 2022 (0%-10%), made determining statistical differences between treatments difficult. Thus, it is challenging to conclude that the chitosan rate tested in 2022 was more effective than the rate tested in 2021. Felipini et al. observed a reduction in foliar apple scab with reagent grade chitosan applied at $0.5 \text{ mg}\cdot\text{ml}^{-1}$ compared to a control in a greenhouse with artificially inoculated leaves (Felipini et al. 2016). Future work is also needed to determine costs per acre of chitosan to identify the most effective and economically feasible chitosan concentrations and be repeated in years when disease pressure is higher.

At the on-farm trials sites, disease suppression by the GS+C treatment was often not significantly different from the GS treatment. However, there were instances in which the GS+C treatments had significantly greater reduction in disease compared to the GS treatment alone. This was specifically prevalent in fruit harvested from trees treated with GS+C. For example, McIntosh and Dabinett fruit treated with GS+C had 65% lower powdery mildew severity and 62% less flyspeck incidence, respectively, compared to the GS treated fruit. Data from this study suggests that chitosan can enhance the efficacy of conventional fungicide programs but may vary

by cultivar. More research is needed to evaluate chitosan on other rootstock and scion cultivar combinations.

Variability in our results may be explained by the modes of action of chitosan which could be heavily influenced by environment and plant genetics. For example, chitosan has been shown to activate plant defenses, thus indirectly reducing disease (Benhamou 1996). The magnitude and effectiveness of the defense response, however, is known to be dependent on plant genotype (Tucci et al. 2011). Chitosan elicits a host response characterized by increased enzymatic activity of chitinase and β -1,3-glucanases, up-regulation of the production of plant-defense related enzymes, such as polyphenoloxidase (PPO) and peroxidase (POD), and enhanced production of callose cell wall appositions in the host's epidermis and outer cortex (Algam et al. 2010; El-Ghaouth et al. 1994a; Benhamou et al. 1998; Li et al. 2015; Zahid et al. 2015; Liu et al. 2007). In-vitro assays have also shown that chitosan can have a direct inhibition of fungal mycelium growth and conidial germination and elongation (DeGenring et al. 2023; Hernández-Lauzardo et al. 2008; El-Ghaouth et al. 2000b). Additionally, research on postharvest applications suggests that chitosan can have direct antimicrobial activity through causing cellular disorganization of the fungal pathogens (Ait Barka et al. 2004; El-Ghaouth et al. 1994b; Benhamou et al. 1998). For direct inhibition to occur, pathogens must come into contact with chitosan. During the season, rain events may wash chitosan away rendering it less effective. In this study chitosan was applied on a schedule based on predicted infection events. Applying chitosan on a calendar schedule or with a spreader sticker product, may lengthen the time chitosan remains on the leaves and fruit. Additional research is needed to determine the ideal timing of chitosan application with respect to predicted infection events. Additionally, this research was limited to commercial chitosan products available in the United States (Romanazzi

et al. 2018), but there are other chitosan products available around the world and technologies, such as chitosan nanoparticles (Kumaraswamy et al. 2018; Ma et al. 2017), that can be evaluated for foliar disease management.

Synergisms between chitosan and biopesticides varied by site, cultivar, and pathogen

In this research, we tested the biopesticide Serenade ASO (*B. subtilis* QST 713). *Bacillus* BCAs have shown to be effective at reducing apple scab compared to a non-treated control (Poleatewich et al. 2012; Travis et al. 2005). However, many studies have found that stand-alone applications of *Bacillus* BCAs are not comparable to a fungicide application (Travis et al. 2005; Yoder et al. 2006; Cromwell et al. 2011). Thus, combining *Bacillus* BCAs with other products or in rotation with fungicides may be a more effective strategy for managing apple scab (Ayer et al. 2021; Poleatewich et al. 2012). To our knowledge, no research has been done on the combined application of *Bacillus* BCAs and chitosan for management of pre-harvest apple diseases. Results from the research orchard experiments suggest that addition of chitosan to the RR treatment was not synergistic as there were no significant differences between RR+C and RR treatments with respect to reduction in disease. This lack of synergism may be mainly due to the low efficacy of this chitosan product observed in our research trials. As previously discussed, the optimal chitosan concentration and application timing still need to be investigated. The chitosan product may have washed off before it was able to serve as a food source for the BCA or stimulate antimicrobial enzymes. Very few studies have examined a co-application of chitosan and a BCA under field conditions (Yu et al. 2008, 2012; Lu et al. 2014; Benhamou et al. 1998). Kokalis-Burelle et al. found that the co-application of reagent grade chitin and *B. cereus* along with a spreader-sticker (SoyDex) enhanced disease suppression likely due to improved foliar colonization of the BCA (Kokalis-Burelle et al. 1992). Future research is needed to evaluate

commercially available chitosan products applied with a surfactant as a strategy to reduce variability.

Results from the on-farm sites suggest that B+C overlaid onto a conventional fungicide program can improve disease suppression for management of apple scab and powdery mildew. The GS+B+C treatment exhibited enhanced disease suppression compared to the GS treatment. However, this efficacy was dependent on cultivar and fungal pathogen. For example, the GS+B+C resulted in up to 50% suppression of powdery mildew on Macoun leaves (but not fruit) and McIntosh fruit (but not leaves) compared to the GS treatment. Overall, McIntosh fruit from GS+B+C treated trees had 35% and 64% lower AUDPC of scab incidence and number of scab lesions, respectively, compared to the apples from GS treated trees. While 35% AUDPC of apple scab is not ideal for growers, it is significantly better than the 57% AUDPC of apple scab incidence observed on GS treated fruit. Kingston Black trees treated with GS+B+C had 47% and 42% lower AUDPC for powdery mildew incidence and general lesion incidence. However, for many of the fungal pathogens and cultivars, there was no effect of treatment on AUDPC. Differences in efficacy of chitosan observed in the on-farm and research orchard sites may be due to the use of two different chitosan products containing different forms of chitosan (extraction method, molecular weight, formulation). Thus, the greater efficacy of the B+C+GS treatments at the on-farms sites may be that ARMOUR-Zen was more effective at reducing disease or enhancing the BCA compared to Tidal Grow. Additional experiments directly comparing these two products are needed to make more definitive conclusions. More research is also needed to test synergisms between chitosan and other BCAs, including different strains of bacterial and fungal-based biopesticides. *Cladosporium cladosporioides* H39 (Köhl et al. 2015)

and *Microphaeropsis* sp. (Carisse et al. 2000), have shown efficacy against apple scab and could be examined in combination with chitosan.

Chitosan did not reduce overwintering spores of V. inaequalis

In the northeastern United States, ascospores produced on diseased leaves in the leaf litter constitute the primary inoculum causing apple scab (MacHardy 1996). Shredding leaf litter with a flail mower and/or treating the leaf litter with urea are common sanitation practices to reduce ascospore production in the orchard (Sutton et al. 2000; Holb 2009). We hypothesized that chitosan would reduce overwintering ascospores through promoting microbial activity leading to enhanced decomposition of leaves and reduction in maturation of pseudothecia (Fan et al. 2023; Riseh et al. 2022; MacHardy 1996). Additionally, the chitosan spray to the leaves could have resulted in a direct antimicrobial activity against the *V. inaequalis* pseudothecia on the leaf. Fall chitosan applications, at the rates tested, did not reduce ascospore production in the spring. Previous research focused on optimizing urea application timing (Sutton et al. 2000; Mac an tSaoir et al. 2010) could be applied to future trials conducted on chitosan, focusing on application rates and timing to better understand if chitosan could play a role in reducing overwintering *V. inaequalis* ascospores.

3.5. Conclusions

While not as effective compared to a conventional fungicide program, pre-harvest application of chitosan significantly reduced apple diseases when applied alone or in combination with a biopesticide or conventional fungicide program. Results also indicate that application rates of chitosan tested do not reduce overwintering ascospores. This research provides evidence that chitosan has potential as an IPM tool, but more research is needed to determine best practices for use in an integrated management program for control of diseases in

apple production in the northeast. Additional research is also needed to identify factors influencing variability and to study co-application of chitosan and other BCAs, including different strains of bacterial and fungal-based biopesticides, for managing diseases. Due to the exploratory nature of this work, sample sizes were small, and trials were conducted in a limited geographical location. Once an optimal chitosan rate and strategy for integration with other IPM products is determined, large scale trials should be conducted.

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CHAPTER 4

Postharvest chitosan sprays reduce bitter rot and blue mold on apple fruit

Liza DeGenring, Kari Peter, and Anissa Poleatewich

Manuscript In Preparation

Abstract

Chitosan is a natural product that has potential use in agriculture for managing diseases. Chitosan has been shown to effectively suppress storage rots when applied postharvest. Application of chitosan pre- and postharvest has potential to manage both latent and postharvest rots but these effects are not well studied. Furthermore, to determine the most effective strategy for using chitosan to manage apple diseases, research on application rates, chitosan molecular weight, phytotoxicity potential, and formulation is needed. The objectives of this study were to (1) identify non-phytotoxic concentrations of chitosan on apple fruit; (2) evaluate commercial chitosan products for reduction of postharvest disease severity on inoculated fruit; (3) evaluate the effect of pre-harvest chitosan applications on suppression of latent infections, postharvest rots, and fruit quality; and (4) evaluate the effect of pre-harvest plus postharvest chitosan applications on suppression of *Penicillium expansum* and *Colletotrichum fioriniae* on inoculated fruit. For objective 3 and 4, pre-harvest chitosan applications of the products Tidal Grow and ARMOUR-Zen 15, and a commercial biopesticide, Serenade ASO, were tested in field studies located at a research orchard in Pennsylvania and two commercial orchards in New Hampshire. Under lab conditions, chitosan products applied at higher rates were more effective at reducing disease but tended to cause phytotoxicity. This phytotoxic effect was remediated when the product's pH was adjusted to ~5. Tidal Grow products applied at 1.0% (v/v) chitosan reduced lesion size caused by *P. expansum* and *C. fioriniae* on inoculated apples up to 86% compared to

a water treatment. Pre-harvest applications of chitosan and Serenade reduced bitter rot up to 85% on immature fruit in the research orchard. There was no interaction between the pre-harvest and postharvest chitosan treatments on postharvest rot disease. The results from this research suggest that Tidal Grow adjusted to pH ~5 can reduce postharvest diseases of apple fruit.

Keywords: chitosan, apple, *Penicillium expansum*, *Colletotrichum fioriniae*, postharvest, preharvest

4.1. Introduction

Management of postharvest diseases of fruits and vegetables has relied on the use of chemical fungicides (Eckert and Ogawa 1988; Gutiérrez-Martínez et al. 2018). Growing concerns over fungicide resistance, increased restrictions on the use of fungicides (Ragsdale and Sisler 1994), and consumer concern over chemical residues has led to the search for alternative methods for managing postharvest diseases (Ragsdale and Sisler 1994; Lamichhane et al. 2016; Pimentel 2005; Ekström and Ekblom 2011). While extensive research has been conducted on managing postharvest disease with microbial biocontrol agents (BCAs) (Mari et al. 2007; Sharma et al. 2009; Janisiewicz and Korsten 2002; Ippolito and Nigro 2000), obstacles to widespread adoption still exist, including inconsistent efficacy (Droby et al. 2009). In addition to microbial biocontrol, research has focused on the use of several natural compounds, such as cellulose, chitin, and chitosan, for management of postharvest diseases of fruits and vegetables (Zhang et al. 2011; Romanazzi and Feliziani 2016; Sharif et al. 2018).

Chitosan is a natural product derived from chitin that is found in insect and crustacean exoskeletons and cell walls (Sharif et al. 2018; Kaur and Dhillon 2014). Chitosan is a β -(1,4)-glucosamine polymer that is more soluble in solution compared to chitin and thus chitosan is predominately found in commercial formulations (Tripathi and Dubey 2004; Sharif et al. 2018).

Chitosan has been shown to promote plant growth and reduce disease and is gaining interest as a product for agricultural use (Zhang et al. 2011; Romanazzi et al. 2018). Chitosan has predominately been evaluated for managing postharvest diseases and extending shelf-life of fruits and vegetables (Romanazzi et al. 2018; Sharif et al. 2018; Bautista-Baños et al. 2006; Zhang et al. 2011). When applied postharvest, chitosan forms a film on the fruit, slowing the ripening process (El Ghaouth et al. 1991a; Bhaskara Reddy et al. 2000) and providing a moisture barrier, thus preventing weight loss and reducing respiration rate (Chien et al. 2007; El Ghaouth et al. 1991; Li et al. 2015). A chitosan post-harvest dip can improve firmness, total soluble solids (TSS) content, titratable acidity, and ascorbic acid (all of which correlate to the quality of the fruit) (Chien et al. 2007). The chitosan barrier on the fruit prevents the outward flux of nutrients, interfering with the establishment of a nutritional relationship between the host and pathogen (El Ghaouth et al. 1994). Studies have shown that chitosan influences fungal spore germination, radial growth, and germ tube elongation (El-Ghaouth et al. 1992; Wang et al. 2014), as well as causes cellular disorganization of fungal pathogens through cell wall loosening, cytoplasm disintegration, and excessive branching and swelling of the cell wall (Ait Barka et al. 2004; El-Ghaouth et al. 1994b; Benhamou et al. 1998). El Ghaouth et al. (1994) found that when a wounded area was treated with a chitosan solution, the host's primary cell walls below the wounded site showed no signs of alteration even when large numbers of fungal cells were in the wounded area. This suggests that chitosan may impair the pathogen's ability to produce macerating enzymes (El-Ghaouth et al. 1994b).

In addition to the inhibition and functional disruption caused to invading pathogens, chitosan stimulates a defense response in the host tissue, including cell wall thickening, formation of hemispherical protuberances along host cell walls, and occlusion of many

intercellular spaces with a fibrillar material (El Ghaouth et al. 1994; El Ghaouth et al. 1997). When applied to harvested fruits, chitosan may stimulate up-regulation and production of defense-related compounds, such as polyphenoloxidase (PPO) and peroxidase (POD) (Li et al. 2015; Liu et al. 2007), as well as antioxidants and enzymatic activity of anti-fungal enzymes, such as β -1,3-glucanase (Wang & Gao, 2013). Chitosan may also cause an increase in the production of compounds associated with carbohydrate catabolism and energy production, aconitase, NADH dehydrogenase, and malate dehydrogenase, which can provide energy for resisting a pathogen (Li et al. 2015). While these studies show promise for the postharvest application of chitosan, there are still gaps in knowledge pertaining to how efficacy is affected by molecular weight, commercial formulation, concentration of chitosan, solution pH, and number of applications (Bautista-Baños et al. 2006; Bhaskara Reddy et al. 2000; DeGenring et al. 2023). For example, previous research suggests that a higher concentration (1-2.5 mg·ml⁻¹ respectively) of chitosan tends to correlate with greater inhibition of fungal growth (Hernández-Lauzardo et al. 2008; Yu et al. 2012; El-Ghaouth et al. 2000b; Muñoz and Moret 2010; El-Ghaouth et al. 1997; Benhamou and Theriault 1992); however, a higher concentration can potentially cause phytotoxicity and have negative effects on plant growth (Ait Barka et al. 2004; DeGenring et al. 2023).

There is limited research on the effect of pre-harvest application of chitosan for managing postharvest diseases (Romanazzi et al. 2002; Bhaskara Reddy et al. 2000). Applied pre-harvest, reagent grade chitosan reduces disease on perishable fruits (apple, citrus, pear, strawberry, grape, and tomato) and vegetables (cucumber and bell pepper) caused by *Botrytis cinerea* (Pers.:Fr), *Rhizopus stolonifer* (Ehrenb.:Fr.) Vuill, *Penicillium expansum* (Link), *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Jarvis and Shoemaker) and *Colletotrichum gloeosporioides* (Penz.) Penz. &

Sacc., (El-Ghaouth et al. 2000a; Li et al. 2015; Zhang et al. 2011; El-Ghaouth et al. 2000b; Romanazzi and Feliziani 2016; Liu et al. 2007). Further research is needed to determine how pre-harvest chitosan applications alone and in combination with other disease management tools can impact postharvest diseases.

Apple (*Malus domestica* Borkh.) fruit production is an excellent model to address these knowledge gaps as there is extensive research on postharvest management of apple diseases. Management of tree fruit diseases is especially challenging in the Northeast, where the moist and warm conditions during the growing season favor disease. While marketing strategies vary in the Northeast, apple orchards in states like New Hampshire, Maine, and Vermont rely predominately on pick-your-own and farm stand markets. Thus, fruit quality is critical as consumers have a low threshold for imperfections caused by disease. Additionally, fruit rot diseases are a significant concern as affected fruit either fall from the tree prematurely, are culled at harvest, or are lost during storage. Several summer fruit rot, such as bitter rot (*Colletotrichum spp.*), and postharvest rots, such as blue mold (*Penicillium spp.*) and gray mold (*B. cinerea*), occur in the Northeast (Sutton et al. 2014).

Bitter rot is caused by several species in the Genus *Colletotrichum* including those in the *C. gloeosporioides* and *C. acutatum* species complexes (Dowling et al. 2020). *C. fioriniae* from the *C. acutatum* species complex is the most common in the Northeast and Mid-Atlantic U.S (Martin and Peter 2021). *C. fioriniae* spreads mostly via rain-splashed conidia (Sutton et al. 2014; Dowling et al. 2020). Bitter rot fungi are hemibiotrophic, meaning that the initial biotrophic infection can have extended latent or quiescent phases before development of necrotrophic rot (Peres et al. 2005; De Silva et al. 2017). This complicates disease management for growers as symptoms on fruit are not present until after harvest and potentially once fruit

have already been put into long term storage (Wenneker and Thomma 2020). Research has shown that the prevalence of some fruit rots, particularly bitter rot, is increasing in Northeastern apple orchards (Rosenberger 2017; Wallhead 2016).

Blue mold is caused by the fungus *Penicillium expansum*, which is considered the biggest postharvest pathogen globally. Conidia dispersal occurs during the harvesting process but may also be present on storage bins and packhouse walls (Sutton et al. 2014). The pathogen mainly enters through wounds or bruises although infection via lenticels may occur (Sutton et al. 2014). Blue mold on apples results in considerable losses during the storage period (Luciano-Rosario et al. 2020). Additionally, *P. expansum* can produce carcinogenic mycotoxins that pose a human health risk if infected apples are processed (Morales et al. 2007). Four postharvest fungicides are currently registered for blue mold disease management, but the emergence of fungicide resistance has greatly decreased their efficacy (Luciano-Rosario et al. 2020). Research has shown that the prevalence of some fruit rots, particularly bitter rot, is increasing in Northeastern apple orchards (Rosenberger 2017; Wallhead 2016). Due to this increase in pathogen prevalence and emergence of fungicide-resistant populations of these pathogens, many growers are seeking alternative tools to manage disease in their orchards and postharvest.

The objectives of this study were to (1) identify non-phytotoxic concentrations of chitosan on apple fruit; assess potential for chitosan phytotoxicity on apple fruit; (2) evaluate commercial chitosan products for reduction of postharvest disease severity on inoculated fruit; (3) evaluate the effect of pre-harvest chitosan applications on suppression of latent infections, postharvest rots, and fruit quality; and (4) evaluate the effect of pre-harvest plus postharvest chitosan applications on suppression of *Penicillium expansum* and *Colletotrichum fioriniae* on inoculated fruit.

4.2. Materials and Methods

4.2.1. Chitosan Products

Two commercially available products were tested in this study. ARMOUR-Zen 15 (15 % chitosan) was obtained from Botry-Zen Ltd (Dunedin, New Zealand). Tidal Grow high molecular weight (MW) 2 % and Tidal Grow low MW 4 % were obtained from Tidal Vision Inc. (Bellingham, WA). The exact MWs of these products are proprietary, but the high MWs are within the range of 310-375 kDa and the low MWs are within the range of 50-190 kDa. The biopesticide Serenade ASO, with the active ingredient *Bacillus subtilis* QST 713, was obtained from Bayer AG (Leverkusen, Germany).

4.2.2. Pathogen Isolate and Inoculum Preparation

Penicillium expansum (Link) isolated from apple (Lancaster County, PA) and *Colletotrichum fioriniae* (Marcelino & Gouli) isolate FREC 79 isolated from apple (Biglerville, PA) were obtained from Dr. Kari Peter (Martin et al. 2022). Isolates were maintained on Difco potato dextrose agar (PDA). To prepare inoculum, *P. expansum* and *C. fioriniae* PDA plates were incubated for 12 days (d) and a spore suspension was obtained by flooding the cultures with 10 mL of sterile reverse osmosis (RO) water containing 0.1 % (v/v) Tween 80 (VWR, Radnor, PA) (El-Ghaouth et al. 2000b). Conidia were dislodged using a sterile FisherBrand cell spreader (Fisher Scientific, Hampton, NH). The resulting suspension was filtered through 4 layers of sterile cheesecloth. Conidial concentration was determined with a hemocytometer (Hausser Scientific, Horsham, PA) and adjusted to 1.0×10^4 spores mL⁻¹ (Janisiewicz et al. 2003; McLaughlin et al. 1990). The water control for non-inoculated fruit was RO water containing 0.1 % (v/v) Tween 80.

4.2.3. *Objective 1. Assessment of Chitosan Phytotoxicity on Apple Fruit (Preliminary Experiment 1)*

Because chitosan can cause phytotoxicity, fruit from ‘Golden Delicious’ trees grown in the University of New Hampshire’s greenhouses were used in preliminary trials to determine if commercial chitosan products prepared at varying chitosan concentrations (v/v) and pH could cause symptoms of phytotoxicity on wounded fruit and to identify non-phytotoxic concentrations of chitosan for future trials. This preliminary experiment 1 (PE-1) consisted of four treatments: three commercial chitosan products (ARMOUR-Zen 15, Tidal Grow high MW, and Tidal Grow low MW) and a water control. ARMOUR-Zen 15 and Tidal Grow low MW 4 % treatments were tested at 0.5, 1.0, 1.5, 2.0, and 3.0 % (v/v) chitosan. Tidal Grow high MW 2 % treatment was tested at 0.5, 1.0, and 2.0 % (v/v) chitosan. Tidal Grow products were prepared as pH non-adjusted (pH = 3.5-4.0) and as pH adjusted to ~5.0 using 1 M NaOH to test the effect of pH on phytotoxicity (Table A-4). Each treatment was applied to three fruit and organized in a randomized complete block design.

Prior to treatment, fruit were washed with soap and tap water. After drying, fruit were sprayed with 70 % ethanol and left to dry for an hour. All fruit were wounded (3 mm by 5 mm deep) using a sterilized six-pence nail to simulate stem punctures or harvest damage (Poleatewich et al. 2012; El-Ghaouth et al. 2000b). Nails were mounted through a rubber stopper to ensure uniformity of wound depth (Poleatewich et al. 2012). Wounds on fruit received 35 μ L of each chitosan treatment (El-Ghaouth et al. 2000b). For the water control, fruit were treated with 35 μ L of sterile MilliQ water.

Fruit were stored in 20-count molded plastic trays with plastic covers, which were drilled with five holes to allow for air exchange (Poleatewich et al. 2012). The trays were kept at

ambient temperature (20-24 °C) and high humidity (greater than 95 % relative humidity). Fruit were evaluated 7 and 14 d post treatment for symptoms of phytotoxicity. To assess phytotoxicity, the lesion diameter was measured laterally and horizontally across the wound site and the total lesion area was estimated using the average of the two measurements (Poleatewich et al. 2012). To compare lesion area over time, the area under the disease progress curve (AUDPC) was calculated for each fruit (Shaner and Finney 1977).

4.2.4. Objective 2: Evaluate Postharvest Application of Commercial Chitosan Products

(Preliminary Experiments 2 and 3)

Two preliminary trials were used to evaluate the efficacy of commercial chitosan products to reduce postharvest diseases. These experiments consisted of a 6 x 3 factorial with six chitosan treatments (a water control, Tidal Grow high MW at 0.5 % and 1.0 %, Tidal Grow low MW at 0.5 % and 1.0 %, and ARMOUR-Zen 15 % at 0.5 % chitosan) and three pathogen inoculation treatments (water control, *P. expansum*, and *C. fioriniae*). The pH of the Tidal Grow products was adjusted to ~5.0 using 1 M NaOH. Each treatment combination was applied to 10 replicate fruit and arranged in a randomized complete block design. In preliminary experiment 2 (PE-2), the chitosan treatments were applied at 0 days post inoculation (DPI), while in preliminary experiment 3 (PE-3), the chitosan treatments were applied at 0 DPI and 1 DPI.

McIntosh fruit from a New Hampshire (NH) commercial farm were harvested from trees that were conventionally managed by the grower using their own disease management practices and equipment. Fruit were cleaned and wounded as described in Section 4.2.3. Wounds on fruit received 35 µL of each chitosan treatment. One hour after the chitosan treatment, fruit were inoculated with a 20 µL spore suspension (200 spores per wound) or water. For PE-3, at 1 DPI, wounds received a second 35 µL of each chitosan treatment. Fruit were stored as described in

Section 4.2.3 and evaluated at 7 and 14 DPI. To assess disease severity, lesion diameter was measured laterally and horizontally across the wound site and the total lesion area was estimated using the average of the two measurements (Poleatewich et al. 2012). To compare expansion of symptomatic lesion area over time, the AUDPC was calculated for each fruit (Shaner and Finney 1977).

4.2.5. Objective 3. Effect of Pre-harvest Chitosan Applications on Suppression of Latent Infections, Postharvest Rots, and Fruit Quality

Experiments were conducted in a research orchard located in Pennsylvania (experiments 1-2) and commercial orchards located in New Hampshire (experiment 3-4).

4.2.5.1. Research Orchard trials (Experiments 1-2)

Experiments 1 and 2 were conducted in 2021 and 2022 in a 0.8-acre research block at the Penn State University (PSU) Fruit Research and Extension Center (FREC) located in Biglerville, Pennsylvania. Results from the first year were used to inform and adjust experiments in the second year. Experiments were conducted on semi-dwarf cultivar ‘Law Rome’ grafted on M.7 rootstock planted in 2015. Maintenance programs for insect pests and fire blight were applied with an airblast sprayer delivering 100 gallon/acre to the entire orchard following commercial production practices. To promote bitter rot infections in the orchard, fruit infected with *C. fioriniae* were hung in the orchard in April and replaced in June as described by Martin & Peter (2022). Briefly, ‘Gold Delicious’ apples inoculated with *C. fioriniae* were placed in onion bags and hung above the tops of trees.

In experiment 1, five pre-harvest treatments (Table 3-1) were evaluated; water control, grower standard (GS), chitosan (C), reduced risk (RR), and reduced risk + chitosan (RR+C). Reduced risk products are classified as having low impact on the environment, high specificity to

target organisms, and low potential for human health risk. The chitosan product, Tidal Grow 2%, was applied at $0.025 \text{ mg}\cdot\text{ml}^{-1}$ (0.0025% (v/v) chitosan). Each treatment was applied 14 times (starting in mid-April until harvest) to six replicate trees arranged in a randomized complete block design with a buffer tree in between each treatment tree. In experiment 2, five treatments were evaluated, with some modifications from experiment 1 (Table 3-2). The rate of chitosan was increased from 473 mL/acre (in experiment 1) to 1893 mL/acre ($0.1 \text{ mg}\cdot\text{ml}^{-1}$ or 0.01% (v/v) chitosan), and the sulfur component of the reduced risk treatment was removed. Each treatment was applied 15 times (starting in mid-April until harvest) to five replicate trees arranged in a randomized complete block design with a buffer tree in between each treatment tree. For both experiments, treatments were applied using a boom sprayer at 400 psi, delivering 100 gallon/acre. A final application was made on mature fruit prior to harvest in early October. At harvest, 25 fruit per replicate tree were harvested and evaluated for incidence of general rots (Figure 4-1B).

4.2.5.2. NH On-farm trials (Experiments 3-4)

Experiments 3 and 4 were conducted in 2022 on two commercial orchard sites in NH. This research relied on natural inoculum. Experiment 3 was conducted on a commercial farm in a 3.0-acre orchard with semi-dwarf cultivars ‘McIntosh’ and ‘Macoun’ grafted on Bud.9 or M.9 rootstock planted in 2017. Each treatment was applied to seven replicate ‘McIntosh’ trees and eight replicate ‘Macoun’ trees. Treatments were applied to trees arranged in a randomized complete block design with a buffer tree in between each treatment tree. Experiment 4 took place in a 0.9-acre orchard with semi-dwarf cider cultivars ‘Kingston Black’, ‘Dabinett’, and ‘Wickson’ grafted on M.26 rootstock planted in 2018. Each treatment was applied to four replicate trees and were arranged in a randomized complete block design.

Three pre-harvest treatments were evaluated in these experiments: grower standard (GS), grower standard + chitosan (GS+C), and grower standard + biopesticide + chitosan (GS+B+C) (Table 3-3 and 3-4). Briefly, the GS treatments consisted of conventional fungicides focused on apple scab prevention. The chitosan (C) treatment was ARMOUR-Zen 15 applied at 3785 mL/acre (0.15% (v/v) chitosan) and the biopesticide (B) treatment was Serenade ASO applied at 3785 mL/acre. The GS treatment was applied using the grower's equipment, a Rears Pul Blast Sprayer (REARS MFG. CO., Coburg, OR), delivering 38-40 gal/A for experiment 3 and a Rears powerblast sprayer (REARS MFG. CO., Coburg, OR), delivering 38-40 gal/A for experiment 4. The C and B+C sprays were applied by the researchers using two Dramm backpack BP-4Li sprayers (Model #BP-4LI and #MS40Li (MUS)) at 150 psi, delivering 23-25 gal/A. Maintenance programs for insect pests and fire blight were applied by the grower to the entire orchard following commercial production practices. Fruit were harvested in late September, prior to the start of pick-your-own on the farm, and placed in cold storage at 4 °C in a cooler located at the University of New Hampshire (UNH) Woodman Research Farm (Durham, NH) until evaluations for disease incidence were conducted.

4.2.5.3. Disease Assessment of Latent Infections and Postharvest Rots

For experiments 2-4, immature fruit were collected in August to measure treatment effects on latent infections or quiescent rots (rots that remain dormant and appear in storage) (Figure 4-1A). Fruit were surface sterilized and then frozen for 24-hours to induce emergence of quiescent infections (Martin and Peter 2023; Børve and Stensvand 2017). After 24-hours, fruit were left at room temperature for 7-10 days and evaluated for disease incidence. At harvest, 25 fruit per replicate tree were harvested and evaluated for incidence of all rots (fruit exhibiting any rot symptoms were counted as diseased regardless of the causal agent) (Figure 4-1B).

To assess effects of pre-harvest treatments on development of rots in storage, clean fruit (~100 fruit per treatment) were selected at harvest and stored in a 1 °C cooler located at FREC or in 4 °C cooler located at UNH Woodman Research Farm. Fruit were stored for 5-months then rated for fruit rot incidence. Fruit were evaluated for disease incidence when taken out of storage and then left at room temperature for 14 d and evaluated again for latent infections (Figure 4-1C).

4.2.5.4. Fruit Quality Evaluations

For experiments 1-4, fifteen fruit per treatment were randomly selected at harvest to assess fruit quality through starch pattern iodine index (SPI) and soluble solids contents (SSC) (Mitcam et al. 1996). SPI was measured as described by Ewing et al. (2019). Briefly, apples were cut along the diameter and the stem-side cross section was sprayed with a 0.22 % iodine and 0.88 % potassium iodine (w/v) solution. Ratings were taken using the Cornell Starch-Iodine Index 1-8 scale, where 1 = 100 % staining (minimal starch hydrolysis) and 8 = 0 % staining (complete starch hydrolysis) (Blanpied and Silsby 1992). SSC is an estimate of sugar content in fruit and was measured using a Hanna digital refractometer (Hanna Instruments, Woonsocket, RI, model HI96801) and reported as percent Brix (weight percentage of sucrose contained in 100 mL of water) (Mitcam et al. 1996). Briefly, the blossom end side cross section of the apple was peeled and cut into pieces using a sterile knife. The pieces were then placed in a sterile garlic press and juiced onto the refractometer prism to read the percent Brix.

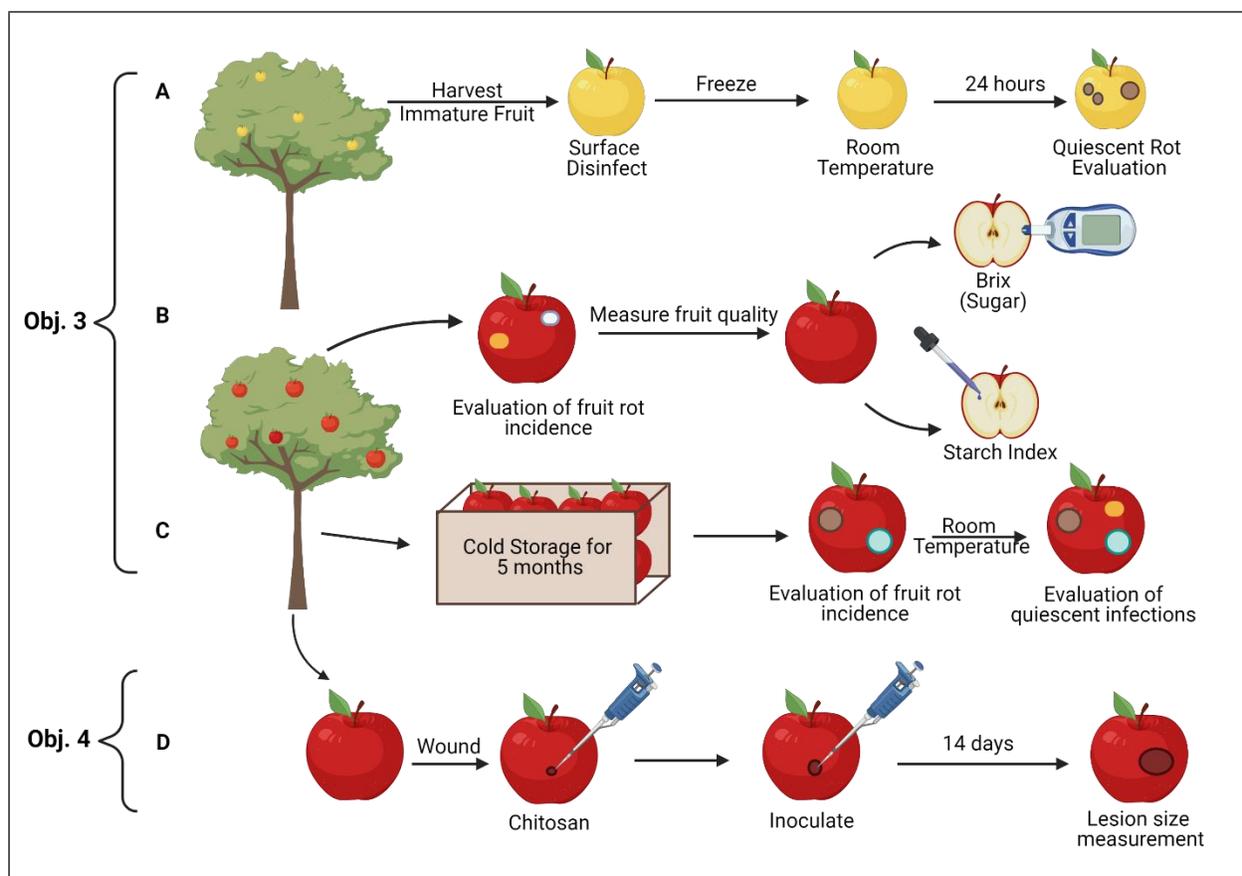


Figure 4-1. Workflow for objective 3; evaluate the effect of pre-harvest chitosan applications on suppression of (A) latent infections on immature fruit, (B) postharvest rots and fruit quality at harvest, and (C) postharvest and latent infections after cold storage. Objective 4 (D) evaluates the effect of pre-harvest and postharvest chitosan applications on suppression of *Penicillium expansum* and *Colletotrichum fioriniae* on inoculated fruit.

4.2.6. Objective 4. Effect of Pre-harvest and Postharvest Chitosan Applications on Suppression of *Penicillium expansum* and *Colletotrichum fioriniae* on Inoculated Fruit (Experiments 5-7)

Fruit harvested from experiments 2-4 were utilized to compare pre-harvest, postharvest, and pre-harvest + postharvest chitosan applications for suppression of postharvest diseases (Figure 4-1D). Experiment 5 utilized ‘Rome’ fruit harvested from experiment 2, the postharvest experiment was arranged as a 5 x 4 x 3 factorial with five pre-harvest treatments, four postharvest treatments (water control, Tidal Grow high MW at 1.0 %, Tidal Grow low MW at 1.0 %, and ARMOUR Zen 15 % at 1.0 % chitosan), and three inoculation treatments (water control, *P. expansum*, and *C. fioriniae*) (Table 4-1). The pH of the Tidal Grow products was

adjusted to ~5.0 using 1 M NaOH. Postharvest treatments were selected based on results from Objective 2. Each treatment combination was applied to five fruit arranged in a randomized complete block design.

Experiment 6 utilized McIntosh and Macoun fruit harvested from experiment 3 and experiment 7 utilized Wickson fruit harvested from experiment 4. Experiments 6 and 7 were arranged as a 3 x 4 x 3 factorial with three pre-harvest treatments, four postharvest treatments, and three inoculations (Table 4-1). Each treatment combination was applied to six fruit arranged in a randomized complete block design.

Fruit were not washed to prevent removing the pre-harvest treatments from the fruit. Fruit were wounded, treated with chitosan, and inoculated following the protocol in Section 4.2.4.

Fruit were stored as described in Section 4.2.3. Disease severity was measured at 5, 9, and 14

DPI and AUDPC calculated as described in Section 4.2.4.

Table 4-1. Treatment list for experiments 5, 6, and 7. Experiment 5 consisted of a 5 x 4 x 3 factorial with five pre-harvest treatments, four postharvest treatments, and three inoculations. Experiments 6 and 7 consisted of a 3 x 4 x 3 factorial with three pre-harvest treatments, four postharvest treatments, and three inoculations.

	Pre-harvest¹	Post-harvest²	Inoculations
Exp. 5	Water Control	Water Control	Water Control
	Grower Standard	Tidal Grow HMW 1.0%	<i>Penicillium expansum</i>
	Chitosan	Tidal Grow LMW 1.0%	<i>Colletotrichum fioriniae</i>
	Reduced Risk	ARMOUR-Zen 15 1.0%	
	Reduced Risk + Chitosan		
Exp. 6 & 7	Grower Standard	Water Control	Water Control
	Grower Standard + Chitosan	Tidal Grow HMW 1.0%	<i>Penicillium expansum</i>
	Grower Standard + Biopesticide + Chitosan	Tidal Grow LMW 1.0%	<i>Colletotrichum fioriniae</i>
		ARMOUR-Zen 15 1.0%	

¹ Pre-harvest treatments for experiment 5 are detailed in Table 3-2. Pre-harvest treatments for experiment 6 are detailed in Table 3-3. Pre-harvest treatments for experiment 7 are detailed in Table 3-4.

² Treatments evaluated were water control, tidal grow high molecular weight (HMW) or low molecular weight (LMW) at 1.0 % chitosan (v/v), or ARMOUR-Zen 15 at 1.0 % chitosan (v/v).

4.2.7. Data Analysis

Data analysis for each experiment was performed using RStudio version 2022.09.6 "Spotted Wakerobin" (RStudio Team 2020). Additionally, each cultivar was analyzed separately. Data were analyzed with an analysis of variance (ANOVA) using partial (type II) sums of squares ('car' package). Post hoc Tukey means separation tests were conducted using least squared adjusted treatment means obtained via the 'emmeans' package in Rstudio. Graphs were created in Rstudio using the package 'ggplot2'.

4.3. Results

4.3.1. Objective 1. Assessment of Chitosan Phytotoxicity on Apple Fruit (PE-1)

Wounded apples treated with higher concentrations of chitosan generally had greater phytotoxicity compared to apples treated with water ($P \leq 0.039$) (Figure A-4). Phytotoxicity was detected on wounded apples treated with Tidal Grow high MW at 1.0 %-1.5 %, Tidal Grow low MW at 1.5 %-3.0 %, and ARMOUR-Zen 15 at 1.5 % -2.0%. (Table A-4). However, when the pH of the Tidal Grow products was adjusted to ~5, no phytotoxicity on chitosan treated apples was observed and lesions measurements were not different from water treated apples ($P \geq 0.158$). Phytotoxicity was not observed on wounded apples treated with Tidal Grow high MW at 0.5 %, Tidal Grow low MW at 0.5 % - 1.0 %, and ARMOUR-Zen 15 at 0.5% - 1.0 % ($P \geq 0.158$). These results were used to select treatments for future experiments.

4.3.2. Objective 2: Evaluate Commercial Chitosan Products for Reducing Postharvest Diseases on Inoculated Fruit (PE-2 and PE-3)

McIntosh fruit inoculated with *P. expansum* and treated with Tidal Grow low MW at 1 % chitosan had 33 % lower AUDPC compared to apples treated with water ($P = 0.014$) (Figure 4-2A) and up to 37 % lower AUDPC compared to apples treated with Tidal Grow high MW at 0.5

% and ARMOUR-Zen at 0.5 % ($P \leq 0.042$) (Figure 4-2A). For fruit that were treated at 0 DPI and 1 DPI (PE-3), apples treated with Tidal Grow low MW at 1 % had 86 % lower AUDPC compared to apples treated with water ($P \leq 0.001$) (Figure 4-2B). Additionally, apples treated with Tidal Grow low MW at 0.5 % and Tidal Grow high MW at 0.5 % treatments had lower AUDPC compared to water control apples ($P \leq 0.011$).

McIntosh fruit inoculated with *C. fioriniae* and treated with Tidal Grow high MW at 1 % and Tidal Grow low MW at 1 % chitosan had the lowest AUDPC out of all the chitosan treated apples (Figure 4-2C) with a reduction in AUDPC up to 55 % compared to the water control ($P \leq 0.001$). For fruit that were treated twice at 0 DPI and 1 DPI (PE-3), apples treated with Tidal Grow low MW at 1% had 34 % lower AUDPC compared to apples treated with water ($P \leq 0.001$) (Figure 4-2D). Additionally, apples treated with Tidal Grow low MW at 0.5 % and Tidal Grow high MW at 0.5 % and 1.0 % all had lower AUDPC compared to apples treated with water ($P \leq 0.043$). Within the non-inoculated apples, there was no effect of treatment on AUDPC when treatments were applied at 0 DPI ($P = 0.2245$) or when treatments were applied at 0 and 1 DPI ($P = 0.516$) (Table A-5).

4.3.3. Objective 3. Effect of Pre-harvest Chitosan Applications on Suppression of Latent

Infections, Postharvest Rots, and Fruit Quality

4.3.3.1. Experiment 1.

At harvest, rot incidence on fruit was low (0 % to 9 %), resulting in no significant differences between treatments ($P = 0.145$) (Table 4-2). Apples treated with GS had significantly lower SPI compared to all other treatments ($P \leq 0.007$) (Table 4-2). Apples treated with the RR+C treatment had the highest SSC and was significantly different from the water control ($P = 0.015$) (Table 4-2).

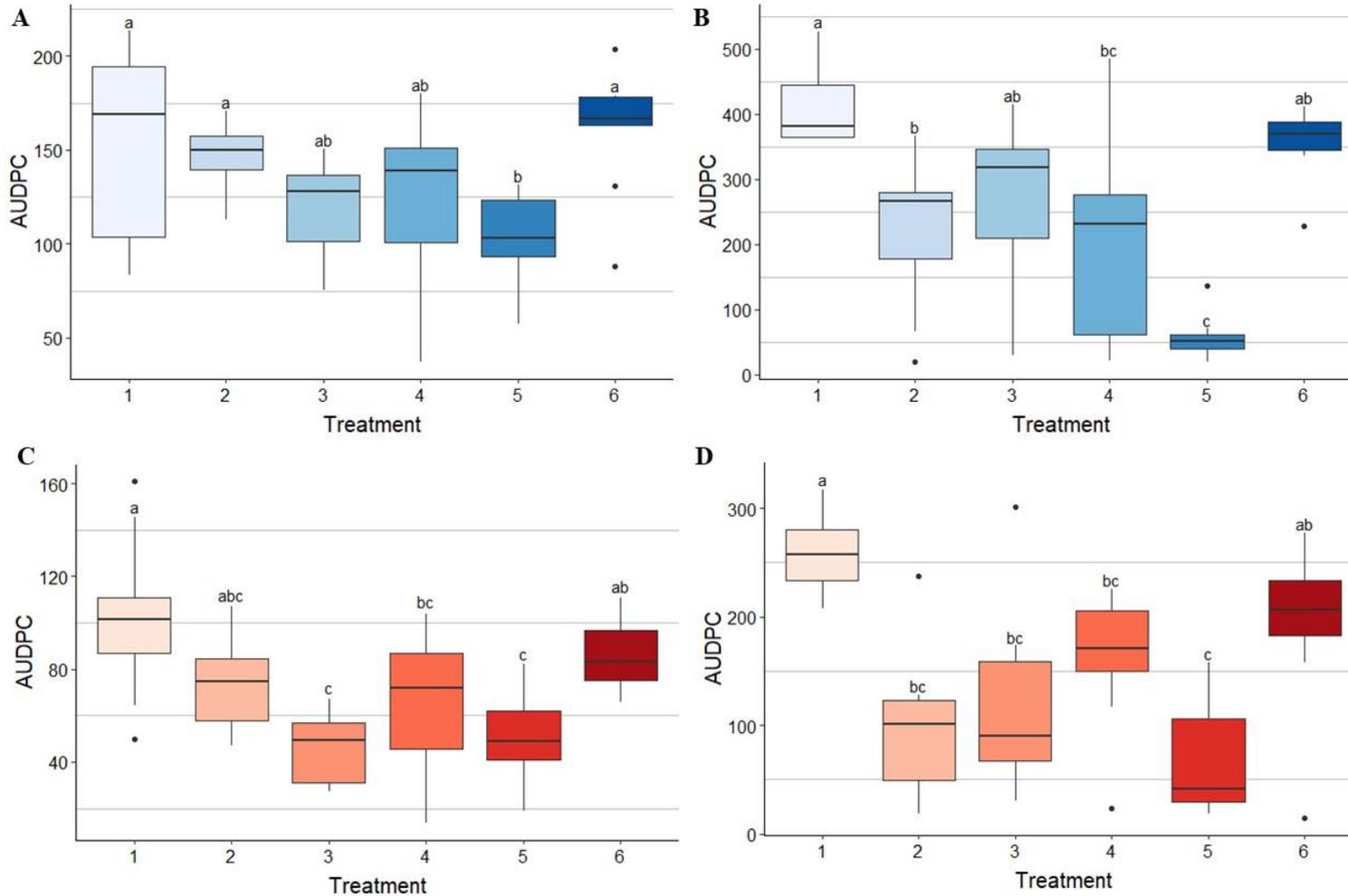


Figure 4-2. Effect of chitosan treatments on area under the disease progress curve (AUDPC) for McIntosh apples inoculated with *Penicillium expansum* in objective 2: (A) preliminary experiment 2 (PE-2) and (B) preliminary experiment 3 (PE-3), and *Colletotrichum fioriniae* in objective 2: (C) PE-2 and (D) PE-3. Treatments were a (1) water control, Tidal Grow 2 % high molecular weight (HMW) at (2) 0.5 % and (3) 1.0 % chitosan, Tidal Grow 4 % low molecular weight (LMW) at (4) 0.5 % and (5) 1.0 % chitosan, and (6) ARMOUR-Zen 15 % at 0.5 % chitosan. Treatment means followed by the same letter are not significantly different ($\alpha=0.05$) as determined by the Tukey HSD Post-hoc test. The lines in the boxplot represent median, while the box represents the Interquartile Range (IQR). The start and end of the lines represent the minimum value in the data ($Q_3 - 1.5 \times \text{IQR}$), while any outliers are represented as dots.

After five months in storage, rot incidence ranged from 7 % to 38 % and the GS treated apples had 79 % less rot incidence compared to the water treated apples ($P = 0.038$) (Table 4-3). The other treatments were not significantly different from the water control. For latent infections, the GS treated apples had significantly lower rot incidence compared to the water treated apples ($P = 0.002$) (Table 4-3). GS treated apples had the lowest overall rot incidence, followed by the C and RR treated apples (Table 4-3).

4.3.3.2. Experiment 2.

Bitter rot incidence ranged from 15 % to 35 % on the water control apples. In August, the GS and RR+C treated apples had 90 % and 85 %, respectively, less bitter rot incidence compared to apples treated with water ($P \leq 0.030$) (Figure 4-3).

At harvest, overall rot incidence on non-treated fruit was low (0 % to 4 %), resulting in no significant differences between treatments ($P = 0.109$) (Table 4-2). Fruit quality, as measured by SPI and SSC, was evaluated at harvest. Apples treated with the GS had significantly lower SPI compared to all other treatments ($P \leq 0.001$) (Table 4-2). Apples treated with the GS treatment had the highest SSC and was significantly different from the water control ($P = 0.015$) (Table 4-2).

After five months in storage, there was no effect of treatment on rot incidence ($P = 0.162$). For latent infections, the GS treated apples had significantly lower rot incidence compared to the water treated apples ($P = 0.045$) (Table 4-3). Overall rot incidence was low, making differences between treatments difficult to observe.

Table 4-2. Mean rot incidence, mean starch pattern iodine index (SPI), or total soluble solids \pm standard error on ‘Rome’ harvested fruit from experiments 1 and 2 (2021 and 2022 FREC research orchard trials).^{1,2}

Year	Measurement	Water Control	GS	C	RR	RR+C
Exp. 1	Rot Incidence (%)	2.2 \pm 1.5	0.0 \pm 0.0	8.7 \pm 3.8	5.5 \pm 1.3	5.7 \pm 3.8
	SPI (0-8)	6.4 \pm 0.5 (a)	3.0 \pm 0.4 (b)	5.2 \pm 0.4 (a)	6.3 \pm 0.4 (a)	5.4 \pm 0.5 (a)
	SSC (%Brix)	11.9 \pm 0.8 (b)	13.8 \pm 0.4 (ab)	13.6 \pm 0.5 (ab)	13.9 \pm 0.4 (ab)	14.5 \pm 0.6 (a)
Exp. 2	Rot Incidence (%)	1.6 \pm 1.6	0.0 \pm 0.0	0.8 \pm 0.8	0.8 \pm 0.8	4.0 \pm 1.3
	SPI (0-8)	7.7 \pm 0.2 (a)	5.6 \pm 0.3 (b)	7.5 \pm 0.3 (a)	7.9 \pm 0.1 (a)	8.0 \pm 0.0 (a)
	SSC (%Brix)	11.5 \pm 0.2 (b)	12.3 \pm 0.1 (a)	11.8 \pm 0.1 (ab)	11.9 \pm 0.1 (ab)	10.8 \pm 0.2 (c)

¹ Treatments evaluated were water control, grower standard (GS), chitosan (C), reduced risk (RR), and a reduced risk and chitosan mixture (RR+C).

² Within a disease measurement, treatment means followed by different letters are significantly different ($\alpha=0.05$) as determined by the Tukey HSD Post-hoc test.

Table 4-3. Mean rot incidence of Rome fruit taken out of storage after 5 months (storage rot incidence), mean rot incidence of fruit left at room temperature for two weeks (quiescent rot incidence), and total rot incidence of stored fruit \pm standard error from experiments 1 and 2 (2021 and 2022 FREC research orchard trials).^{1,2}

Year	Measurement	Water Control	GS	C	RR	RR+C
Exp. 1	Storage Rot Incidence (%)	32.5 \pm 6.9 (a)	6.7 \pm 3.0 (b)	32.7 \pm 6.6 (a)	30.5 \pm 5.0 (ab)	38.0 \pm 10.4 (a)
	Quiescent Rot Incidence (%)	38.8 \pm 8.9 (a)	3.5 \pm 2.3 (b)	14.1 \pm 5.0 (ab)	18.3 \pm 7.0 (ab)	26.7 \pm 6.9 (a)
	Total Rot Incidence (%)	71.4 \pm 11.7 (a)	10.2 \pm 4.6 (b)	46.8 \pm 8.4 (ab)	48.8 \pm 8.7 (ab)	63.0 \pm 14.3 (a)
Exp. 2	Storage Rot Incidence (%)	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	1.0 \pm 1.0	2.0 \pm 1.2
	Quiescent Rot Incidence (%)	17.0 \pm 2.5 (a)	4.0 \pm 1.9 (b)	9.0 \pm 2.9 (ab)	130 \pm 1.2 (ab)	20.0 \pm 2.2 (a)
	Total Rot Incidence (%)	17.0 \pm 2.5 (a)	4.0 \pm 1.9 (b)	9.0 \pm 2.9 (ab)	14.0 \pm 1.9 (a)	22.0 \pm 2.5 (a)

¹ Treatments evaluated were water control, grower standard (GS), chitosan (C), reduced risk (RR), and a reduced risk and chitosan mixture (RR+C).

² Within a disease measurement, treatment means followed by different letters are significantly different ($\alpha=0.05$) as determined by the Tukey HSD Post-hoc test.

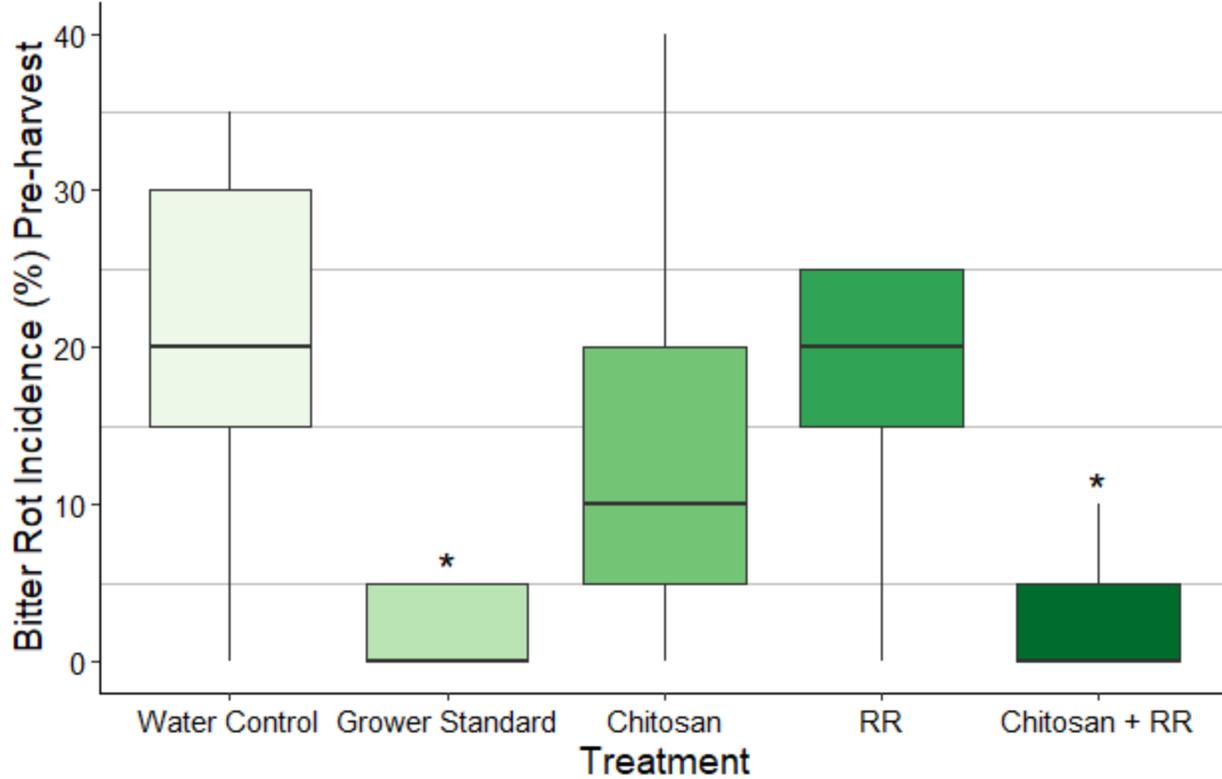


Figure 4-3. Effect of pre-harvest treatment on bitter rot incidence on Rome fruit harvested in August from objective 3 experiment 2 (2022 FREC research orchard trial). Treatments included a water control, grower standard, chitosan (Tidal Grow 2% chitosan at 1893 mL/acre), reduced risk (RR), and chitosan + reduced risk (chitosan + RR). Treatment means followed by an asterisk (*) differ statistically from the water control according to Dunnett’s test at $\alpha=0.05$. The lines in the boxplot represent median, while the box represents the Interquartile Range (IQR). The start and end of the lines represent the minimum value in the data ($Q_3 - 1.5 \times IQR$), while any outliers are represented as dots.

4.3.3.3. Experiments 3-4.

In both experiments 3 and 4, across cultivars, there was no effect of treatment on rot incidence of harvested fruit ($P \geq 0.318$) (Table 4-4). Overall, rot incidence was low (0 % to 7 %). Macoun fruit treated with GS+C had lower SPI compared to fruit treated with water ($P = 0.047$) (Table 4-4). Dabinett fruit treated with GS+B+C had the highest SPI but did not significantly differ from fruit treated with water ($P = 0.127$), and the lowest SSC ($P \leq 0.001$). There was no effect of treatment on SPI for Wickson ($P = 0.682$) or McIntosh ($P = 0.090$). McIntosh fruit treated with GS had the highest SSC ($P \leq 0.001$) (Table 4-4). There was no effect of treatment on SSC for apples from Wickson ($P = 0.968$) or Macoun ($P = 0.396$). Unfortunately, one of the cultivars in Farm #2, Kingston Black, did not have enough fruit at harvest for evaluations.

After five months in storage, there was no effect of pre-harvest treatment on rot incidence on Macoun or Wickson apples ($P \geq 0.169$) (Table 4-5) or latent rot incidence on Wickson fruit ($P = 0.723$). On McIntosh fruit, the GS+B+C treated apples had significantly less rot incidence compared to the GS and GS+C treated apples ($P \leq 0.049$) (Table 4-5). There was no effect of pre-harvest treatment on latent rot incidence of McIntosh fruit ($P = 0.798$). On Dabinett fruit, the GS+C treated apples had significantly less rot incidence and latent rot incidence compared to the GS and GS+B+C treated apples ($P \leq 0.003$) (Table 4-5).

Table 4-4. Mean rot incidence, mean starch pattern iodine index (SPI), or total soluble solids \pm standard error on fruit harvested from experiments 3 and 4 (NH 2022 on-farm trials).^{1,2}

	Cultivar	Measurement	GS	GS+C	GS+B+C
Exp. 3	Macoun	Rot Incidence (%)	3.2 \pm 1.5	4.0 \pm 1.5	2.0 \pm 1.2
		SPI (0-8)	7.2 \pm 0.2 (a)	6.5 \pm 0.2 (b)	7.0 \pm 0.2 (ab)
		SSC (%Brix)	14.3 \pm 0.3	14.6 \pm 0.3	14.8 \pm 0.1
	McIntosh	Rot Incidence (%)	6.6 \pm 3.1	4.0 \pm 4.0	1.7 \pm 1.0
		SPI (0-8)	7.7 \pm 0.1	8.0 \pm 0.0	7.7 \pm 0.1
		SSC (%Brix)	15.2 \pm 0.2 (a)	13.2 \pm 0.4 (b)	13.4 \pm 0.3 (b)
Exp. 4	Dabinett	Rot Incidence (%)	5.3 \pm 2.7	3.0 \pm 1.9	1.0 \pm 1.0
		SPI (0-8)	5.8 \pm 0.5 (ab)	4.8 \pm 0.3 (b)	6.1 \pm 0.3 (a)
		SSC (%Brix)	14.3 \pm 0.2 (a)	14.4 \pm 0.2 (a)	13.2 \pm 0.1 (b)
	Wickson	Rot Incidence (%)	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
		SPI (0-8)	6.4 \pm 0.2	6.6 \pm 0.2	6.5 \pm 0.2
		SSC (%Brix)	14.3 \pm 0.2	14.3 \pm 0.2	14.3 \pm 0.3

¹ Treatments evaluated were grower standard (GS), grower standard + chitosan (GS+C), grower standard + biopesticide + chitosan (GS+B+C).

² Within a disease measurement, treatment means followed by different letters are significantly different ($\alpha=0.05$) as determined by the Tukey HSD Post-hoc test. Measurements with significant differences are visually represented by the shaded cells.

Table 4-5. Mean rot incidence of fruit taken out of storage after 5 months (storage rot incidence), mean rot incidence of fruit left at room temperature for two weeks (quiescent rot incidence), and total rot incidence of stored fruit \pm standard error from experiments 3 and 4 (NH 2022 on-farm trials).^{1,2}

	Cultivar	Measurement	GS	GS+C	GS+B+C
Exp. 3	Macoun	Storage Rot Incidence (%)	7.3 \pm 2.6	20.4 \pm 5.8	19.5 \pm 6.5
		Quiescent Rot Incidence (%)	15.9 \pm 3.5	16.4 \pm 6.5	4.5 \pm 3.1
	McIntosh	Storage Rot Incidence (%)	22.9 \pm 4.5 (a)	14.9 \pm 2.6 (a)	4.9 \pm 2.4 (b)
		Quiescent Rot Incidence (%)	9.8 \pm 2.8	13.1 \pm 4.5	9.8 \pm 3.2
Exp. 4	Dabinett	Storage Rot Incidence (%)	61.1 \pm 3.2 (a)	26.4 \pm 4.4 (b)	60.6 \pm 6.3 (a)
		Quiescent Rot Incidence (%)	73.3 \pm 3.8 (a)	45.9 \pm 3.4 (b)	72.8 \pm 3.3 (a)
	Wickson	Storage Rot Incidence (%)	1.5 \pm 0.9	3.2 \pm 0.8	0.8 \pm 0.8
		Quiescent Rot Incidence (%)	4.5 \pm 2.8	3.5 \pm 1.9	1.7 \pm 1.7

¹ Treatments evaluated were grower standard (GS), grower standard + chitosan (GS+C), grower standard + biopesticide + chitosan (GS+B+C).

² Within a disease measurement, treatment means followed by different letters are significantly different ($\alpha=0.05$) as determined by the Tukey HSD Post-hoc test. Measurements with significant differences are visually represented by the shaded cells.

4.3.4. Objective 4. Effect of Pre-harvest and Postharvest Chitosan Applications on Suppression of *Penicillium expansum* and *Colletotrichum fioriniae* on Inoculated Fruit

4.3.4.1. Experiment 5.

On Rome apples harvested from FREC in 2022 (experiment 2), there was no interaction between pre-harvest and postharvest treatments on AUDPC of *P. expansum*, *C. fioriniae*, or water inoculated fruit ($P \geq 0.281$). Thus, the simple effects of the pre-harvest and postharvest treatments were analyzed. Apples treated pre-harvest with C, RR, and RR+C pre-harvest had significantly lower *P. expansum* AUDPC compared to GS treated apples ($P \leq 0.026$) (Table A-6). Regardless of pre-harvest treatment, apples treated postharvest with Tidal Grow low MW at 1 % chitosan had significantly lower AUDPC of *P. expansum* compared to apples treated with ARMOUR Zen at 1 % or Tidal Grow high MW at 1 % chitosan ($P \leq 0.001$) but did not significantly differ from the apples treated with water ($P = 0.860$) (Table 4-6).

Pre-harvest and postharvest treatments did not have an effect on AUDPC of *C. fioriniae* ($P \geq 0.615$) (Table 4-6; Table A-6). Within apples not inoculated, there was no effect of pre-harvest treatment on AUDPC of naturally present pathogens ($P = 0.593$), however, apples treated with C or RR+C had 29 % to 37 % lower AUDPC compared to apples treated with water or GS (Table A-6). Regardless of pre-harvest treatment, apples treated postharvest with Tidal Grow low MW at 1 % chitosan had significantly lower AUDPC of naturally present pathogens compared to apples treated with ARMOUR Zen at 1 % ($P = 0.010$) but did not significantly differ from apples treated with Tidal Grow high MW at 1 % or water ($P \geq 0.535$) (Table 4-6).

Table 4-6. Effect of postharvest treatments on area under the disease progress curve (AUDPC) \pm standard error of *Penicillium expansum*, *Colletotrichum fioriniae*, and water control on Rome fruit in experiment 5.
1,2

Infestation	Postharvest Treatment			
	Water Control	Tidal Grow HMW 1.0 %	Tidal Grow LMW 1.0 %	ARMOUR- Zen 1.0 %
<i>Penicillium expansum</i>	115.3 \pm 15.1 (b)	158.9 \pm 11.6 (a)	102.7 \pm 13.5 (b)	196.9 \pm 9.5 (a)
<i>Colletotrichum fioriniae</i>	49.6 \pm 7.8	41.7 \pm 2.9	46.1 \pm 3.5	49.1 \pm 3.9
Water control	11.6 \pm 2.3 (b)	33.8 \pm 4.2 (ab)	21.52 \pm 1.7 (b)	50.9 \pm 11.5 (a)

¹ Treatments evaluated were water control, tidal grow high molecular weight (HMW) or low molecular weight (LMW) at 1.0 % chitosan (v/v), or ARMOUR-Zen 15 at 1.0 % chitosan (v/v).

² Within a disease measurement, treatment means followed by different letters are significantly different ($\alpha=0.05$) as determined by the Tukey HSD Post-hoc test.

4.3.4.2. Experiment 6.

On Macoun harvested apples, there was no interaction between pre-harvest treatments and postharvest treatments on AUDPC of *P. expansum*, *C. fioriniae*, or water inoculated fruit ($P \geq 0.203$). Apples treated with the GS+B+C treatment pre-harvest had significantly lower AUDPC of *P. expansum* compared to the GS treated apples ($P = 0.008$) (Table A-7; Figure A-5). Regardless of the pre-harvest treatments, apples treated postharvest with Tidal Grow high MW or Tidal Grow low MW at 1 % chitosan had significantly lower AUDPC of *P. expansum* compared to apples treated with water ($P \leq 0.001$) (Figure 4-4A). Within apples inoculated with *C. fioriniae*, apples treated with the GS+B+C treatment pre-harvest had the lowest AUDPC, although not significantly different from apples treated with GS ($P = 0.528$) (Table A-7; Figure A-6). Regardless of pre-harvest treatments, apples treated postharvest with Tidal Grow high MW or Tidal Grow low MW at 1 % chitosan had significantly lower AUDPC of *C. fioriniae* compared to apples treated with water ($P \leq 0.042$) (Figure 4-4C). Within apples not inoculated, apples treated with GS+B+C treatment pre-harvest had significantly lower AUDPC of naturally present pathogens compared to the apples treated with GS ($P = 0.033$) (Table A-7). Across, the pre-harvest treatments, apples treated with Tidal Grow high MW or Tidal Grow low MW at 1 %

had the lowest AUDPC of naturally present pathogens but did not significantly differ from the apples treated with water ($P \geq 0.056$) but were different from the apples treated with ARMOUR-Zen at 1 % ($P \leq 0.015$) (Table A-8).

On McIntosh harvested apples, there was no interaction between pre-harvest treatments and postharvest treatments on AUDPC of *P. expansum*, *C. fioriniae*, or water inoculated ($P \geq 0.065$). There was no effect of pre-harvest treatment on AUDPC of *P. expansum* ($P = 0.735$) (Table A-7; Figure A-7). Regardless of the pre-harvest treatments, apples treated postharvest with Tidal Grow high MW or Tidal Grow low MW at 1 % chitosan had significantly lower AUDPC of *P. expansum* compared to apples treated with water ($P \leq 0.001$) (Figure 4-4B). Within apples inoculated with *C. fioriniae*, apples treated with the GS+C treatment pre-harvest had the lowest AUDPC, although not significantly different from apples treated with GS ($P = 0.456$) (Table A-7; Figure A-8). Regardless of pre-harvest treatments, apples treated postharvest with Tidal Grow low MW at 1 % chitosan had significantly lower AUDPC of *C. fioriniae* compared to apples treated with water ($P = 0.011$) (Figure 4-4D). Additionally, apples treated with Tidal Grow high MW at 1 % chitosan had lower AUDPC but did not significantly differ from the apples treated with water ($P = 0.875$). Within apples not inoculated, there was no effect of pre-harvest treatment on AUDPC of naturally present pathogens ($P = 0.230$) (Table A-7). Across the pre-harvest treatments, apples treated with Tidal Grow high MW or Tidal Grow low MW at 1 % had the lowest AUDPC of naturally present pathogens but did not significantly differ from the apples treated with water ($P \geq 0.522$) but were different from the apples treated with ARMOUR-Zen at 1 % ($P \leq 0.031$) (Table A-8).

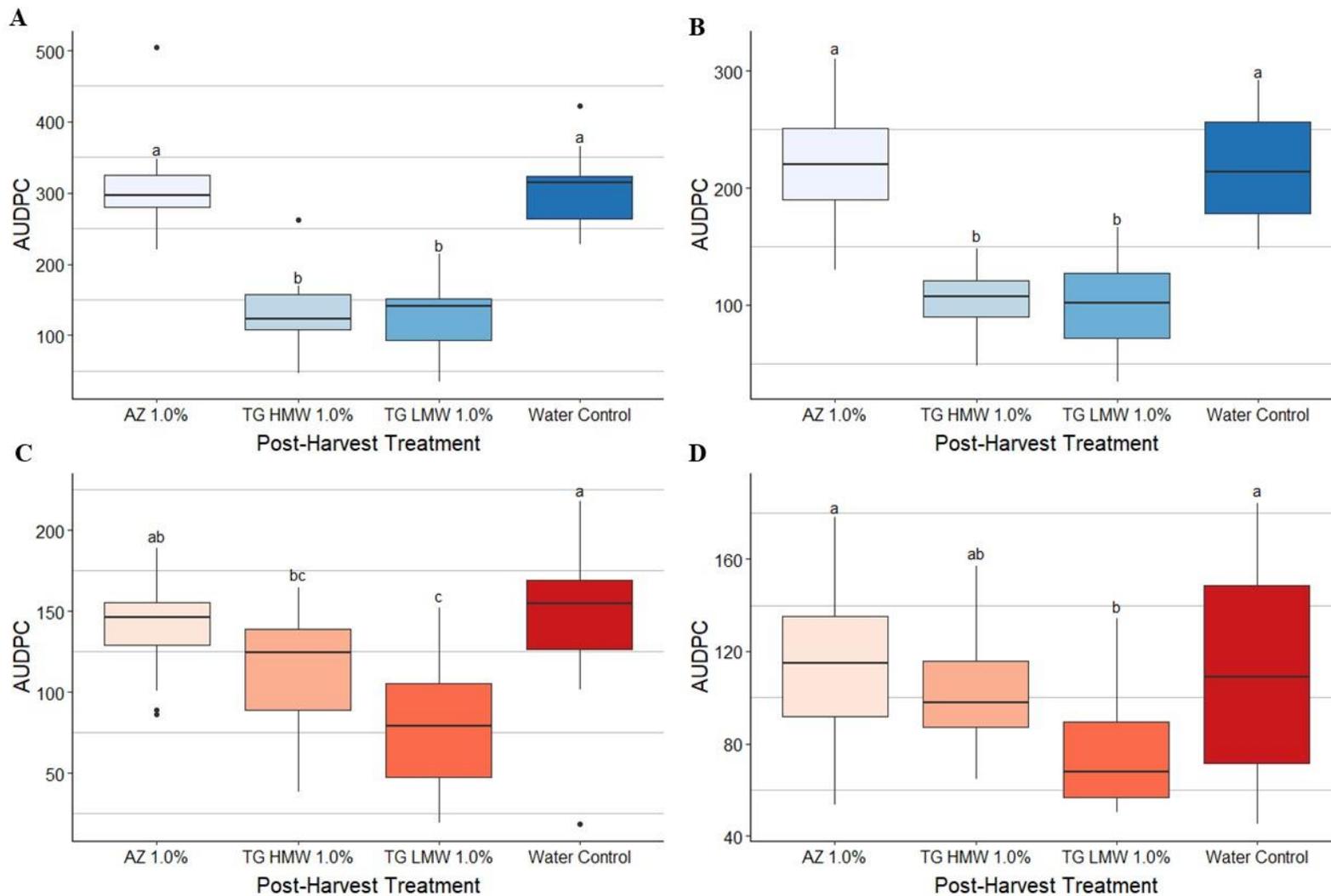


Figure 4-4. Effect of postharvest chitosan treatments on area under the disease progress curve (AUDPC) of *Penicillium expansum* on (A) Macoun and (B) McIntosh apples, and AUDPC of *Colletotrichum fiorinia* on (C) Macoun and (D) McIntosh apples in experiment 6. Treatments were ARMOUR-Zen 15 % at 1.0 % chitosan, Tidal Grow 2 % high molecular weight (HMW) at 1.0 % chitosan, Tidal Grow 4 % low molecular weight (LMW) at 1.0 % chitosan, and a water control. Treatment means followed by the same letter are not significantly different ($\alpha=0.05$) as determined by the Tukey HSD Post-hoc test. The lines in the boxplot represent median, while the box represents the Interquartile Range (IQR). The start and end of the lines represent the minimum value in the data ($Q_3 - 1.5 \times \text{IQR}$), while any outliers are represented as dots.

4.3.4.3. Experiment 7.

On Wickson harvested apples, there was no interaction between pre-harvest treatments and postharvest treatments on AUDPC of *P. expansum*, *C. fioriniae*, or water inoculated ($P \geq 0.482$). Within apples inoculated with *P. expansum*, apples treated with the GS+C treatment pre-harvest had significantly lower AUDPC compared to the GS+B+C treated apples ($P = 0.025$) (Table A-7). Regardless of the pre-harvest treatments, apples treated postharvest with Tidal Grow high MW at 1 % chitosan had significantly lower AUDPC of *P. expansum* compared to apples treated with water ($P < 0.001$) (Figure 4-5A). Within apples inoculated with *C. fioriniae*, apples treated with the GS treatment pre-harvest had the lowest AUDPC, although did not significantly differ from apples treated with GS+C or GS+B+C ($P \geq 0.052$) (Table A-7). Regardless of pre-harvest treatments, apples treated postharvest with ARMOUR-Zen at 1 % had the greatest AUDPC and were significantly different compared to apples treated with water ($P < 0.001$) (Figure 4-5B). Within apples not inoculated, apples treated with GS and GS+C had the lowest AUDPC of naturally present pathogens and differed from apples treated with the GS+B+C ($P \leq 0.001$) (Table A-7). Across the pre-harvest treatments, apples treated with Tidal Grow low MW at 1 % had low AUDPC of naturally present pathogens but did not significantly differ from the apples treated with water ($P = 0.558$) but were different from the apples treated with ARMOUR-Zen at 1 % ($P = 0.035$) (Table A-8).

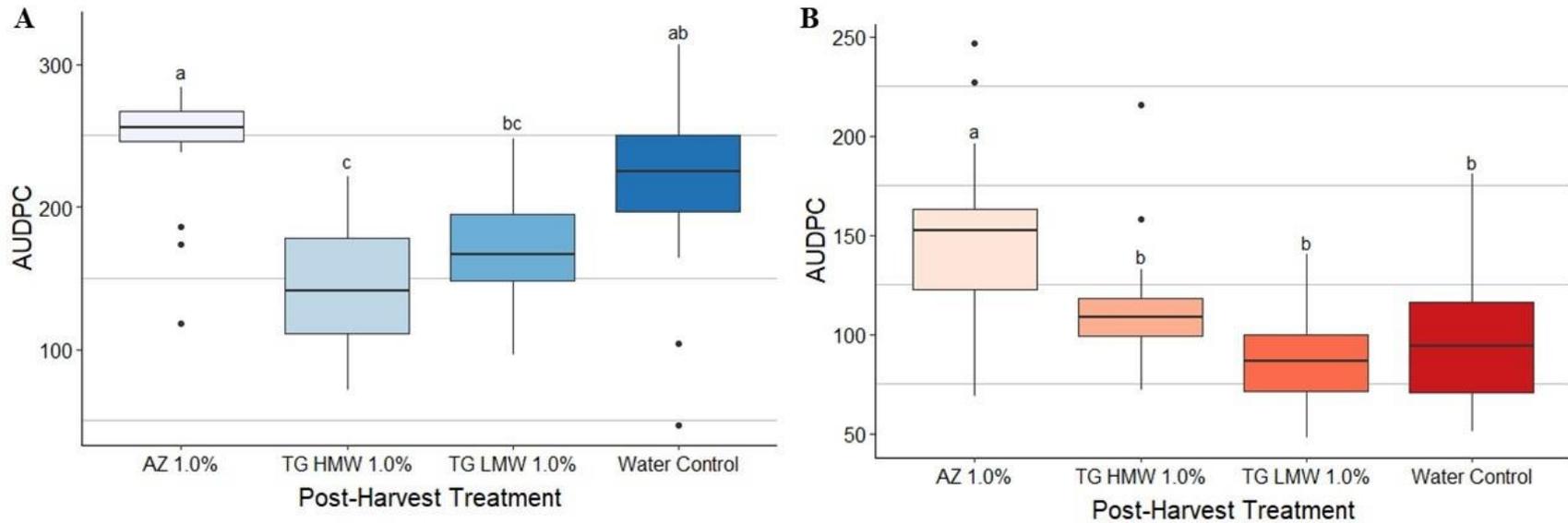


Figure 4-5. Effect of postharvest chitosan treatments on area under the disease progress curve (AUDPC) of **(A)** *Penicillium expansum* and **(B)** *Colletotrichum fioriniae* on Wickson apples in experiment 7. Treatments were ARMOUR-Zen 15 % at 1.0 % chitosan, Tidal Grow 2 % high molecular weight (HMW) at 1.0 % chitosan, Tidal Grow 4 % low molecular weight (LMW) at 1.0 % chitosan, and a water control. Treatment means followed by the same letter are not significantly different ($\alpha=0.05$) as determined by the Tukey HSD Post-hoc test. The lines in the boxplot represent median, while the box represents the Interquartile Range (IQR). The start and end of the lines represent the minimum value in the data ($Q3 - 1.5 \times IQR$), while any outliers are represented as dots.

4.4. Discussion

Results from this research suggest that commercial chitosan products can reduce severity of postharvest diseases compared to a water control. This supports the on-going body of evidence that chitosan is a potential option for managing postharvest diseases of fruit. In this study, the efficacy of chitosan varied by product, molecular weight, concentration of chitosan, application timing, and cultivar. The greatest reduction of lesion size caused by *P. expansum* and *C. fioriniae* across apple cultivars tested was observed on apples treated postharvest with Tidal Grow low MW at 1.0 % chitosan (v/v) when the product's pH was adjusted to ~5.

While apples treated with Tidal Grow low and high MW products had the greatest suppression of symptoms caused by *P. expansum* and *C. fioriniae*, the low MW product was more consistent across cultivars. There are conflicting reports in the literature on the role of MW on chitosan efficacy. Low MW chitosan has been shown to be more effective at reducing fungal growth of *R. stolonifer* while high MW chitosan affected spore shape, sporulation, and germination (Hernández-Lauzardo et al. 2008). However, Younes et al. (2014) found that the influence of MW on the antifungal activity of chitosan was dependent on the type of fungus. Inhibition of *F. oxysporum* growth correlated with increasing MW, while inhibition of *Aspergillus niger* van Tieghem growth was correlated with decreasing MW (Younes et al. 2014). Based on our research, it appears the low MW chitosan does have greater disease suppression on apples for the postharvest diseases of blue mold and bitter rot.

In addition to MW, concentration is another important factor influencing disease control efficacy. In this research, higher chitosan concentrations were more effective at reducing disease symptoms compared to lower concentrations, which has also been reported in the literature (Hernández-Lauzardo et al. 2008; Yu et al. 2012; El-Ghaouth et al. 2000b; Muñoz and Moret

2010; El-Ghaouth et al. 1997; Benhamou and Theriault 1992). However, higher concentrations can cause phytotoxicity and have negative effects on plant growth (Ait Barka et al. 2004; DeGenring et al. 2023). This was observed in PE-1 (objective 1), where both Tidal Grow products and ARMOUR-Zen 15 caused phytotoxicity on wounded apples at chitosan concentrations greater than 1.0 %. Thus, this research focused on a concentration of 1.0 % (v/v) chitosan. Additionally, the phytotoxicity seen at higher chitosan concentrations may be related to the pH of the product. We hypothesize that the low pH of the Tidal Grow treatments contributed to the observed phytotoxicity on wounded apples. Chitosan is not water-soluble and must be dissolved in acid, resulting in solutions with pH ranging from 3-5. Tidal Grow products, when mixed at the various chitosan rates (0.5% to 2.0%) have a pH from 3.5-4.0 which can cause phytotoxicity on plants (DeGenring et al. 2023). Adjusting Tidal Grow to pH ~5 reduced phytotoxicity on wounded apples.

While Tidal Grow low MW reduced blue mold and bitter rot symptom severity, it is important to note that there is a zero-tolerance threshold for some postharvest pathogens. For example, because *P. expansum* can produce the mycotoxin patulin, any evidence of infection will result in a rejection of a batch for processing (Morales et al. 2017). The methods used in this study to evaluate chitosan were designed to create optimal disease conditions (fruit were wounded, inoculated with high inoculum load, and not refrigerated). Under natural conditions, a wounded apple may not encounter such a high inoculum load and fruit are placed in cold storage shortly after harvest. Thus, the next steps should be to determine if Tidal Grow low MW chitosan prevents *P. expansum* under commercial settings and natural inoculation conditions.

In contrast to Tidal Grow, ARMOUR-Zen 15 treatments did not reduce lesions caused by *P. expansum* or *C. fioriniae* on apples when applied postharvest. While ARMOUR-Zen was not

effective in this research, it has shown promise in other studies. Calvo-Garrido et al. (2013) reported that a pre-harvest ARMOUR-Zen application was effective in reducing Botrytis bunch rot on grapes at harvest compared to a water control and fungicide treatments. These researchers adjusted the pH of ARMOUR-Zen to ~7 with sodium bicarbonate which could have influenced the efficacy of the product. Additionally, Calvo-Garrido et al. (2013) applied ARMOUR-Zen at 0.144 % (w/v), which is significantly lower than the 1.0 % applied in this research. Effect of application rate may vary by host and pathogen however, because ARMOUR-Zen applied at 0.5 % on tomato plants resulted in reduced *Alternaria solani* Sorauer foliar disease incidence under both greenhouse and field conditions (Ramkissoon et al. 2016). Feliziani et al. (2013) found that ARMOUR-Zen at 1.0 % was effective at reducing *B. cinerea* on non-inoculated grapes but was not effective under inoculated conditions. The variability reported in the literature and observed in this study indicate that more research is needed to determine what factors influence chitosan efficacy, such as host plant, pathogen, application timing, and disease pressure.

Overall, it is unclear if pre-harvest applications of chitosan provides protection once fruit are harvested and stored under natural inoculum conditions. Unfortunately, low levels of natural rot incidence at harvest made determining differences between treatments difficult. Tidal Grow high MW was used at FREC and was incorporated into an IPM program designed predominately for managing pre-harvest foliar diseases. While bitter rot incidence was less on apples treated with a RR+C mixture, latent rot incidence was only lower on apples treated with GS treatments in 2022. The lack of efficacy in reducing postharvest diseases with a pre-harvest application could also be due to the low chitosan concentrations applied pre-harvest (0.0025 % in 2021 and 0.01 % in 2022). Similar results were observed by Chien & Chou (2006) in which Tankan citrus fruit treated with chitosan products at 0.1 % and 0.2 % had lower postharvest fungal growth but

that treatment with chitosan at 0.05 % was not effective. The rates and treatments in our research were selected to evaluate reduction of pre-harvest foliar disease and were found to be effective (Chapter 3). Thus, while the pre-harvest application at lower rates of chitosan may not be effective at reducing postharvest disease, chitosan may still provide value as it reduces other diseases in the orchard. An additional explanation for the lack of efficacy in reducing postharvest diseases could be due to coverage. For example, pre-harvest application of chitosan to apple trees in the orchard may have covered less tissue area compared to a postharvest application, such as a dip or spray of fruit. Apple trees were sprayed the day before harvest, but applying the treatment with a boom sprayer may not coat the fruit as effectively as a postharvest dip. Research suggests that a chitosan film on the fruit is essential for reducing postharvest losses due to pathogens and weight loss (El-Ghaouth et al. 1994b; Romanazzi and Feliziani 2016; Gutiérrez-Martínez et al. 2018). A pre-harvest treatment of chitosan, followed by a postharvest dip of chitosan, may be more effective to reduce both pre-harvest and postharvest diseases.

In contrast to the natural inoculum experiments, the pre-harvest chitosan treatments reduced symptoms on apple fruit inoculated with *P. expansum* and *C. fioriniae*. For inoculated fruit harvested from FREC, apples treated with the pre-harvest C application had smaller lesion size caused by *P. expansum* and *C. fioriniae* compared to apples treated with the water and GS treatments. These results highlight the efficacy of Tidal Grow high MW at reducing postharvest diseases as in some cases, Tidal Grow high MW was more effective than the GS. Additionally, for inoculated apples from experiments 6 and 7, McIntosh and Wickson apples treated with the pre-harvest GS+C application had smaller lesion size caused by *C. fioriniae* and *P. expansum*, respectively, compared to the GS treated apples. However, the inconsistencies of pre-harvest chitosan application leading to reduced lesion size on inoculated apples may be due to the

reduction in efficacy of chitosan over time. Chitosan has shown to prime the defense mechanisms of the host to reduce disease (Bautista-Baños et al. 2006; Zhang et al. 2011). Additionally, the longevity of the chitosan film around the fruit is still unknown (Romanazzi and Feliziani 2016). While the pre-harvest chitosan products were sprayed on the trees at harvest, the time in-between harvest and fruit inoculations could have reduced the efficacy of the pre-harvest treatment.

Macoun apples treated with the pre-harvest GS+B+C application had lower lesion size for all wounded fruit compared to the GS treated apples in experiment 6. The same results were not seen for McIntosh apples, even though the pre-harvest treatments were the same. This suggests that the pre-harvest chitosan + biopesticide application's ability to reduce lesion size on inoculated fruit may vary based on the cultivar. Additionally, the efficacy of these chitosan + biopesticide applications could be influenced by the other products applied in combination with these treatments. For inoculated Rome fruit from experiment 5, apples treated with the pre-harvest RR+C applications had smaller lesion size of both *P. expansum* and *C. fioriniae* compared to apples treated with the water and GS treatments. However, this reduction in lesion size was the same on apples treated with the C and RR pre-harvest treatments, suggesting that the combined treatments did not result in a synergistic effect. The RR treatment in experiment 5 and the B treatments in experiment 6 were selected to be Serenade ASO due to our experiments also examining the efficacy of these products in reducing pre-harvest foliar diseases (Chapter 3). *Bacillus subtilis* QST 713, the active ingredient in Serenade, has been predominantly researched for management of foliar and soil diseases (Shafi et al. 2017; Nagorska et al. 2007; Kloepper et al. 2004). However, some studies have found that it can be effective as a pre-harvest spray (Gava et al. 2019) or a postharvest treatment when combined with a disinfectant (Kittemann et al. 2010)

to reduce postharvest rots. Poleatewich et al. (2012) found that *Bacillus* spp. applied as a pre- and postharvest application was effective at reducing bitter rot. Future research could include a postharvest application of chitosan and a biopesticide to examine the potential synergistic effects of this combination on managing postharvest diseases.

Storage conditions are important for reducing symptoms caused by postharvest apple diseases and can affect efficacy of disease control products. It is important to note that the inoculated fruit were left at room temperature and high RH once inoculated, creating high disease pressure. Chitosan and the chitosan + biopesticide pre-harvest treatments may be more effective at reducing disease under lower temperatures (between 1 to 3 °C) and humidity (Prange and Wright 2023) typical of many cold storage facilities. Many postharvest treatments made in combination with chitosan have been evaluated, such as essential oils, beneficial microbes, and heat treatments (Romanazzi and Feliziani 2016). The combination of heat treatments and a 1.0 % chitosan treatment was effective at reducing brown rot caused by *Monilinia fructicola* (Wint.) on peaches (Casals et al. 2012) and blue mold and gray mold on inoculated apples (Shao et al. 2012). While a combination of cold storage at 4-6 °C and a chitosan treatment was effective at reducing diseases caused by *Cladosporium* spp. and *Rhizopus* spp. on inoculated strawberry fruit (Park et al. 2006). There was a synergistic effect of chitosan and *C. laurentii* on reducing *P. expansum* infections on apples (Yu et al. 2007, 2012). Future research should focus on the combination of postharvest management strategies. Overall, this research suggests that there is a role for chitosan and a chitosan + biopesticide mixture in reducing postharvest diseases, however research is still needed to determine how to maximize efficacy and consistently reduce disease.

Overall, fruit quality was not influenced by chitosan products. SPI was decreased by only GS treatments in Rome apples from FREC, meaning that only the GS treatments resulted in

lower starch content. However, the SPI measurements were not directly correlated to SSC measurements, as RR+C increased SSC measurement in 2021 while GS only increased SSC measurement in 2022. Li et al. (2015) and Arnon et al. (2014) found no difference in SSC or titratable acidity in apples or citrus fruit treated with chitosan or a water control. Our research did show that ARMOUR-Zen 15 treatments did have some influence on SPI and SSC measurements, but it was dependent on cultivar. Bautista-Baños et al. (2006) review found that the effect of chitosan treatment on total soluble salts, a similar measurement as SSC, varied by the commodity. Our research suggests that it may also vary by cultivar but a study with more cultivars would be necessary to confirm this hypothesis. While chitosan may play a role in enhancing fruit quality, it is not consistent and should be studied further to determine the role of chitosan.

5. Conclusions

The results from this research suggest that commercial chitosan products may enhance postharvest disease management. Future research should focus on optimizing product formulation and application strategies to enhance efficacy. This could include integrating chitosan with other management strategies, such as optimal storage parameters, additional postharvest treatments, such as curing, combined application of chitosan with reduced rates of fungicides, or combined application of chitosan with biopesticides. Chitosan may have a role as a pre-harvest disease management tool but under the parameters of our research, its efficacy was not consistent. Future research should investigate the application of chitosan to other commodities to determine the scale at which chitosan can be used for postharvest disease management. This research provides a foundation for future researchers and a steppingstone

towards providing farmers with additional tools for managing postharvest diseases and for reducing food loss in an economical and sustainable way.

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CHAPTER 5

Conclusion

The overall goal of this research was to investigate if biopesticide-chitosan synergisms exist and if the combination leads to improved biological control of pre- and post-harvest above-ground plant diseases. Two model systems, greenhouse ornamentals and tree fruit, were used to answer these questions in this research. These models were chosen to represent two plant types (herbaceous and woody) and two production industries (greenhouse production and perennial field production).

Results from this research suggest that chitosan can reduce severity of above-ground, fungal plant diseases. Specifically, our results add to the growing body of research that chitosan molecular weight (MW), pH, concentration, and formulation influence chitosan efficacy (Figure 5-1). Additionally, disease control efficacy appears to depend on the pathogen.

	Gaps in knowledge addressed:	Gaps in knowledge that remain:
1. Molecular Weight 	All chitosan molecular weight's reduced disease but low molecular weight appears to be the most effective	Is chitosan low molecular weight the most effective when applied to other model systems?
2. pH 	Chitosan pH needs to be adjusted to ~5 + add a surfactant for improved coverage.	Would adjusting the pH and adding a spreader-sticker to the pre-harvest chitosan sprays change efficacy?
3. Concentration 	For petunia: desired chitosan concentration was ~0.4%; for postharvest: desired chitosan concentration was ~1.0%	How would desired chitosan concentration change with a different model?
4. Formulation 	ARMOUR-Zen 15 was effective at reducing <i>B. cinerea</i> on petunia while Tidal Grow was the most effective at reducing postharvest rots.	Is variation in chitosan formulation efficacy due to the product OR the model system?

Figure 5-1. Summary of the gaps in knowledge related to chitosan as a crop protection tool addressed by this research and the gaps in knowledge that remain.

1. Effect of molecular weight on efficacy. While medium and high MW reduced disease, low MW chitosan was the most effective. MW is the sum of the atomic weights of atoms in a molecule and is an important factor that influences properties of a polymer. Essentially, MW is an indication of the length of individual polymer chains. Chitosan MW is influenced by the length of the deacetylation process used to convert chitin to chitosan. The shorter the deacetylation process, the more hydroxyl groups and potentially amino acids are present, resulting in higher MW (Kaur and Dhillon 2014). MW is intertwined with degrees of deacetylation (DD), molecular charge, and viscosity (Raafat and Sahl 2009). A high molecular weight chitosan product will also have lower DD, lower molecular charge, and is more viscous; while a low MW chitosan product will have a high DD, high molecular charge, and is less viscous (Raafat and Sahl 2009). Thus, it is important to note that while we investigated effects of MW, the confounding factors of DD, charge, and viscosity may have also affected our results. Furthermore, little is known about how chitosan MW is related to its mode of action in crop protection. Hernandez-Lauzardo et al. (2008) found that low MW chitosan inhibited *Rhizoctonia solinifer* mycelial growth while high MW chitosan affected spore shape, sporulation, and germination. Other studies have found the low MW chitosan has high antimicrobial and anti-viral properties, but this is predominantly linked to the higher molecular charge that binds to the negatively charged bacterial surface (Ma et al. 2017; Younes et al. 2014; Gutiérrez-Martínez et al. 2018). My research found that regardless of MW, chitosan was able to reduce fungal disease, suggesting that other variables, such as chitosan concentration and formulation, are more important for fungal disease suppression than MW.

2. Effect of chitosan solution pH on efficacy. I found that the pH of the chitosan solution needs to be adjusted to ~5 to reduce phytotoxicity on plant parts. However, it is interesting to

note that the Tidal Grow treatments not adjusted for pH (~3.5) suppressed growth of *Botrytis cinerea* in vitro greater than the Tidal Grow treatments with the adjusted pH (~5). Some researchers have found that the antimicrobial activity of chitosan is linked to the solution pH and as pH increases, antimicrobial activity of chitosan decreases (Kong). This may be why the lower pH treatments had greater suppression of fungal growth. However, regardless of antimicrobial activity, any product used for disease management cannot cause phytotoxicity — which can be caused by low solution pH. Phytotoxicity can result in senescence of plant tissue, leading to weaker tissue that can be colonized by pathogens (as seen in our research). Thus, adjusting the chitosan solution pH to at least 5 provides a pH that is low enough to prevent chitosan from precipitating out of solution but maintains antimicrobial properties and reduces risk of phytotoxicity. In the FREC pre-harvest sprays, the pH of the Tidal Grow solution was not adjusted. Adjustment of pH to reduced phytotoxicity may not be as big of a concern in apple as it was on petunia because apples have waxy leaves that may not be as susceptible to phytotoxicity as the non-waxy petunia leaves. However, the pre-harvest applications of Tidal Grow to apple trees were applied at very low concentrations so it is unclear if the pH of Tidal grow at higher concentrations would cause phytotoxicity on apple leaves. Based on my research, I think it is critical for commercial producers of chitosan to formulate products with pH in mind, specifically to prevent phytotoxicity so that the products could be applied across many cropping systems.

In addition to pH, the mixing of a surfactant when preparing chitosan products likely resulted in better coverage and decreased phytotoxicity in the petunia experiments. The lower efficacy observed in the apple orchard experiments compared to the ornamental greenhouse experiment may be related to the use of a surfactant in the greenhouse but not in the FREC and NH on-farm trials. Surfactants or spreader stickers are important to include in spray programs as

they help products spread and stick to the leaves for longer periods (Yates 2015). This is especially important in apple orchards when spraying for apple scab based on NEWA modeling because farmers are often spraying before a rain event. The spreader-sticker prevents the product from being washed away during a rain event. It would be important for future research to examine the use of chitosan + a spreader-sticker / surfactant for management of apple foliar diseases when applied pre-harvest. In conclusion, I suspect that the lack of a spreader sticker may have played a role in the lack of disease suppression provided by the pre-harvest chitosan sprays, but chitosan concentration may also have been too low to be effective.

3. Effect of chitosan concentration on efficacy. Higher chitosan concentrations often correlate to greater reduction in disease as more chitosan molecules are present to activate plant defense responses or to interact with the pathogen. However, it can be difficult to maintain a high concentration of chitosan due to its insolubility in water. With reagent grade chitosan products, the pH must be maintained below ~5.5 or chitosan will begin to precipitate out of the solution. ARMOUR-Zen 15 must have a unique formulation for the product to have a pH of ~5.0 and a chitosan concentration of 15%. This suggests that there are new innovations being used by manufacturers in the formulation of chitosan products to achieve higher chitosan concentrations at a pH above 5. In my experience, even with the pH adjustment to ~5, chitosan concentration of 0.4% was the highest concentration that could be applied to petunia leaves before phytotoxicity was observed. When treating apple fruit, the chitosan concentration of 1.0% was the desired concentration for disease suppression without causing phytotoxicity. My research highlights that apple fruit, with waxy skin, can tolerate higher concentrations of chitosan compared to the non-waxy petunia leaves. More research is needed to make specific recommendations for chitosan

concentration in other crops, considering the plant part being sprayed and the plant pathogen being targeted.

Future work should also include a cost analysis of chitosan. The higher the concentration of chitosan needed to suppress disease – the higher the cost. Higher concentrations of chitosan may be more economical in a greenhouse or postharvest setting where product is applied to a relatively small area, but in field settings, whole tree or whole field applications require a lot of product over a larger area of land. Thus, an economic assessment of chitosan is critical to identify the most effective and economically feasible chitosan concentration (and use patterns) for disease management. Additionally, chitosan cost varies by product and our research suggests that the chitosan product is critical for disease suppression. Thus, including a cost-benefit analysis for each chitosan product may be critical for growers to make decisions based on chitosan cost and level of disease control.

4. Effect of chitosan formulation on efficacy. I observed different results for the two chitosan products tested in this research. The commercial product, ARMOUR-Zen 15, reduced lesion size of *Botrytis cinerea* on petunia leaves but was not effective at reducing lesion size of *Colletotrichum fioriniae* and *Penicillium expansum* on apples. Conversely, Tidal Grow products reduced severity of postharvest rots on apples but not gray mold on petunia. These differences may be explained by chitosan source, formulation, and manufacturing conditions – as every batch of chitosan can have slight variations due to the deacetylation process (Kaur and Dhillon 2014). Unfortunately, due to the proprietary nature of these variables, I was not able to compare formulation of the two products. Thus, it is unclear to what extent these variations in chitosan production and formulation can explain the differences in product efficacy. Additionally, the differences in product efficacy may be explained by the differences in the two model systems

(petunia and apple) including the cropping system and the fungal pathogen. Future research should evaluate these commercial products under other model systems to determine best practice for each product.

Tidal Grow products reduced *B. cinerea* growth in-vitro but were not effective at reducing symptoms in-vivo. Conversely, ARMOUR-Zen was less effective at reducing *B. cinerea* in-vitro but was effective at reducing symptoms in-vivo. Based on this, I hypothesized that Tidal Grow products reduce disease through direct antagonistic interactions with the pathogen. To my knowledge, Tidal Grow has not been tested in other research and thus there is more work that needs to be done to evaluate this hypothesis. Interestingly, Tidal Grow's high MW formulation is similar in consistency and appearance to that of the reagent grade compounds I tested. The Tidal Grow low MW is slightly different from Tidal Grow high MW, and I suspect that it contains inert ingredients to achieve a chitosan concentration of 4% while maintaining solubility.

ARMOUR-Zen 15 was most effective at reducing *B. cinerea* when applied to the whole petunia plant compared to *in vitro*. Based on this observation, I hypothesize that ARMOUR-Zen 15's mode of action may be to activate the plant's defense response. There is variability in the efficacy of ARMOUR-Zen 15 product reported in the literature (Calvo-Garrido et al. 2013; Feliziani et al. 2013; Ramkissoon et al. 2016) as discussed in Chapter 4. To my knowledge previous research has not examined the mode of action of ARMOUR-Zen 15 to explain disease control. Additionally, there are inert ingredients in the ARMOUR-Zen 15 formulation that could play a role in disease reduction. ARMOUR-Zen 15 is not viscous and is a dark brown color and has a 15% chitosan concentration with a pH of 5, which I found impossible to do when working with reagent grade compounds. Thus, there are many unknown variables that need to be

considered when speculating about the mode of action employed by ARMOUR-Zen 15 for disease control.

Production practices can influence MW, DD, charge, and solubility which all have a direct influence on the biological properties of chitosan and its ability to suppress disease (Orzali et al. 2017; Kaur and Dhillon 2014). Thus, it is unclear what components of these commercial products influence disease suppression as we do not have the whole picture when the formulations are proprietary. Additionally, there are a broad range of commercially available chitosan products (Romanazzi et al. 2018) that are not registered in the United States that should be included in future research.

This research is the first to examine commercial chitosan products available to growers in the United States for their efficacy under commercial conditions. While our research suggests that chitosan products are effective tools at managing fungal diseases, these results are steppingstones for future researchers and there is still much to learn about chitosan and its role in disease management.

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APPENDIX



Figure A-1. Petunia plants four days post chitosan application in preliminary growth room trials. Phytotoxicity was observed at higher concentration of low molecular weight reagent grade chitosan (v/v) and on the acetic acid control. Chlorotic spots developed along spray patterns or along leaf margins, suggesting that the chitosan treatment was not spreading evenly over the leaf due to its high viscosity. The nonionic surfactant CapSil (4oz/100gal) was added to chitosan solutions for in planta experiments to improve coverage and reduce phytotoxicity.

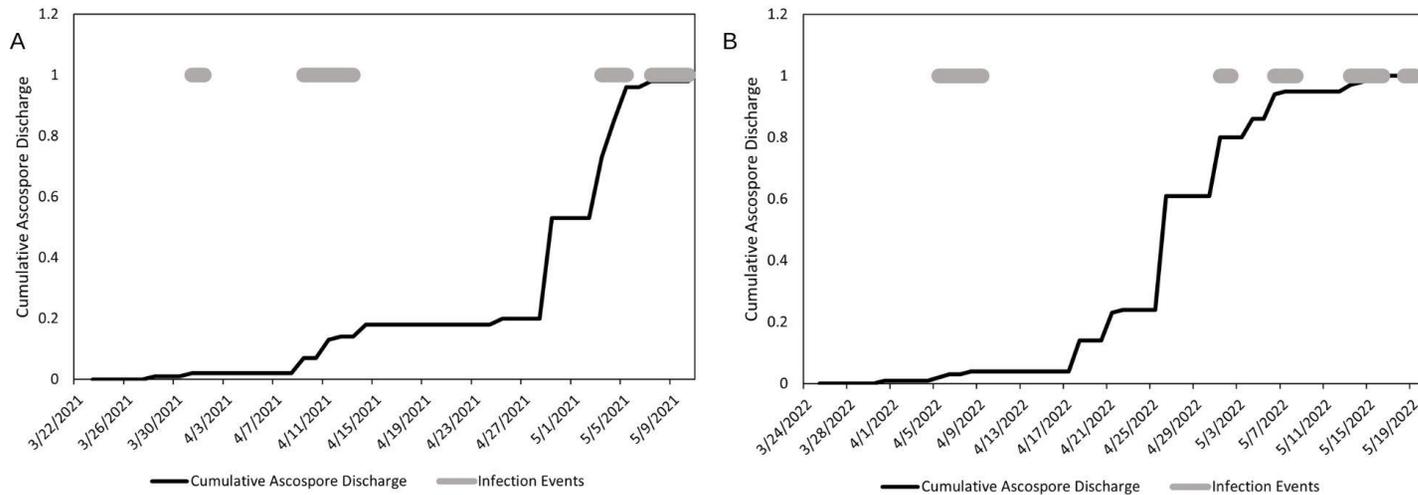


Figure A-2. Cumulative *Venturia inaequalis* ascospore discharge and primary scab infection events (of more than one day) data from Network for Environment and Weather Application’s apple scab models based on data collected from the weather station located at Penn State University Fruit Research and Extension Center: (A) experiment 1 and (B) experiment 2.

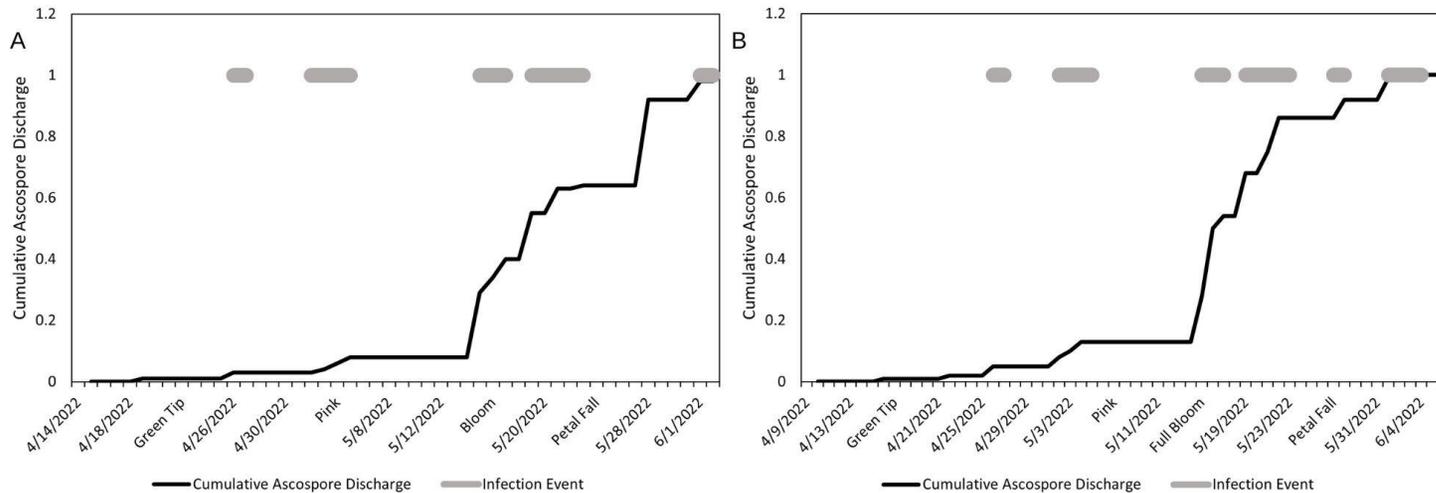


Figure A-3. Cumulative *Venturia inaequalis* ascospore discharge and primary scab infection events (of more than one day) data from Network for Environment and Weather Application’s apple scab models based on data collected from the weather station located at New Hampshire sites: (A) experiment 3 and (B) experiment 4.

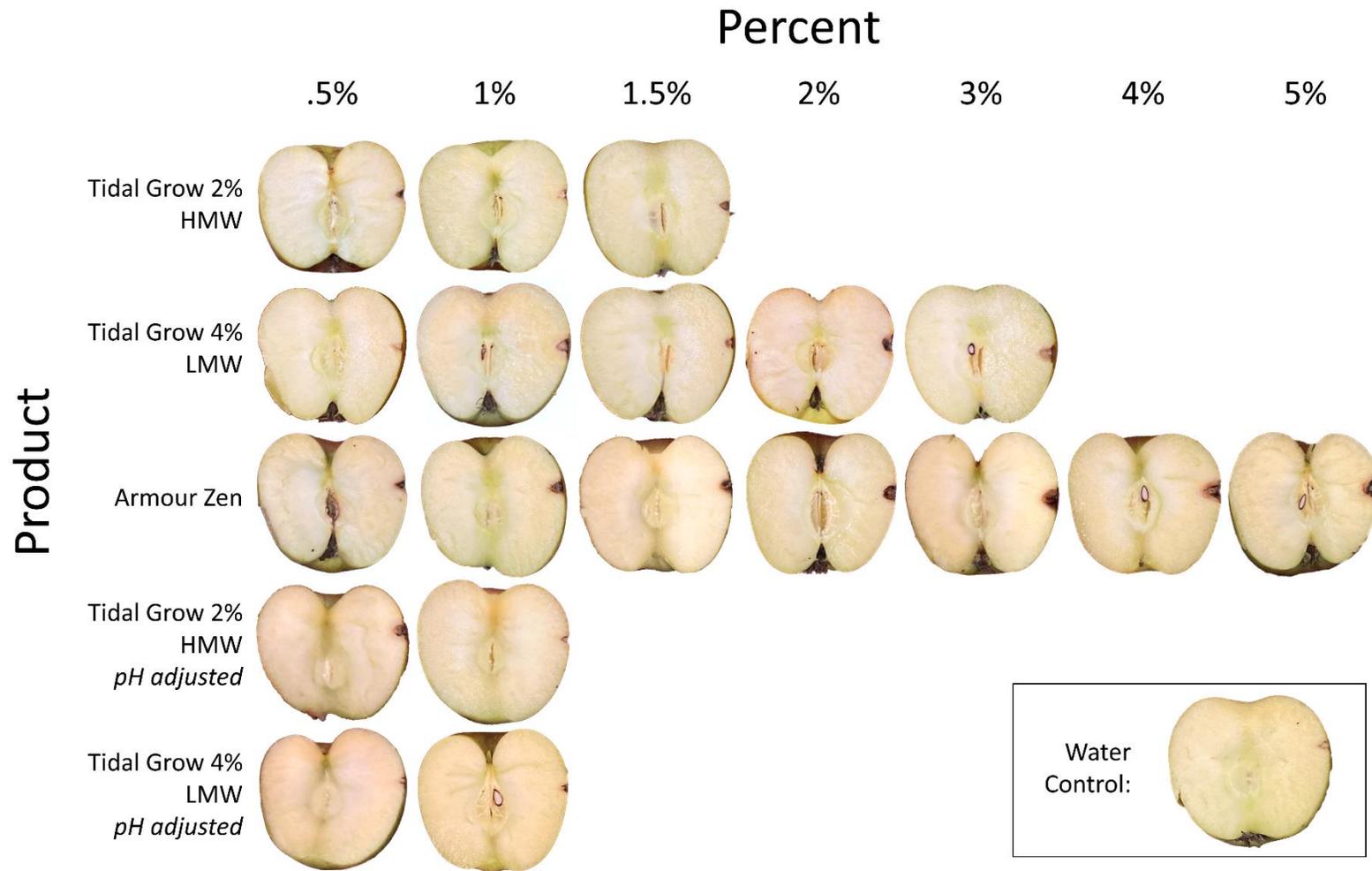


Figure A-4. ‘Golden Delicious’ apples wounded on the right-side diameter and treated with a commercial chitosan product compared to a water treated apple. The darkness of the wound correlated to the severity of phytotoxicity caused by the chitosan treatment on the exterior of the apple.

Penicillium 14DPI

Post-Harvest Treatment

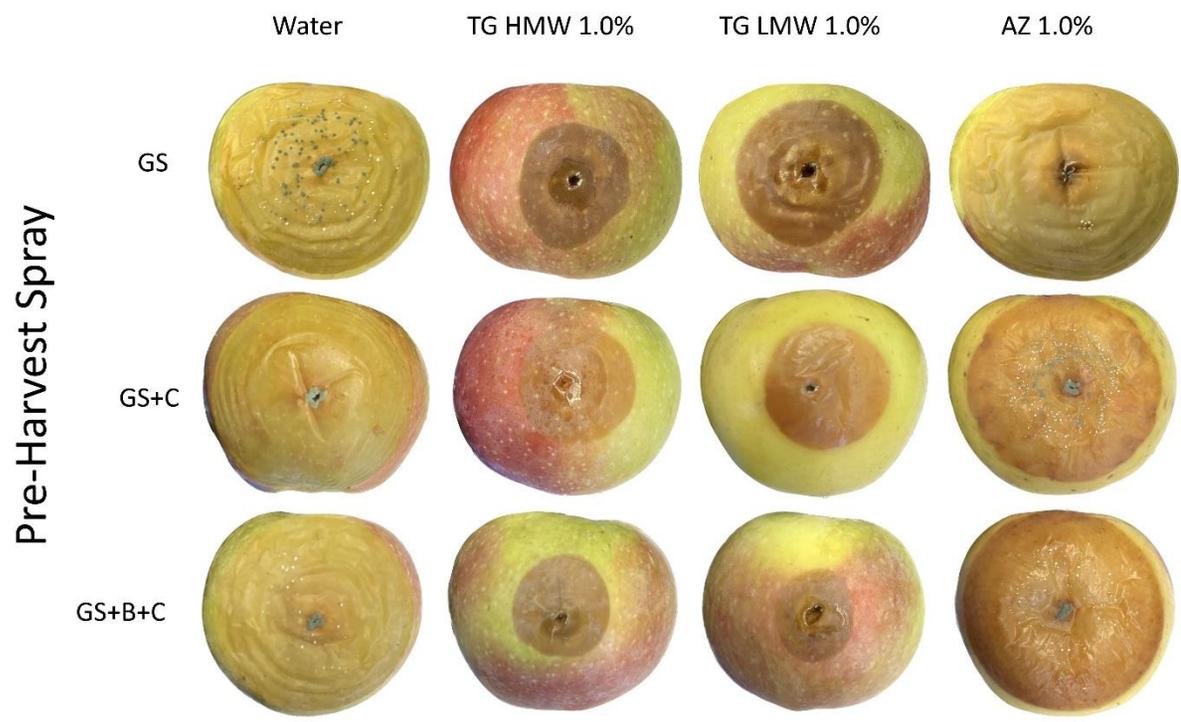


Figure A-5. Representative Macoun apples from pre- and postharvest treatments 14 days post infestation (dpi) with *Penicillium expansum* in experiment 6. Pre-harvest sprays included grower standard (GS), grower standard + chitosan (GS+C), and grower standard + biopesticide + chitosan (GS+B+C). Postharvest treatments include water control, Tidal Grow high molecular weight (TG HMW) at 1.0 %, Tidal Grow low molecular weight (TG LMW) at 1.0 %, and ARMOUR-Zen 15 (AZ) at 1.0 %.

Colletotrichum 14DPI

Post-Harvest Treatment

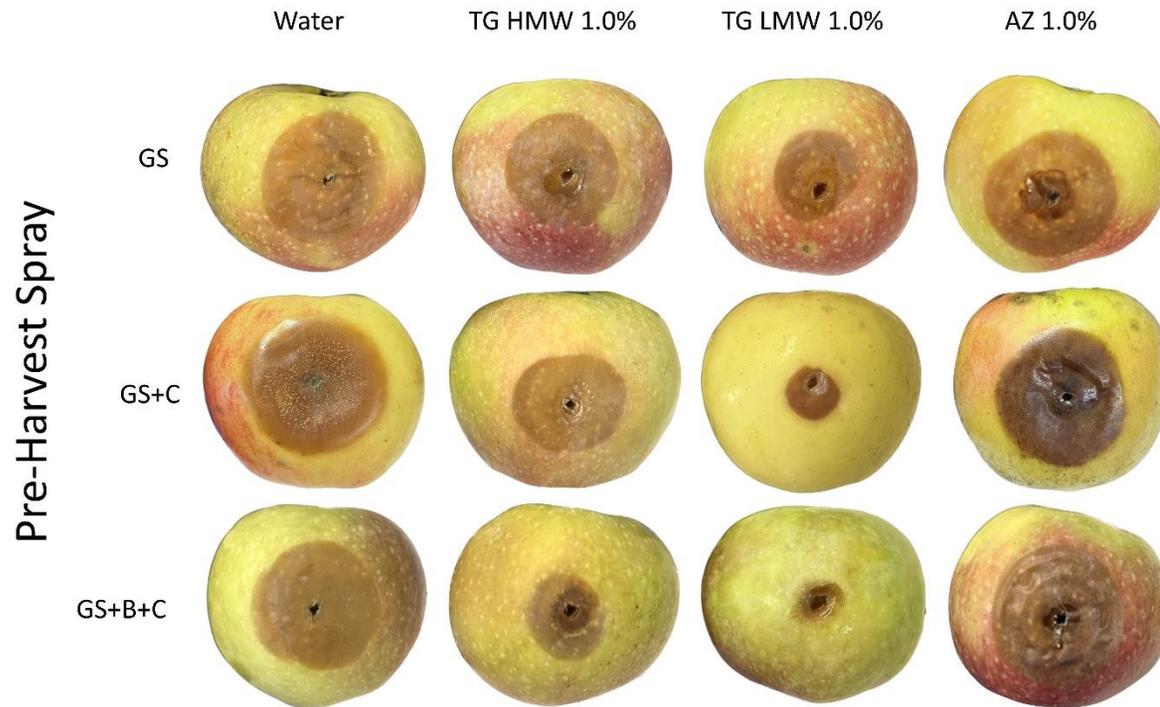


Figure A-6. Representative Macoun apples from pre- and postharvest treatments 14 days post infestation (dpi) with *Colletotrichum fioriniae* in experiment 6. Pre-harvest sprays included grower standard (GS), grower standard + chitosan (GS+C), and grower standard + biopesticide + chitosan (GS+B+C). Postharvest treatments include water control, Tidal Grow high molecular weight (TG HMW) at 1.0 %, Tidal Grow low molecular weight (TG LMW) at 1.0 %, and ARMOUR-Zen 15 (AZ) at 1.0 %.

Penicillium 14DPI

Post-Harvest Treatment

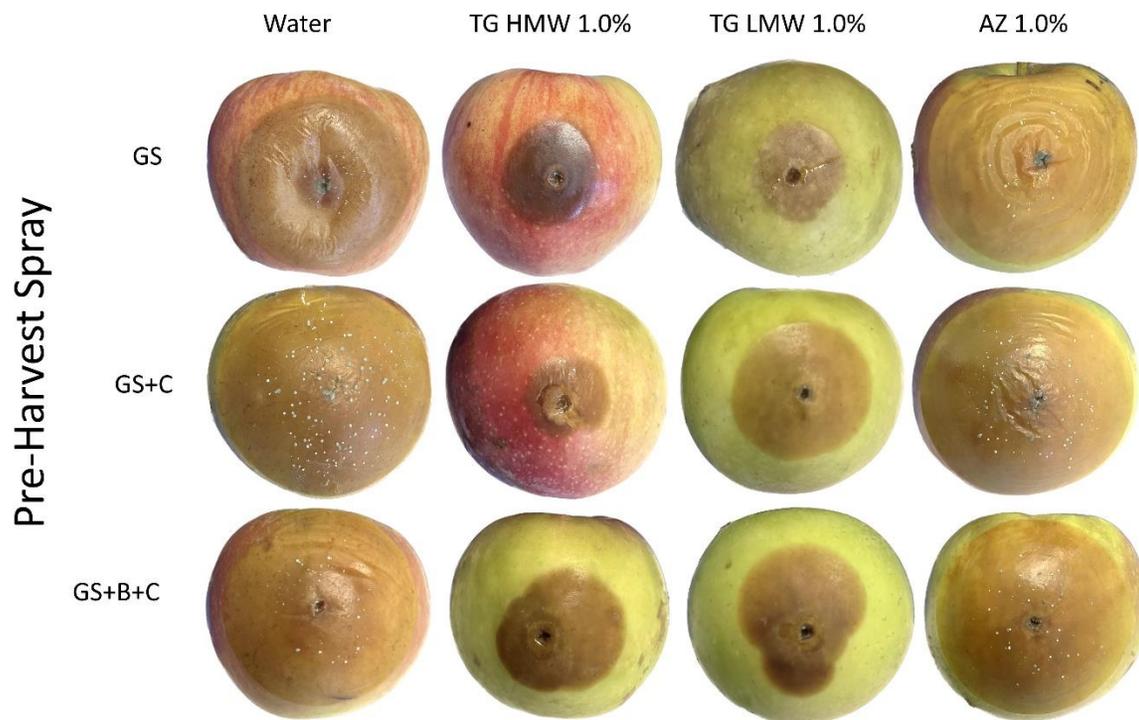


Figure A-7. Representative McIntosh apples from pre- and postharvest treatments 14 days post infestation (dpi) with *Penicillium expansum* in experiment 6. Pre-harvest sprays included grower standard (GS), grower standard + chitosan (GS+C), and grower standard + biopesticide + chitosan (GS+B+C). Postharvest treatments include water control, Tidal Grow high molecular weight (TG HMW) at 1.0 %, Tidal Grow low molecular weight (TG LMW) at 1.0 %, and ARMOUR-Zen 15 (AZ) at 1.0 %.

Colletotrichum 14DPI

Post-Harvest Treatment

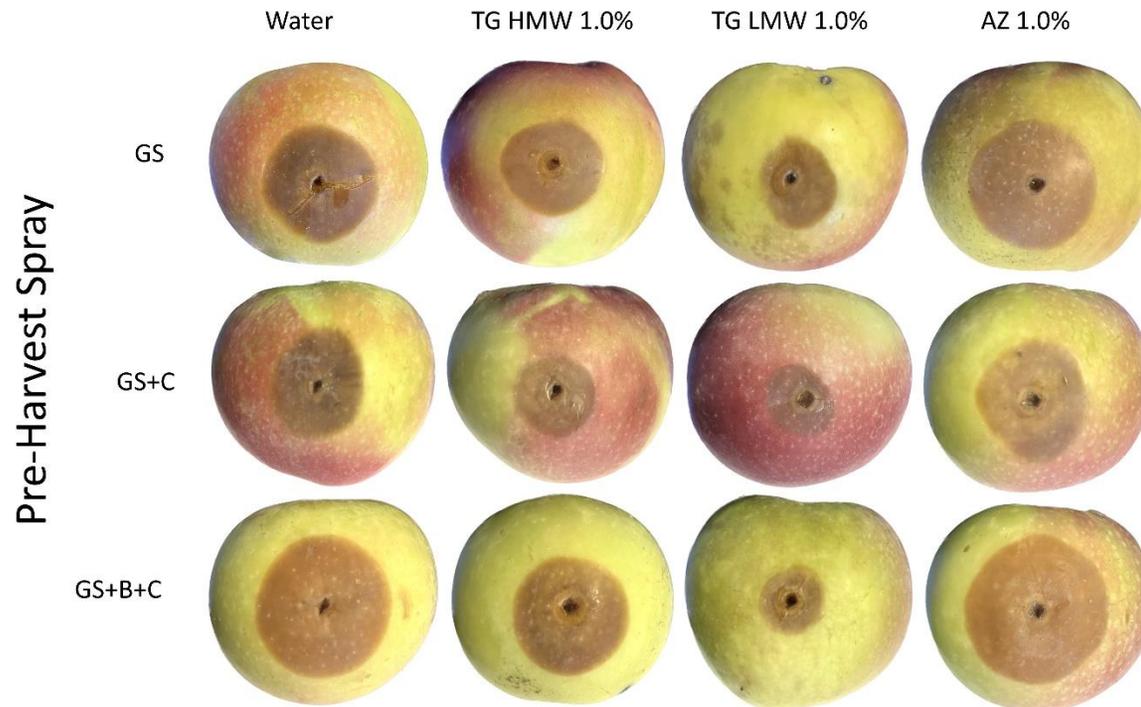


Figure A-8. Representative McIntosh apples from pre- and postharvest treatments 14 days post infestation (dpi) with *Colletotrichum fioriniae* in experiment 6. Pre-harvest sprays included grower standard (GS), grower standard + chitosan (GS+C), and grower standard + biopesticide + chitosan (GS+B+C). Postharvest treatments include water control, Tidal Grow high molecular weight (TG HMW) at 1.0 %, Tidal Grow low molecular weight (TG LMW) at 1.0 %, and ARMOUR-Zen 15 (AZ) at 1.0 %.

Table A-1. Apple scab infection events and rainfall collected from Network for Environment and Weather Application’s apple scab models based on data collected from the weather station located at each site (NEWA 2023; <https://newa.cornell.edu/>).

	Primary Scab Infection Events	Secondary Scab Infection Events	Rain (in) (April – Oct)
Experiment 1: FREC 2021	5	25	25.22
Experiment 2: FREC 2022	8	21	24.62
Experiment 3: NH On-Farm #1	6	13	15.91
Experiment 4: NH On-Farm #2	7	17	21.34

Table A-2. Mean disease incidence \pm standard error on ‘Rome’ leaves from the 2021 Penn State University Fruit Research and Extension Center research trial (Experiment 1).^{1,2}

	Water Control	GS	C	RR	RR+C
Powdery Mildew Incidence (%)	31.8 \pm 1.8	17.4 \pm 1.5	25.7 \pm 1.8	21.9 \pm 1.9	22.7 \pm 2.1
Rust Incidence (%)	0.3 \pm 0.1	0.2 \pm 0.2	1.3 \pm 0.4	1.0 \pm 0.3	1.3 \pm 0.4

¹ Treatments evaluated were water control, grower standard (GS), chitosan (C), reduced risk (RR), and a reduced risk and chitosan mixture (RR+C).

² Within a disease measurement, treatment means followed by different letters are significantly different ($\alpha=0.05$) as determined by the Tukey HSD Post-hoc test.

Table A-3. Mean disease incidence or russet severity (score 0-6) \pm standard error on ‘Dabinett’ and ‘Wickson’ harvested apples from the 2022 New Hampshire on-farm site #2 (Experiment 4).^{1, 2}

		Dabinett			Wickson		
		GS	GS+C	GS+B+C	GS	GS+C	GS+B+C
Harvested Fruit	Powdery Mildew Incidence (%)	18.7 \pm 8.1	8.0 \pm 3.7	24.0 \pm 6.7	85.3 \pm 6.7	73.0 \pm 10.1	76.0 \pm 2.3
	Russet Score (0-6)	0.2 \pm 0.1	0.2 \pm 0.05	0.3 \pm 0.1	1.6 \pm 0.1	1.4 \pm 0.1	1.3 \pm 0.1
	Flyspeck Incidence (%)	5.3 \pm 2.7	2.0 \pm 2.0	6.0 \pm 3.5	2.7 \pm 1.3	1.0 \pm 1.0	0.0 \pm 0.0

¹ Treatments evaluated were grower standard control (GS), grower standard + chitosan (GS+C), and grower standard + biopesticide + chitosan combination (GS+B+C).

² Within a disease measurement and within a cultivar (Dabinett, Wickson), treatment means followed by different letters are significantly different ($\alpha=0.05$) as determined by the Tukey HSD Post-hoc test.

Table A-4. Effect of commercial chitosan products on area under the disease progress curve (AUDPC) \pm standard error on phytotoxicity seen on Golden Delicious fruit in preliminary experiment 1. Treatment means followed by an asterisk (*) differ statistically from the water control according to Dunnett's test at $\alpha=0.05$.

Treatment	Chitosan rate (%)	Solution pH ^x	AUDPC	Standard Error
Water Control	0	5.2	0.0	0.0
Tidal Grow HMW ^z	0.5	3.6	10.9	4.1
	1.0	3.6	33.3 *	3.8
	1.5	3.5	44.9 *	11.0
	0.5	5.1	8.4	1.9
	1.0	4.8	10.8	8.7
	Tidal Grow LMW	0.5	4.1	0.0
1.0		4.0	22.2	3.1
1.5		4.0	33.0 *	2.4
2		4.0	47.3 *	3.3
3		3.9	48.2 *	1.3
0.5		5.2	1.2	1.2
ARMOUR-Zen 15	1.0	5.0	10.6	2.0
	0.5	5.0	26.6	13.6
	1.0	5.0	19.1	6.8
	1.5	5.0	27.6 *	13.3
	2	5.0	31.8 *	7.2
	3	5.0	24.7	0.9

^x Additional treatments for Tidal Grow products were added in which the pH was adjusted to ~5.0 using 1 M NaOH

^z Two Tidal Grow products were tested, high molecular weight (HMW) and low molecular weight (LMW)

Table A-5. Effect of commercial chitosan products on area under the disease progress curve (AUDPC) \pm standard error of water inoculated McIntosh fruit for preliminary experiments 2 and 3. Means were not significantly different ($\alpha=0.05$) as determined by the Tukey HSD Post-hoc test.

Treatment ^z	AUDPC	
	Experiment 1	Experiment 2
Water Control	34.4 \pm 12.9	6.5 \pm 3.6
Tidal Grow HMW 0.5 %	20.4 \pm 2.4	21.9 \pm 1.4
Tidal Grow HMW 1.0 %	34.8 \pm 10.3	48.5 \pm 4.1
Tidal Grow LMW 0.5 %	18.6 \pm 4.5	20.6 \pm 4.7
Tidal Grow LMW 1.0 %	18.3 \pm 4.3	34.5 \pm 3.2
ARMOUR-Zen 0.5 %	15.2 \pm 1.7	23.2 \pm 2.7

^z Two Tidal Grow products were tested, high molecular weight (HMW) and low molecular weight (LMW).

Table A-6. Effect of pre-harvest treatments on area under the disease progress curve (AUDPC) \pm standard error of *Penicillium expansum*, *Colletotrichum fioriniae*, and water control on Rome fruit in experiment 5.^{1,2}

Infestation	Pre-harvest Treatment				
	Water Control	GS	C	RR	RR+C
<i>Penicillium expansum</i>	146.8 \pm 14.5 (ab)	188.7 \pm 8.3 (a)	134.5 \pm 18.1 (b)	126.1 \pm 16.0 (b)	121.1 \pm 18.8 (b)
<i>Colletotrichum fioriniae</i>	47.9 \pm 3.9	52.02 \pm 4.7	43.3 \pm 3.8	44.78 \pm 5.2	44.8 \pm 8.6
Water control	33.3 \pm 11.2	36.8 \pm 8.3	23.4 \pm 4.6	30.7 \pm 8.6	23.1 \pm 2.9

¹ Treatments evaluated were water control, grower standard (GS), chitosan (C), reduced risk (RR), and a reduced risk and chitosan mixture (RR+C).

² Within a disease measurement, treatment means followed by different letters are significantly different ($\alpha=0.05$) as determined by the Tukey HSD Post-hoc test.

Table A-7. Effect of pre-harvest treatments on area under the disease progress curve (AUDPC) \pm standard error of *Penicillium expansum*, *Colletotrichum fioriniae*, and water control on Macoun, McIntosh, and Wickson fruit in experiments 6 and 7.^{1,2}

Cultivar	Infestation	Pre-harvest Treatment		
		GS	GS+C	GS+B+C
Exp. 3	<i>Penicillium expansum</i>	236.9 \pm 22.2 (a)	217.9 \pm 18.8 (ab)	192.6 \pm 21.3 (b)
	<i>Colletotrichum fioriniae</i>	118.8 \pm 9.0 (ab)	133.6 \pm 8.9 (a)	106.8 \pm 10.2 (b)
	Water Control	125.7 \pm 13.1 (a)	111.2 \pm 13.1 (ab)	93.4 \pm 7.8 (b)
Exp. 3	<i>Penicillium expansum</i>	156.9 \pm 14.3	165.3 \pm 17.5	161.4 \pm 11.7
	<i>Colletotrichum fioriniae</i>	100.9 \pm 6.9 (ab)	89.5 \pm 6.9 (b)	111.8 \pm 6.8 (a)
	Water Control	116.8 \pm 7.6	97.5 \pm 10.1	109.8 \pm 7.7
Exp. 4	<i>Penicillium expansum</i>	196.4 \pm 11.7 (ab)	211.0 \pm 12.1 (b)	173.5 \pm 13.2 (a)
	<i>Colletotrichum fioriniae</i>	101.3 \pm 7.2	113.3 \pm 7.4	123.5 \pm 10.5
	Water Control	88.7 \pm 8.2 (b)	92.6 \pm 8.4 (b)	137.6 \pm 12.9 (a)

¹ Treatments evaluated were grower standard (GS), grower standard + chitosan (GS+C), grower standard + biopesticide + chitosan (GS+B+C).

² Within a disease measurement, treatment means followed by different letters are significantly different ($\alpha=0.05$) as determined by the Tukey HSD Post-hoc test.

Table A-8. Effect of postharvest chitosan application on area under the disease progress curve (AUDPC) \pm standard error of water (control) inoculated Macoun, McIntosh, and Wickson fruit in experiments 6 and 7. Within each cultivar, treatment means followed by different letters are significantly different ($\alpha=0.05$) as determined by the Tukey HSD Post-hoc test.

AUDPC			
Treatment ^z	Macoun	McIntosh	Wickson
Water Control	113.7 \pm 11.7 (ab)	105.8 \pm 11.5 (ab)	76.0 \pm 15.2 (c)
Tidal Grow HMW 1.0 %	102.9 \pm 7.6 (b)	113.2 \pm 10.5 (ab)	119.2 \pm 8.9 (ab)
Tidal Grow LMW 1.0 %	75.9 \pm 10.9 (b)	88.0 \pm 7.0 (b)	94.9 \pm 8.5 (bc)
ARMOUR-Zen 1.0 %	147.8 \pm 11.4 (a)	124.9 \pm 8.7 (a)	135.1 \pm 12.8 (a)

^z Two Tidal Grow products were tested, high molecular weight (HMW) and low molecular weight

