Use of Biorational Products for Botrytis Management in Floriculture Crops

Josselyn Calidonio
jcalido@clemson.edu

Follow this and additional works at: https://tigerprints.clemson.edu/all_theses

Part of the Plant Pathology Commons

Recommended Citation
Calidonio, Josselyn, "Use of Biorational Products for Botrytis Management in Floriculture Crops" (2023). All Theses. 4139.
https://tigerprints.clemson.edu/all_theses/4139

This Thesis is brought to you for free and open access by the Theses at TigerPrints. It has been accepted for inclusion in All Theses by an authorized administrator of TigerPrints. For more information, please contact kokeefe@clemson.edu.
EVALUATION OF DIFFERENT BIORATIONAL PRODUCTS FOR BOTRYTIS MANAGEMENT IN FLORICULTURE CROPS

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Plant and Environmental Sciences

by
Josselyn Gabriela Calidonio Cabrera
August 2023

Accepted by:
Dr. James E. Faust, Committee Chair
Dr. Guido Schnabel
Dr. Hehe Wang
Botrytis cinerea is an important pathogen that has a significant economic impact on the floriculture industry from propagation to the postharvest environment. Chemical fungicide applications have been one of the main approaches that growers use for botrytis blight management; however, studies have shown that the indiscriminative use of these chemicals leads to fungicide resistance (Brent and Hollomon, 1998; Fillinger and Elad 2016). This thesis explores the potential use of biorational products for botrytis blight management. Biorationals are defined as compounds that have low or no direct mammalian toxicity and few effects on the environment (Paulitz and Belanger 2001). A review of the literature is provided in Chapter 1, which focuses on the importance of Botrytis cinerea in the floriculture industry, common cultural and chemical practices employed by growers, fungicide resistance, and the use of biorational products employed against fungal diseases. Chapter 2 evaluates biorational products which offers an alternative approach to manage botrytis blight in cut rose flowers and petunia flowers including biological control agents, botanicals, plant nutrients, microorganism-derived compounds, and system-acquired resistance inducers. Chapter 3 evaluates combinations of the most effective biorational products observed in Chapter 2.
DEDICATION

To God, for giving me this opportunity, to help me walk through this journey, for making me capable of reaching my goals, for giving me the strength and faith to keep moving, and for all the angels that he put around me to show me his love and that he is always with me.

To all my family, which is my great support, my mom and my dad who always taught me to never give up, to follow my dreams, and to always give the best of me, to my grandmother who taught me not to be scared and to keep doing what I love.

To people who create opportunities for others, because through this, they help us to reach our dreams and help us to prepare for the next stage of our life.

To all the people that have a dream and work very hard to accomplish them, because they are an example of perseverance and inspiration.
ACKNOWLEDGEMENTS

To Dr. James Faust, for giving me this opportunity, and for all the trust you give me. Thank you for all your patience and for all that you taught me since day one, to help me grow professionally. Thank you so much for believing and for making me part of your lab group. You are a great advisor, who led us to become the best version of ourselves.

To Dr. Guido Schnabel, for all your guidance, thank you so much for allowing me to be part of your lab. For all your comments and words of motivation. Thank you so much for your guidance.

To Dr. Hehe Wang, thanks for being part of my committee and for your help in this journey.

To Dr. William Bridges, for always being there every time I have a question, thank you so much for your patience and for sharing your knowledge.

To the American Floral Endowment, who funded my project, thank you so much for creating this opportunity, I am very grateful, thanks to this amazing project I have learned a lot personally and professionally.

To Melissa Muñoz, for all the technical things you taught me through this journey, thank you so much for all your patience and for always being there, I learned a lot from you. Thank you also for your friendship, I am very grateful to meet you here.
To Julia Gelain, thank you so much for your help in the lab, and for your patience and kindness every time I needed help.

To the Faust’s and Schnabel’s lab graduate students, thank you so much for always being there, for all your support, and for the help you give me to improve in the grad student life.

To all the undergraduate students that help me with my experiments, thank you for taking care of my plants and for all your effort in this project. I really value that.

To Taylor Martin, for all your help around the greenhouses, and for being there always. Thank you so much for your kindness.

To all the growers in Colombia that help with my project. Thank you so much for being there and your collaboration.

To all the labor people that work in greenhouses around the world. Thank you for your help in the process of growing the flowers needed for this project. I really appreciate and admire all the effort that you put into your job.

To my uncle Jose Cabrera and my aunt Thelma Calidonio, for all the support and for always keeping in touch with me while I was on this journey.

To Emily, Dr. Faust, and his family, who always open the doors of their home for every student, making us feel at home far from our country home. Thank you so much for all those times, I really enjoyed it.
To Fabian Rodriguez, my favorite person, thank you for all the things you thought me. Thank you so much for lifting me up every time that I was down. Thank you so much for all your patience and for reminding me that everything will be fine, you are a great and kind person, I admire you so much and I can’t be more grateful to have you in my life. Your help was very important to accomplish this project, making it lighter.

To Amalia Diaz, thank you so much for your help in my experiments. Thank you so much for all the coffees and late-night talks at home, and thanks for becoming my unconditional friend here in Clemson.

To Daniela Negrete, thanks for your friendship. Thank you so much for your support and motivation.

To Melissa Muñoz and David, thank you so much for all the help that you gave me through this journey, to opening the door of your home for me, you became my family here in Clemson. Thank you so much for all your kindness and love.

To all the people and friends that I didn’t mention and who have contributed in some way to this project, thank you so much for being around, and for your support.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>x</td>
</tr>
</tbody>
</table>

**CHAPTER**

I. LITERATURE REVIEW .............................................................................. 1

- *Botrytis cinerea* .............................................................................. 1
- Botrytis management in floriculture ............................................ 2
- Fungicide resistance development ............................................... 3
- Use of biorationals for *B. cinerea* management ............................. 4
- References .......................................................................................... 10

II. EVALUATION OF BIORATIONAL PRODUCTS FOR BOTRYTIS BLIGHT MANAGEMENT ........................................ 23

- Abstract ............................................................................................. 23
- Introduction ....................................................................................... 24
- Materials and Methods ...................................................................... 27
- Results and Discussion .................................................................... 32
- References ......................................................................................... 44
Table of contents (Continued)

III. EVALUATION OF THE COMBINATION OF BIORATIONAL PRODUCTS ON BOTRYTIS BLIGHT IN PETUNIA FLOWERS AND CUT ROSE FLOWERS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>52</td>
</tr>
<tr>
<td>Introduction</td>
<td>53</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>55</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>58</td>
</tr>
<tr>
<td>References</td>
<td>66</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Active ingredients and application rate of biorational products and commercial fungicides used in experimentation</td>
<td>38</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Botrytis blight severity on detached petunia flowers treated with spray application of biorational products, prior to inoculation with spore suspension $10^4$ spores/mL. Captan and Miravis Prime were used as fungicide controls. An inoculated and non-inoculated control is included. Data show results of 72 h after inoculation. Lettering indicates significant differences between the treatments using Fisher’s least significant difference (LSD) test ($\alpha = 0.05$). Error bars represent ±1 SE. Data from three replications using 10 flowers per treatment. ........................................... 39</td>
</tr>
<tr>
<td>2.2</td>
<td>Botrytis blight damage on petunia flowers 72 h after inoculation Flowers treated with spray applications. Showing results of the controls and best-performing treatments. ........................................................................... 40</td>
</tr>
<tr>
<td>2.3</td>
<td>Botrytis blight severity on cut rose flowers treated with a dip application of 15 biorational products. Captan and Miravis Prime were fungicide controls. Inoculated and non-inoculated controls are included. Botrytis blight severity is expressed as the area under the disease progression curve (AUDPC) including the severity ratings from day 3, 5 and 7 after inoculation with a spore suspension of $10^5$ spores/mL. Data are from three replications using 8 cut rose flowers per treatment per replication. Lettering indicates significant differences between the treatments using Fisher’s least significant difference (LSD) test ($\alpha = 0.05$). Error bars represent ±1 SE. ..................................................................................... 41</td>
</tr>
<tr>
<td>2.4</td>
<td>Botrytis blight damage on cut rose flowers 7 d after inoculation. Botrytis blight damage on cut rose flowers 7 d after inoculation. Flowers treated with dip applications. Showing results of controls and best-performing treatments. ........................................................................................................ 42</td>
</tr>
</tbody>
</table>
List of Figures (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>Botrytis blight severity on cut rose flowers treated with dip application of biorational products. Miravis Prime is used as fungicide control. An inoculated and non-inoculated control is included. Botrytis blight severity is expressed as the area under the disease progression curve (AUDPC) including the severity ratings from day 3, 5 and 7 after inoculation with spore suspension 10^5 spores/mL. Data are from two replications using 10 cut rose flowers per treatment per replication. Lettering indicates significant differences between the treatments using Fisher’s least significant difference (LSD) test (α = 0.05). Error bars represent ±1 SE.</td>
</tr>
<tr>
<td>3.1</td>
<td>Botrytis blight damage on petunia flowers 72 h after inoculation. Flowers treated with spray application using the combined biorational products.</td>
</tr>
<tr>
<td>3.2</td>
<td>Botrytis blight severity on detached petunia flowers treated with spray application of biorational products and its combinations, prior to inoculation with spore suspension 10^4 spores/mL. Captan and Miravis Prime were used as fungicide controls. An inoculated and non-inoculated control is included. Data shows results 72 h after inoculation. Lettering indicates significant differences between the treatments using Fisher’s least significant difference test (α = 0.05). Error bars represent ±1 SE. Data from three replications using 10 flowers per treatment per replication.</td>
</tr>
<tr>
<td>3.3</td>
<td>Botrytis blight damage on petunia flowers 72 h after inoculation. Flowers treated with spray application using the combined biorational products.</td>
</tr>
<tr>
<td>3.4</td>
<td>Botrytis blight severity rating on cut rose flowers treated with dip application of four biorational products and their six combinations. Miravis Prime (fludioxonil + pydiflumetofen) was used as a fungicide control. An inoculated and non-inoculated control is included. Botrytis blight severity is expressed as the area under the disease progression curve (AUDPC) which included the severity ratings from day 3, 5 and 7 after inoculation with a spore suspension of 10^5 spores/mL. Lettering indicates significant differences between the treatments using Fisher’s least significant difference test (α = 0.05). Error bars represent ±1 SE. Data from three replications using 6 rose flowers per treatment per replication.</td>
</tr>
</tbody>
</table>
CHAPTER ONE
LITERATURE REVIEW

BOTRYTIS CINEREA

*Botrytis cinerea*, is the causal agent of botrytis blight, also known as gray mold. It is an important pathogen of nursery, vegetable, ornamental, field, and fruit crops (Dik and Wubben 2007), being particularly aggressive on mature tissue of dicotyledonous hosts. The pathogen commonly enters the plant tissue in earlier stages of crop development and remains latent for a considerable period until the environment is conducive for fungal growth. Significant damage occurs following the harvest of apparently healthy crops, causing important losses in horticultural crops in the postharvest environment (Williamson et al. 2007).

*B. cinerea* propagules including conidia, mycelium, or sclerotia can remain in the greenhouse for long periods of time on living or decaying tissue (Williamson et al. 2007). The disease starts when conidia are released and spread in the greenhouses through the air, water, insects, or humans. The optimal condition for fungus development are temperatures between 15-25 °C and relative humidity >93% (Williamson et al. 1995). Moist conditions help the conidia germinate, promoting the production of germ tubes and appressoria (Ravi et al. 2021) that help the fungus penetrate into plant tissue (Van Kan 2005). The infection can also occur without the help of appressoria via natural openings, wounds, or by colonizing dead plant tissue in contact with healthy tissue (Daugthtrey et al.1995). Plants with a nutrient deficiency will be more vulnerable to *B. cinerea* infection.
Dense mycelial structures called sclerotia form on plant tissue and are key for the long-term survival of *B. cinerea*, which under optimal conditions will germinate and produce conidia (Daughtrey et al. 1995).

The production of flowering plants is hindered by their susceptibility to *B. cinerea*. In cut flowers, such as roses and gerbera, botrytis blight is a severe disease (Dik and Wubben 2007). The infection can be observed as necrotic spots on flower petals, but also the leaves and stems can be affected (Pikovskyi et al. 2018). These symptoms reduce the quality and the vase life of cut flowers (Dik and Wubben 2007).

**BOTRYTIS MANAGEMENT IN FLORICULTURE**

* Cultural practices. Growers employ different approaches to control diseases.
Sanitation includes the elimination of dead and infected plant tissue, which is the primary source of inoculum for *B. cinerea* (Hausbeck and Pennypacker 1991). The fungi can live as a saprophyte on dead plant debris. During greenhouse production, plants tend to be grown close to each other, which creates a humid canopy that is optimal for the growth of this pathogen. Therefore, the use of aeration or ventilation and increasing the spacing between the plants provides more air movement and light penetration, helping to reduce the relative humidity in the canopy, which is not favorable for *B. cinerea* (Dik and Wubben 2007). Cleaning the boots, tools, and clothes of greenhouse workers and disinfecting benches and greenhouse surfaces is vital for reducing the dispersal of conidia (Ravi et al. 2021). Additionally, plant nutrient management plays an important role in the susceptibility to *B. cinerea*. For example, high amounts of nitrogen result in weaker cells
and denser canopies, promoting favorable conditions for disease infection in the host plant (Hubber and Watson 1974).

**Chemical management.** Preventive chemical fungicide applications are one of the main strategies for botrytis management during greenhouse production and in the postharvest environment. Multi-site fungicides, e.g., captan (FRAC M3) and thiram (FRAC M4), and some site-specific such as dicarboximide fungicide (FRAC 2) and anilio-pyrimide fungicide (FRAC 9) (Fernandez-Ortuño et al. 2015). Throughout the years, *B. cinerea* has developed resistance to many single-site fungicides because of its high adaptability (Muñoz et al. 2019). The floriculture industry relies on the use of fungicides for the control of this disease, but due to the high occurrence of fungicide resistance management success has declined. Resistance management strategies must be implemented to achieve successful disease management (Ravi et al. 2021).

**Fungicide Resistance Development**

Fungicide resistance development is a result of the adaptation of the pathogen to fungicides due to a heritable change that leads to the emergence and spread of mutant strains with reduced fungicide sensitivity (Delp and Dekker 1985). *B. cinerea* isolates can develop simultaneous resistance to different chemical classes (Katan 2007). Fungicide-resistance development depends on many important factors such as physiology, morphology, and reproductive ability of the pathogen, frequency of fungicide application, and the mode of action of the fungicide compound (Brent and Hollomon, 2007).

**Use of Biorationals for *B. Cinerea* Management**
The call for new and sustainable alternatives for managing diseases is more pronounced nowadays (Shrestha and Hausbeck 2021). A potential option for managing diseases is the use of biorational products. Biorational products are defined as compounds that have low or no direct mammalian toxicity and little to no negative effects on the environment. Biorationals include biological control agents (BCA), botanical extracts, minerals, microorganism-derived compounds, and systemic acquired resistance inducers (Copping and Menn, 2000; Paulitz and Belanger 2001).

**Biological control agents.** BCAs are living organisms that can kill or suppress plant pathogens. Biological control agents can be bacteria, fungi, nematodes, or viruses (Usta 2013; Adriaens et al. 2007). BCAs are considered friendly to the environment and attractive resources to protect plants against diseases (Calderon et al. 2019). Multiple modes of action are accredited to BCAs used for *B. cinerea* control, e.g., competition, hyperparasitism, induction of host resistance, and antibiosis (Haidar et al. 2016; Pal and gardener 2006). Successful competition depends on colonization where microorganisms compete for available nutrients and space. This mode of action is described as an indirect antagonistic effect (Spadaro and Droby 2016). Biological control agents can also have a direct antagonism through hyperparasitism. This occurs when a pathogen is attacked by a non-pathogenic microorganism that kills it or its structures (Boosalis 1964; Pal and Gardener 2006). Antibiosis occurs when BCAs release toxins capable of poisoning or killing the pathogen (Landa et al. 2002). Some biologicals such as *Bacillus* and *Pseudomonas* (Kloepper et al. 2004) induce host resistance by triggering plant defense against pathogens (Ramamoorthy et al. 2001). There are two types of enhanced resistance...
in the host plant, induced systemic resistance (ISR) and systemic acquired resistance (SAR) (Choudhary et al. 2007). ISR occurs when host plant defense is triggered beneficial microorganisms, while SAR is enhanced resistance that is activated in the plant after being exposed to pathogenic microbes or molecules such as salicylic acid or chitosan (Pieterse et al. 2014).

Multiple factors can influence the efficacy of BCAs. They are most effective at optimal growth conditions (temperature, light, humidity) (Tatagiba et al. 1998) and their development and ultimately their efficacy against pathogens such as *B. cinerea* is also dependent on the age and type of flower, inoculum concentration, free surface water availability, and colonization of host tissue by other microbes (Sutton et al. 1997).

In strawberries (*Fragaria ×ananassa*), *Trichoderma harzianum* has shown success against *B. cinerea* when applied as a aerial spray under greenhouse conditions (Freeman et al. 2004). Reduction of the infection was not observed when *B. cinerea* and the antagonistic fungus *Ulocladium atrum* were applied in combination or when *U. atrum* was applied after inoculation with *B. cinerea* on cyclamen (*Cyclamen persicum*) leaves, but efficacy was achieved when *U. atrum* was applied 48 h or more before inoculation (Kessel et al. 2002). More research is needed for improving the understanding of the mode of action and properties of the different BCAs (Calderon et al. 2019).

Enhancing the performance of the microorganisms can be achieved by mixing two or more compatible BCAs that have different properties, e.g., *B. cinerea* infection was reduced by 80% to 99% when two microorganisms *Pichia guilermondii*, a yeast, and *Bacillus mycoides*, a bacterium, were used in combination, while they suppressed only
74% of the disease when applied separately, this experiment was evaluated in detached strawberry leaves. (Guetsky et al. 2001).

**Botanicals.** Botanicals are plant extracts and products derived from plant oils. These compounds have been considered due to their inhibitory effects against pathogens (Isman 2000; Tabassum and Vidyasagar 2013) and several modes of action were identified, including reduction of cell growth, inhibition of biofilm, and disruption of the cell wall of the pathogen (Nazzaro et al. 2017). Some studies report that essential oils of some plant species such as thyme (*Thymus vulgaris* L) may trigger host defense against *B. cinerea* in apple (*Malus x domestica* Borkhausen ‘Red Fuji) fruit (Banani et al. 2018). Soybean (*Glycin max*) oil was reported to be effective against *B. cinerea* when applied as spray in field grown grapes, affecting the pathogen by cell damage (*Vitis vinifera*) (Wurms et al. 2021). The extract of giant knotweed a botanical known for activating host defense (*Reynoutria sachalinensis*), showed an anti-fungal effect on *B. cinerea* on young tomatoes (*Solanum lycopersicum*) and pepper (*Capsicum annuum*) plants (Schmitt et al. 1996).

**Plant nutrients.** Plant nutrients are important inorganic compounds required for plant growth. These nutrients are divided into two categories, macronutrients and micronutrients (Gianquinto et al. 2013). Macronutrients are minerals needed in greater amounts. These include nitrogen, potassium, calcium, magnesium, phosphorus, and sulfur. Micronutrients are needed in smaller amounts. These minerals are iron, zinc, manganese, copper, boron, chlorine, molybdenum, and nickel (Grusak, 2001). The use of nutrients is key for the growth and development of crops and microbes, and they play an
important role in disease management (Agrios, 2005). Plant nutrients have a direct effect on the physiology, the integrity of the cell walls of the host plant, and plant biochemistry, e.g., concentrations of phenolics can be affected by boron deficiency (Graham and Webb 1991). The development of mechanical barriers, through the formation of thicker cell walls and the synthesis of natural defense compounds such as antioxidants, flavonoids, and phytoalexins that protect the tissue against plant pathogens, are the two ways of primary resistance that mineral nutrients can offer to control diseases (Bhaduri et al. 2014; Gupta et al. 2017). Several studies have been performed to evaluate the effect of nutrients against diseases, e.g., calcium applications at different doses were applied on petunia (*Petunia × hybrida*) plants to observe its effect against *B. cinerea*. It was found that the use of calcium reduced botrytis blight severity in petunias (Bennett et al. 2020) and cut rose (*Rosa × hybrida*) flowers (Muñoz 2022). In strawberries, powdery mildew severity was reduced by 85% when potassium silicate was applied (Kanto et al. 2006). Low severity of botrytis blight and delay of symptoms were observed when silicon was applied to cucumber (*Cucumis sativus*) leaves (Silva et al. 2020).

**Microorganism-derived compounds.** Microorganism-derived compounds are fungicides that are obtained from the fermentation of some microorganisms such as *Streptomyces* (Copping and Menn, 2000). An example is natamycin, which is derived from *Streptomyces natalientes* (Oostendorp, 1981). Natamycin has antifungal properties and works by binding to the ergosterol of fungi inhibiting the growth of the pathogen (Aparicio et al. 2016). Natamycin does not work against bacteria, because bacteria do not have ergosterol (Delves-Broughton et al. 2006). Natamycin has been used to preserve
Natamycin has been effective against postharvest diseases on citrus such as *Geotrichum cancidum* and *Penicillium digitatum*, this was observed on limes after being inoculated and stored for four weeks. (Yigiter et al. 2014). Gray mold on stored blueberries was reduced when natamycin was applied (Saito et al. 2022). Resistance has never been reported for this product (Aparicio et al. 2016; Haack et al. 2018). Another compound derived from microorganisms is polyoxin-D zinc salts isolated from *Streptomyces cacaoi* var. *asoensis*. Polyoxins are known for their efficient antifungal activity, but they don’t have an effect against bacteria (Zhang, 1999). Polyoxins suppress chitin formation in the cell wall of the fungi, inhibiting the growth of mycelia and germination of spores (Endo et al. 1970; Bartnicki-Garcia 1972). Polyoxin D was effective against botrytis blight in cut rose flowers obtained from a greenhouse, roses were treated with polyoxin D and storage at 20 °C and evaluated disease severity 13 days after treatment application (Elad, 1988).

**Systemic acquired resistance inducers (SAR).** Systemic acquired resistance inducers are plant metabolites, chemical compounds, or microorganisms, such as salicylic acid, that trigger a defense response in the plant, activating pathogen-resistant signaling pathways (Kessman et al. 1994; Achuo et al. 2004). Resistance to diseases can be activated in the host plant by applying chemicals such as salicylic acid or compounds that mimic the pathway of silicic acid such as acibenzolar-S-methyl (Oostendorp et al. 2001). This chemical is the first SAR inducer developed (Walters et al. 2005). Studies have
shown that acibenzolar-S-methyl reduced *Phytophthora cactorum* on strawberries and phytophthora blight on peppers (Eikemo et al. 2003; Matheron and Porchas, 2002).
REFERENCES


https://doi.org/10.1002/1526-4998(200008)56:8%3C651::aid-ps201%3E3.0.co;2-u.


https://doi.org/10.1007/978-1-4020-2626-3_17.


Kessel GJT, De Hass BH, Van Der Werf W, Köhl J. 2002. Competitive substrate colonization by *Botrytis cinerea* and *Ulocladium atrum* in relation to biological
https://doi.org/10.1017/s0953756202005956.


https://doi.org/10.1094/pdis.2002.86.3.292.


Muñoz M. 2022. Unveiling the potential of calcium and natamycin for botrytis blight management on cut rose flowers (PhD Diss). Clemson University. Clemson, South Carolina, United States.


https://doi.org/10.2174/1381612805666230109204948.
CHAPTER TWO

EVALUATION OF BIORATIONAL PRODUCTS FOR BOTRYTIS BLIGHT MANAGEMENT ON PETUNIA AND CUT FLOWER ROSES

ABSTRACT

The use of biorational products offers an alternative approach to managing botrytis blight. Biorationals are products derived from natural resources and are believed to have fewer adverse effects on the environment when compared with commercial fungicides. We evaluated 15 biorational products on detached petunia (Petunia × hybrida) flowers and cut rose (Rosa × hybrida) flowers for botrytis management. In the first experiment, the biorational products were applied as a spray on detached petunia flowers and compared with commercial fungicides. Five products showed a reduction in disease severity (reduction percentages shown in parentheses are relative to the inoculated control): Howler—a formulation of Pseudomonas chlororaphis strain AFS009 (51%), ON-Gard Calcium—a product derived from soy protein and calcium chloride (66%), Zivion (natamycin)—a natural fermentation of Streptomyces natalensis (97%), Affirm—a polyoxin D zinc salt (36%), and Regalia—an extract from giant knotweed (Reynoutria sachalinensis) (36%). In the second experiment, the same 15 biorational products were applied as a dip application on cut rose flowers. Applications were made 1 or 8 d prior to inoculation with B. cinerea spores. When biorational products were applied 1 d before inoculation, Actigard (acibenzolar-S-methyl), Affirm (polyoxin D zinc salts), OnGard Calcium (calcium), and Zivion (natamycin) showed a reduction in disease severity of
23%, 63%, 33%, and 18%, respectively. No significant effect was observed with the other biorational products.

**INTRODUCTION**

*Botrytis cinerea* is an important plant pathogen known for causing gray mold in nursery, vegetable, ornamental, field, and orchard crops (Dik and Wubben 2007). The pathogen is ubiquitous and can be spread in greenhouses through the air, water, insects, or humans. Once inside a greenhouse, *B. cinerea* can remain as conidia, mycelia, or sclerotia for long periods of time on living or decaying tissue (Williamson et al. 2007). *B. cinerea* can enter plant tissue during the earlier stages of crop development, and it remains latent for a considerable period until the environment is conducive for fungal growth. The optimal conditions for fungus development are temperatures between 15-25 °C and relative humidity >93% (Williamson et al. 1995). When these conditions are present, the conidia germinate, produce germ tubes, and form appressoria that help the pathogen penetrate the plant surface (Van Kan 2005; Ravi et al. 2021). The dominant symptom observed on flowering plants is necrotic spots on flower petals, and although not as common, the leaves and stems can also be affected (Pikovskyi et al. 2018). Symptom development often occurs during the postharvest environment after visually healthy plants are harvested, processed, and packaged for storage and transport (Williamson et al. 2007). These symptoms compromise the quality and durability of cut flowers (Dik and Wubben 2007).

Growers employ different approaches that involve cultural practices, use of biological control agents and chemical management to control the disease. Sanitation
involves the disinfestation of tools, clothing, and workspace, as well as eliminating the primary source of inoculum for *B. cinerea*, e.g., dead, and infected plant tissue (Hausbeck and Pennypacker 1991; Ravi et al. 2021). Implementing a combination of these practices can result in decreased disease pressure. Fungicides for botrytis management include multi-site fungicides, e.g., captan (FRAC M3) and thiram (FRAC M4), and site-specific fungicides such as dicarboximide fungicide (FRAC 2) and anilio-pyrimide fungicide (FRAC 9) (Fernandez-Ortuño et al. 2015), but throughout the years, due to its high adaptability, *B. cinerea* has developed resistance to many single-site fungicides (Muñoz et al. 2019). In the floriculture industry, fungicides have been essential for disease control, but resistance management is required for their sustainable use (Ravi et al. 2021, Shrestha and Hausbeck 2021).

Biorational products provide an alternative disease-management tool. They are believed to have few to no effects on the environment and encompass biological control agents (BCA), botanical extracts, plant nutrients, microorganisms-derived compounds, and systemic acquired resistance inducers (Copping and Menn 2000; Paulitz and Belanger 2001). BCAs are living organisms such as bacteria, fungi or nematodes that can kill or suppress plant pathogens (Usta 2013; Adriaens et al. 2007). Botanicals consist of plant by-products such as extracts and oils that possess anti-fungal activity with different mechanisms that include reduction of cell growth, inhibition of biofilm, and disruption of the cell wall of the pathogen (Nazzaro et al. 2017). Plant nutrients, such as calcium, help by giving strength to the cell walls, making the plant more resistant to fungal penetration (Gislerød 1997). Cell walls are an important structure present in plants and act as a first
layer of defense against pathogens’ attack (Shi et al. 2019). Calcium plays an important role in the middle lamella forming cross bridges with polygalacturonic acid of pectic chains resulting in the development of gel which provides stability and strength to the plant (Fry 2004) Microorganism-derived compounds are fungicides that are obtained as a natural product of the fermentation of microorganisms such as *Streptomyces* (Copping and Menn 2000). Systemic acquired resistant inducers (SAR) consist of plant metabolites, chemical compounds, or pathogens that trigger a defense response in the plant (Kessman et al. 1994; Achuo et al. 2004). SARs can induce and activate resistance to diseases of host plants by applying chemicals such as salicylic acid, but also by using a compound that mimics the salicylic acid metabolic pathway such as acibenzolar-S-methyl (Oostendorp et al. 2001).

In the case of botanicals, studies have pointed out that essential oils of some plant species like thyme (*Thymus vulgaris* L) may trigger host defense when applied against *B. cinerea* in apple (*Malus x domestica* Borkhausen ‘Red Fuji) fruit (Banani et al. 2018). Soybean oil was reported to be highly efficient in controlling *B. cinerea* in grapes (*Vitis vinifera*). The extract of the plant giant knotweed exhibited an anti-fungal effect on *B. cinerea* when used in young tomatoes (*Solanum lycopersicum*) and pepper (*Capsicum annuum*) plants (Schmitt et al. 1996; Wurms et al. 2021). In cucumber (*Cucumis sativus*) leaves, silicon has been shown to decrease the disease severity and delay the symptoms of botrytis blight (Silva et al. 2020). Polyoxin D has proven effectiveness against botrytis blight in flowering plants such as cut rose flowers (Elad, 1988) and geraniums (*Pelargonium × hortorum*) (Webster, 2005). Natamycin is a compound used to preserve
food that has been effective against gray mold on blueberries (*Vaccinium corymbosum*), sour rot (*Geotrichum candidum*), and blue mold (*Penicillium digitatum*) on citrus with no resistance ever reported (Yigiter et al. 2014; Aparicio et al. 2016; Haack et al. 2018; Saito et al. 2022). Studies have shown that acibenzolar-S-methyl reduced *Phytophthora cactorum* on strawberries and phytophthora blight on peppers (Eikemo et al. 2003; Matheron and Porchas 2002) making it also a possible treatment in controlling *B. cinerea*. All these examples work as a building block for implementing biorational products in the management of botrytis in flowering plants.

*B. cinerea* is an ever-present pathogen that plays an important role in the floriculture industry as a major threat from propagation to the postharvest environment. Fungicide applications have always been the staple approach for growers to manage this disease but an increase in fungicide resistance opens new pathways for the management of *B. cinerea*. Biorational products have shown potential in controlling fungal pathogens; therefore, the objective of this study was to evaluate 15 biorational products as potential alternatives to manage *B. cinerea* in rose and petunia flowers.

**MATERIALS AND METHODS**

Two replicated studies were conducted to evaluate the effect of biorational products on *Botrytis cinerea* infection using detached petunia flowers and cut flower roses as model crops. In the first study, the effect of spray application of biorational products was evaluated on detached petunia flowers that were inoculated with *B. cinerea* 1 d after treatments. In the second experiment, the effect of dip application of biorational
products was evaluated on cut rose flowers where the application occurred 1 d (Expt. 2A) or 8 d (Expt. 2B) prior to inoculation with *B. cinerea* spores.

**B. cinerea culture maintenance and preparation of spore suspension.** Two *B. cinerea* isolates were used for the experiments: PDRK3 (Bennett et al. 2020) was used for petunia inoculation and S4GBR4O (Muñoz et al. 2019) was used for rose inoculation. For each isolate, one 5-mm diameter potato dextrose agar (PDA; Difco Laboratories, Sparks, MD, USA) plug with actively growing mycelium was transferred to a Petri dish with PDA medium and incubated in the dark for 8-10 d until sporulation occurred. The spore suspension was prepared by placing 7 mL of sterile deionized water in the Petri dish. Spores were then scraped with a loop to allow the spores to release into the water. Then, the spore suspension was pipetted from the Petri dish to a beaker with sterile deionized water to measure spore suspension using a hemocytometer (Bright-line 3110, Hausser Scientific, Horsham, PA, USA) by placing 10 µL on each side of the hemocytometer. A final spore suspension of $1 \times 10^4$ spores/mL was prepared for petunia and $1 \times 10^5$ spores/mL for rose.

**Biorational products and fungicide controls.** Fifteen biorational products were evaluated using the recommended concentration on the label: Actigard® 50WG (Syngenta Crop Protection, NC, USA), Affirm™ WDG (Cleary Chemicals, IL, USA), Botector® (Westbridge Agricultural Products, CA, USA), Botrystop™ (Bioworks, Inc. NY, USA), Cease® (Bioworks, Inc. NY, USA), Howler™ (Agbiome Inc. NC, USA), 20 ml/L ON-Gard® Calcium (Bioworks Inc. NY, USA), potassium silicate (Agri-Neo Inc. Quebec, PureCrop 1 (West Coast AG Products, CA, USA), Regalia CG™ (Marrone Bio
Innovations, Inc. CA, USA), Revitalize® (Bonide Products, Inc. NY, USA), RootShield® WP (Bioworks, Inc. NY, USA), Triathlon® BA (OHP, Inc. SC, USA), *Trichoderma asperellum* (Experimental Isolate from Dr. Hehe Wang lab, SC, USA), Zivion™ (DSM, Heerlen, Netherlands). The following commercial fungicides were used as controls: Captan 50 WP (Southern Agricultural Insecticides, Inc., NC, USA) Miravis® Prime (Syngenta, NC, USA). Non-inoculated and inoculated untreated controls were also used.

Biorational products were prepared at the middle of the recommended concentration range in a total volume of 500 mL (Table 2.1).

**Expt. 1. Evaluation of biorational products on detached petunia flowers.**

‘Dreams Burgundy Picotee’ petunia plugs were received from a commercial supplier (Tagawa, CA, USA) and transplanted into 1.4 L pots (1 plug per container) filled with a peat-based growing medium (Fafard 3B, Sun Gro, Anderson, SC, USA). Eighty petunia plants were grown for this experiment in a glass greenhouse (Clemson University, SC, USA) with a computer-controlled environment system (Argus Control Environmental Systems, White Rock, BC, Canada). Plants were fertigated with 250 ppm N with 15N-5P₂O₅-15K₂O (Peters Excel CalMag Special, OH, USA). All open flowers were removed the day before the experiment began to allow the harvest of newly opened flowers for the experiment.

A total of 180 flowers were harvested and immediately placed into cell trays, 30 flowers per tray. The trays containing the petunia flowers were placed into a larger plastic tray with 1 L of water to keep the flowers hydrated. Sets of ten flowers were taken from the trays and were sprayed with the biorational products using a mist sprayer (118.3 mL
clear polyethylene terephthalate plastic bottle, The Cary Company, IL, USA) and allowed to dry for 24 h. Treatments were sprayed just in the front of the petunia flower. After 24 h, the flowers were inoculated with a spore suspension (1 × 10⁴ spores/mL) by spraying 1 mL using a 118.29 mL clear polyethylene terephthalate plastic bottle. Flowers were placed in 30.4-cm width × 20.7-cm depth × 16.8-cm height plastic storage containers (HMS Mfg., MI, USA). A total of 32 plastic storage containers were used for this experiment, each one of them were labeled with 6 different treatments, to randomize the flowers. Inside each container was a piece of polystyrene with six 10-mm diameter holes in which one flower was placed per hole. Water (600 ml) was placed in the bottom of each container to hydrate the flowers and to humidify the boxes once the lids were put into place. Ten flowers were used per treatment. Flowers were incubated in the plastic storage containers for 72 h with lids closed. Disease progression data were collected at 72 h after inoculation. Each flower was rated from 0 to 8 based on a botrytis severity scale that evaluates the area of the infected petal where 0 = no infection, 1 = 1-2%; 2 = 3-5%; 3 = 6-10%; 4 = 11-25%; 5 = 26-50%; 6 = 51-75%; 7 = 76-99% of the flower petal was affected with necrotic spots, and 8 = whole flower is affected for botrytis blight (modified from Bennett, 2020). This experiment was performed three times.

**Expt. 2A. Evaluation of the biorational products applied to roses 1 d prior to inoculation.** A quantity of 152 ‘Orange Crush’ roses were obtained from an Ecuadoran grower through a wholesale distributor (Carolina Florist Supply, SC, USA). The flowering stems were placed in cardboard boxes in a 5 °C cooler for 24 h prior to the start of the experiment. Then the roses were removed from the cooler and 10 cm was cut from
the base of the stem to improve hydration. Any damaged leaves were removed. Roses were randomly selected and placed in groups of 8 roses per treatment. Each flower bud was dipped for 15 s into the treatment solution, moving the head of the rose gently to improve contact of the solution with the multiple layers of flower petals. The roses were then placed in a 0.55-m length × 0.12-m width × 0.12-m height humid chamber covered with clear heavy duty, polyethylene plastic. Ten trays were placed in the bottom of each humid chamber. Each tray contained 1 L water to provide hydration to the roses and to humidify the chamber. Each tray held a 35-cm length × 20-cm width × 0.22-cm height PVC structure with plastic mesh netting to hold the stems upright. Roses were randomized within the open humid chamber for 24 h to allow the treatment solutions to dry. Then, the roses were inoculated with a 1×10⁵ spores/mL suspension using a 118 mL clear polyethylene terephthalate plastic bottle with a mist sprayer. After inoculation, plants remained at 96%-100% relative humidity until disease assessment. Humidity was measured with a psychrometer (RH300, Extech Instruments, Nashua, NH, USA). This experiment was performed three times.

**Expt. 2B. Evaluation of the biorational products applied to roses 8 d prior to inoculation.** In this experiment the dip applications of the bioproducts were performed at two commercial greenhouses in Cundinamarca, Colombia (lat. 4°59’16.9"N and lat. 4°48’03.3"N). Individual ‘Orange Crush’ rose stems were covered with clear plastic bags for the 8 weeks of growth prior to harvest to avoid contact with chemical applications. A total of 150 cut rose flowers were used for this experiment. Then, these flowers were sent to Clemson University to perform inoculation and evaluation of the different treatments.
The following treatments were tested. Actigard50WG (acibenzolar-S-methyl), Affirm WDG (polyoxin D zinc salts), BotryStop (Ulocladium oudemansii), Howler (Pseudomonas chlororaphis), ON-Gard® Calcium (calcium), PureCrop 1 (soybean oil + corn oil), Regalia CG (Reynoutria sachalinensis), Revitalize (Bacillus amyloliquefaciens), RootShield WP (Trichoderma harzianum), Triathlon B (Bacillus amyloliquefaciens), and Zivion (natamycin). After harvest, the stems were randomized and organized in a set of 10 roses. Then, the flower buds were submerged in the treatments as described in Expt. 2A. Immediately after the treatment applications, roses were grouped into their respective treatments in bunches and packed in plastic bouquets. The bouquets were labeled and placed in a cardboard box and cooled to 2 °C in a forced-air cooler. Then, these boxes were stored at 5 °C for 24 h before being shipped by airfreight to Clemson University, Clemson, SC. Boxes arrived after 7 d. Upon arrival, the cut rose flowers were placed in the cooler at 5 °C for 24 h. Then, the stems were taken out of the boxes and 15 cm were cut from the base of the stems to improve water uptake. Flowers were inoculated with the same spore suspension as used in Expt. 2A, labeled, randomized, and placed in the humid chambers. Roses where inoculated 8 days after the treatment application.

Visually ratings were performed at day 3, 5 and 7 after inoculation based on a total flower area affected by botrytis using a 0 to 8 botrytis severity scale where 0 is no symptoms, 1 = necrotic spots on in 1-3 petals of the rose covering the 1-5% of the petal tissue, 2 = necrotic spots present in 4-6 petals of the rose covering 6-15% of the petal tissue, 3 = lesions present in 7-9 petals of the rose covering the 16-29% of the petal
tissue, 4 = necrotic spots present in 10-11 petals of the rose covering 30-50% of the petal tissue, 5 = lesion present in the half of the petals of the rose covering 51-60% of the tissue, 6 = lesion present in the 51-65% of the petals of the rose covering 61-70% of the petal tissue, 7 = necrotic spots present in the 66-90% of the petals of the rose covering 71-95% of the petal tissue, 8 = 96-100% of the petals of the rose affected by the disease, covering the entire petal tissue (Muñoz 2022).

**Statistical analysis.** Data analysis was performed using JMP pro version 16.0.0 (SAS Institute Inc., Cary, NC, USA). Treatment effects were assessed using analysis of variance (ANOVA) and Fisher’s LSD student’s T-test was used to compare means between treatments at p<0.05. For rose, the cumulative effect of the bioproducts over time was analyzed using the area under the disease progression curve (AUDPC) calculated with the data collected at 3, 5 and 7 d after inoculation using the calculation described by Bennett et al. (2020).

**RESULTS AND DISCUSSION**

In petunia flowers, the application of Howler (*Pseudomonas chlororaphis*) and Zivion (natamycin) reduced disease severity by 51% and 66%, respectively, in comparison to the inoculated control (Fig. 2.1 and Fig. 2.2). ON-Gard Calcium (calcium) reduced disease severity by 97%, which was not significantly different from Miravis Prime (fludioxonil + pydiflumetofen), the single-site commercial fungicide control. Regalia (*Reynoutria sachalinensis*) and Affirm (polyoxin D zinc salts) also showed a 23% reduction in botrytis severity, but results varied between experimental replications.
No significant differences were observed between the other 10 treatments and the inoculated control (Fig. 2.1 and Fig. 2.2).

In rose flowers treated 1 day before inoculation, Zivion (natamycin) showed an 18% reduction in botrytis severity in comparison to the inoculated control (Fig. 2.3). The application of Actigard (acibenzolar-S-methyl) and ON-Gard Calcium (calcium) showed a disease severity reduction of 23% and 33%, respectively. Affirm (polyoxin D zinc salts) reduced botrytis blight severity by 63%, similar to the Miravis Prime fungicide control. The other biorational treatments had no significant effect on disease severity. The best performing biorational products applied 1 d before inoculation are showed in Fig. 2.4.

In rose flowers treated 8 d before inoculation, Actigard (acibenzolar-S-methyl) and Zivion (natamycin) reduced disease severity by 16% in comparison to the inoculated control (Fig. 2.5). The use of ON-Gard Calcium (calcium) showed a 31% reduction in disease severity. Affirm (polyoxin D zinc salts) reduced disease severity by 54%. Cease (*Bacillus subtilis*), Howler (*Pseudomonas chlororaphis*) and RootShield (*Trichoderma harzianum*) showed a reduction of 11%.

ON-Gard Calcium (calcium), Howler (*Pseudomonas chlororaphis*), and Zivion (natamycin) reduced disease severity on detached petunia flowers. Regalia (*Reynoutria sachalinensis*) and Affirm (polyoxin D zinc salts) reduced botrytis blight severity, but they but results varied between experimental replications.

On roses, the use of Actigard (acibenzolar-S-methyl), Affirm (polyoxin D zinc salts), Cease (*Bacillus subtilis*), ON-Gard Calcium (calcium) and Zivion (natamycin) reduced disease severity on cut rose flowers when they were applied 1 day and 8 days...
before inoculation. Howler (*Pseudomonas chlororaphis*), and RootShield reduce Botrytis blight severity when they were applied 8 days before inoculation.

Spray applications of ON-Gard Calcium (calcium) on detached petunia flowers and dip applications on cut rose flowers reduced Botrytis blight symptoms. The performance of Calcium was comparable to the fungicide Miravis Prime. Previous studies have shown that the use of calcium chloride at concentrations of 800 mg·L⁻¹ and 1200 mg·L⁻¹ had a positive effect in the reduction of *B. cinerea* on petunia flowers (Bennet et al. 2020). Calcium forms bonds with the pectic material located in the middle lamella, which serves to strengthen the cell wall. This strengthening process effectively increases the resistance of the tissue to fungal penetration (Gislerød, 1997).

The use of *P. chlororaphis* showed a reduction of 51% of botrytis blight on detached petunia flowers. This bacterium was reported to have different modes of action, such as cell wall degrading, antibiotic activity (Chin-A-woeng et al. 1998) or inducing host resistance (Han et al. 2006).

The application of natamycin as a spray on detached petunia flowers and dip application in cut rose flowers was able to reduce botrytis blight severity. Previous studies have shown that when natamycin was use 24 h before inoculation as a dip application in cut rose flowers was able to reduce botrytis severity (Muñoz 2022).

Acibenzolar-S-methyl induces plant defense mechanisms against diseases (Lawton et al. 1996). When it was used as postharvest treatment in strawberries, Actigard reduced botrytis severity (Terry and Joyce 2000). In grapes, botrytis was reduced when acibenzolar-S-methyl was applied as a spray, the product had higher efficacy to reduce
botrytis in grapes when the fruits were immersed in the product in comparison when the product was applied as a spray (Youssef et al. 2019).

Howler (*Pseudomonas chlororaphis*), and RootShield on cut rose flowers reduced Botrytis blight severity only when they were applied 8 days before inoculation. We hypothesize that this could be related to induced resistance in the host plant, because when they were applied 24 hours before inoculation, they did not show any effect.

The other five treatments (Botector (*Aerobasidium pullulans*), BotryStop (*Ulocladium oudemansii*), potassium silicate (silicon), PureCrop 1 (soybean oil + corn oil), Revitalize (*Bacillus amyloliquefaciens*), RootShield WP (*Trichoderma harzianum*), Triathlon B (*Bacillus amyloliquefaciens*), and *Trichoderma asperellum*) had no effect on disease severity. Studies have reported that these BCAs yield variable results because they require the proper environmental conditions (temperature, light, humidity) for growth and to achieve their antagonistic effect against the pathogen (Tatagiba et al. 1998). For example, *B. cinerea* control has been effective when the BCA is capable of surviving and adapting to the environment. For example, in the flowering plant cyclamen (*Cyclamen persicum*), a reduction of the botrytis infection was achieved when applying the *Ulocladium atrum* 48 h before inoculation, whereas no effect was observed when *U. atrum* was applied at the same time as the pathogen (Kessel et al. 2002). Further investigation is needed to understand why these microorganisms did not show any effect against botrytis or how to improve them to create a more viable product.

The results of this research show that the ON-Gard Calcium (calcium), Howler (*Pseudomonas chlororaphis*) and, Zivion (natamycin) hold promise for Botrytis blight
control in petunia and cut rose flowers. Similarly, Actigard (acibenzolar-S-methyl) and Affirm (polyoxin D zinc salts) show potential for their incorporation in an integrated disease management program.
Table 2.1 Active ingredients and application rate of biorational products and commercial fungicides used in experimentation.

<table>
<thead>
<tr>
<th>Product</th>
<th>Active ingredient(s)</th>
<th>Application rate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biorational</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actigard® 50WG</td>
<td>acibenzolar-S-methyl</td>
<td>0.04 g/L</td>
</tr>
<tr>
<td>Affirm™ WDG</td>
<td>polyoxin D zinc salts</td>
<td>0.5 g/L</td>
</tr>
<tr>
<td>Botector®</td>
<td><em>Aureobasidium pullulans</em></td>
<td>0.75 g/L</td>
</tr>
<tr>
<td>BotryStop™</td>
<td><em>Ulocladium oudemansii</em></td>
<td>3.6 g/L</td>
</tr>
<tr>
<td>Cease®</td>
<td><em>Bacillus subtilis</em> strain QST 713</td>
<td>10 ml/L</td>
</tr>
<tr>
<td>Howler™</td>
<td><em>Pseudomonas chlororaphis</em> strain AFS009</td>
<td>6.23 g/L</td>
</tr>
<tr>
<td>ON-Gard® Calcium</td>
<td>calcium</td>
<td>20 ml/L</td>
</tr>
<tr>
<td>Potassium silicate</td>
<td>silicon</td>
<td>394 µl/L</td>
</tr>
<tr>
<td>PureCrop 1</td>
<td>soybean oil and corn oil</td>
<td>15.6 ml/L</td>
</tr>
<tr>
<td>Regalia CG™</td>
<td><em>Reynoutria sachalinensis</em></td>
<td>6.34 ml/L</td>
</tr>
<tr>
<td>Revitalize®</td>
<td><em>Bacillus amyloliquefaciens</em> strain D747</td>
<td>1.3 ml/L</td>
</tr>
<tr>
<td>RootShield® WP</td>
<td><em>Trichoderma harzianum</em></td>
<td>7.39 g/L</td>
</tr>
<tr>
<td>Triathlon® BA</td>
<td><em>Bacillus amyloliquefaciens</em> strain D747</td>
<td>25 ml/L</td>
</tr>
<tr>
<td>Zivion™</td>
<td>natamycin</td>
<td>4.84 ml/L</td>
</tr>
<tr>
<td><strong>experimental isolate</strong></td>
<td><em>Trichoderma asperellum</em></td>
<td>1×10^7 spores/L</td>
</tr>
<tr>
<td><strong>Commercial fungicide</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Captan 50 WP</td>
<td>captan</td>
<td>2.4 g/L</td>
</tr>
<tr>
<td>Miravis® Prime</td>
<td>fludioxonil + pydiflumetofen</td>
<td>350 µl/L</td>
</tr>
</tbody>
</table>
Figure 2.1. Botrytis blight severity on detached petunia flowers treated with spray application of biorational products applied 24 h prior to inoculation with spore suspension $10^4$ spores/mL Captan (captan) and Miravis Prime (fluoxonil + pydiflumetofen) used as fungicide controls. An inoculated and non-inoculated control is included. Data show results of 72 h after inoculation. Lettering indicates significant differences between the treatments using Fisher’s least significant difference (LSD) test ($\alpha = 0.05$). Error bars represent $\pm 1$ SE. Average data from 3 independent replications from three replications using 10 flowers per treatment.
Figure 2.2. The effect of the best performing spray application treatments and control groups on botrytis blight damage on petunia flowers. Pictures taken 72 h after inoculation.
Figure 2.3. Botrytis blight severity on cut rose flowers treated with a dip application of 15 biorational products 1 d before inoculation. Captan (captan) and Miravis Prime (fludioxonil + pydiflumetofen) were fungicide controls. Inoculated and non-inoculated controls are included. Botrytis blight severity is expressed as the area under the disease progression curve (AUDPC) including the severity ratings from day 3, 5 and 7 after inoculation with a spore suspension of $10^5$ spores/mL. Average data from 3 independent replications using 8 cut rose flowers per treatment per replication. Lettering indicates significant differences between the treatments using Fisher’s least significant difference (LSD) test ($\alpha = 0.05$). Error bars represent ±1 SE.
Figure 2.4. Botrytis blight damage on cut rose flowers treated with dip applications 1 d before inoculation. Pictures of the best performing products and controls taken 7 d after inoculation.
Figure 2.5. Botrytis blight severity on cut rose flowers treated 8 d before inoculation with a dip application of biorational products. Miravis Prime (fludioxonil + pydiflumetofen) is used as fungicide control. An inoculated and non-inoculated control is included. Botrytis blight severity is expressed as the area under the disease progression curve (AUDPC) including the severity ratings from day 3, 5 and 7 after inoculation with spore suspension $10^5$ spores/mL. Average data from 3 independent replications using 10 cut rose flowers per treatment per replication. Lettering indicates significant differences between the treatments using Fisher’s least significant difference (LSD) test ($\alpha = 0.05$). Error bars represent ±1 SE.
REFERENCES


*Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium 
https://doi.org/10.1094/mpmi.1998.11.11.1069.

Copping LG, Menn JJ. 2000. Biopesticides: a review of their action, applications and 
https://doi.org/10.1002/1526-4998(200008)56:8%3C651::aid-ps201%3E3.0.co;2-u.

Diseases. APS Press, St. Paul, MN.

Dik AJ, Wubben JP. 2007. Epidemiology of *Botrytis cinerea* diseases in greenhouses, p 
319-333. In: Elad Y, Williamson B, Tudzynski P, Delen N (eds). *Botrytis: Biology, 
https://doi.org/10.1007/978-1-4020-2626-3_17.

Eikemo H, Stensvand A, Tronsmo AM. 2003. Induced resistance as a possible means to 
control diseases of strawberry caused by *Phytophthora* spp. *Plant Disease* 87:345-

Elad Y. 1988. Latent infection of Botrytis cinerea in rose flowers and combined chemical 

resistance to seven chemical classes of fungicides in *Botrytis cinerea*.


Muñoz M. 2022. Unveiling the potential of calcium and natamycin for botrytis blight management on cut rose flowers (PhD Diss). Clemson University. Clemson, South Carolina, United States.


https://doi.org/10.1007/s42161-020-00643-x.

https://doi.org/10.1094/php-01-21-0010-rs.


https://doi.org/10.2174/1381612805666230109204948.
CHAPTER THREE

EVALUATION OF THE COMBINATION OF BIORATIONAL PRODUCTS ON BOTRYTIS BLIGHT IN PETUNIA FLOWERS AND CUT ROSE FLOWERS

ABSTRACT

A select few biorationals were shown to have consistent efficacy against botrytis blight of petunia and cut flower roses. In this study we evaluated the combination of these promising biorational products for disease management. In the first experiments, individual and combination treatments of calcium, *Pseudomonas chlororaphis* and natamycin were applied to detached petunia flowers. In the second experiments individual and combination treatments of acibenzolar-S-methyl, calcium, natamycin and polyoxin D zinc salts were applied to cut rose flowers. On petunia flowers, the combination of natamycin + *P. chlororaphis* and calcium + *P. chlororaphis* reduced disease severity by 77% and 79%, respectively, compared to the inoculated control, while calcium + natamycin showed a 91% reduction. While applied alone calcium, natamycin and *P. chlororaphis* reduced botrytis severity of 56%, 62% and 68% respectively. The combination of calcium + natamycin showed a synergistic effect while natamycin + *P. chlororaphis* and calcium + *P. chlororaphis* showed an antagonistic effect. On cut rose flowers the combinations of acibenzolar-S-methyl + natamycin, calcium + natamycin, and acibenzolar-S-methyl + calcium reduced botrytis blight by 53%, 62% and 66%, respectively, while natamycin + polyoxin D zinc salts, calcium + polyoxin D zinc salts, and acibenzolar-S-methyl + polyoxin D zinc salts reduced disease severity by 76%, 77%
and 85%, respectively. When applied alone acibenzolar-S-methyl, calcium, natamycin and polyoxin D zinc salts reduced botrytis of 49%, 33%, 40% and 61% respectively. All this combination showed synergistic effects except for acibenzolar-S-methyl + natamycin that show and antagonistic effect. Tank mixes containing different modes of action that provide significant reductions in disease severity can be effective as chemical fungicides.

INTRODUCTION

*B. cinerea* is pathogen that causes significant losses in floriculture industry (Vrind 2005). The disease can affect the whole plant, but flower petals are often the most susceptible tissue. Symptoms start as necrotic spots that expand over time provided the proper environment (Pikovskyi et al. 2018) and often render plants as unsaleable (Dik and Wubben 2007).

The use of chemical fungicides is one of the primary strategies for the management of *B. cinerea* in greenhouse production and postharvest of the crops. Development of fungicide resistance in pathogenic microbes and reduction on fungicidal effectivity has been reported, *B. cinerea* is a good example of fungi studied because of the rise of its fungicide resistance development (Bollen and Scholten 1971; Hanh 2014). The persistent use of active ingredients with similar modes of action leads to the pathogen resistance (Fillinger and Elad 2016). Different commercial products have been developed for manufactures where they have combined fungicides with different modes of action such as Switch (cyprodinil + fludioxonil) from and Pristine (pyraclostrobin + boscalid) (Wedge et.al. 2007). However, impact in the environment due to discriminative use of chemicals fungicides is a concern (Fillinger and Elad 2016).
The use of biorational products for disease management provides a means of avoiding fungicide resistance. Biorationals include biological control agents, botanical extracts plant nutrients, microorganism derived compounds and systemic acquired resistance inducers. These compounds have no or low mammalian toxicity and few effects on the environment (Copping and Menn 2000; Paulitz and Belanger 2001).

Variability in their efficacy is one of the main issues of the use of biorationals, mainly in the use of biological control agents (Sylla et al. 2015). Mixture of two or more microorganisms has been recommended to address this issue (Sylla et al. 2015) or apply them as combination or in rotation with chemical fungicides (Shtienberg and Elad 1997). For example, reduction on sour rot on citrus was observed when mixture of natamycin and fludioxonil and propiconazole (Chen et al., 2021). The improvement in the efficacy and less variability in the use of combined products could be the result of different modes of actions of the different compounds (Muñoz 2022; Guetsky et al. 2001).

Previous work has demonstrated that many biorational products failed to protect flowers from botrytis infection, while a few products reduced disease severity but were not as effective as conventional fungicides (Chapter 2). Thus, the objective of this study was to take the five biorational products that were previously identified as effective for botrytis management and provide them in combination with each other to see if the additive effect may be comparable to conventional fungicides.

**MATERIALS AND METHODS**

Two experiments were conducted to evaluate the efficacy of the combination of biorational products on detached petunia and cut rose flowers. In the first experiments,
the effect of spray application of the combination of biorational products were evaluated on detached petunia flowers. In the second experiments, the effect of dip application of the biorational product combinations were evaluated on rose flowers. The products were selected based on the results of the Chapter 2.

**Expt. 1. Evaluation of the combination biorationals in detached petunia flowers.** Seventy ‘Dreams Burgundy Picotee’ plugs (Pan American Seed Co., IL, USA) were transplanted into 1.4 L pots (1 plug per container) filled with a peat-based growing medium (Fafard 3B, Sun Gro, Anderson, SC, USA) and grown in a glass greenhouse at Clemson University (Clemson, SC, USA) with a computer-controlled environment system (Argus Control Environmental Systems, White Rock, BC, Canada). Plants were fertigated with 250 ppm N using 15N-5P₂O₅-15K₂O (Peters Excel CalMag Special, OH, USA). All open flowers were removed the day before the experiment began to allow the harvest of newly opened flowers for the experiment.

Three biorational products Howler (*Pseudomonas chlororaphis*), ON-Gard Calcium (calcium), Zivion (natamycin), and their combinations were evaluated on detached petunia flowers. The following combinations were performed: calcium + natamycin, calcium + *P. chlororaphis*, natamycin + *P. chlororaphis* and calcium + natamycin + *P. chlororaphis*. Two commercial fungicides were used as control groups. These included the single-site fungicide Miravis Prime (fludioxonil + pydiflumetofen) and the multi-site fungicide Captan (captan). Non-inoculated and inoculated untreated controls were also used. Ten petunia flowers per treatment were evaluated and a total of 100 flowers were harvested and the pedicel was immediately placed into trays containing
water. Sets of 10 flowers were taken from the trays and the open flowers were sprayed on
the open, upper side with the biorational products using a mist sprayer (118 mL clear
polyethylene terephthalate plastic bottle, The Cary Company, IL, USA) and allowed to
dry for 24 h. Then the flowers were inoculated with a spore suspension (1 X 10^4
spores/mL) by spraying 1 mL per flower. Flowers were then placed in one of 17 plastic
storage containers (30.4-cm width × 20.7-cm depth × 16.8-cm height, HMS Mfg., MI,
USA). Six flowers from different treatments were placed randomly in each container.
Inside each container was a piece of polystyrene with six 10-mm-diameter holes in which
one flower was placed per hole. Water (600 ml) was placed in the bottom of each
container to hydrate the flowers and to humidify the boxes once the lids were put into
place. Flowers were incubated in the plastic storage containers for 72 h. Disease
progression data were collected every 24 h. Each flower was rated from 0 to 8 using a
botrytis severity scale described in Chapter 2, Expt. 1 (modified from Bennett 2020). This
experiment was performed three times.

**Expt. 2. Evaluation of the combination of biorational products applied as a dip
to roses one day prior to inoculation.** Seventy-eight ‘Orange Crush’ roses were obtained
from an Ecuadoran grower through a wholesale distributor (Carolina Florist Supply, SC,
USA). The cut rose flowers were placed in cardboard boxes in a 5 °C cooler for 24 h
prior to the start of the experiment. Then the roses were removed from the cooler and 10
cm was cut from the base of the stem to improve hydration. Any damaged leaves were
removed. Roses were randomly selected and placed in groups of 6 roses per treatment.
Then the roses were treated with the different biorational products. Four biorational
products Actigard (acibenzolar-S-methyl), Affirm (polyoxin D zinc salts), ON-Gard calcium (calcium), Zivion (natamycin), and their six combinations were evaluated: acibenzolar-S-methyl + calcium, acibenzolar-S-methyl + natamycin, acibenzolar-S-methyl + polyoxin D zinc salts, calcium + natamycin, calcium + polyoxin D zinc salts and natamycin + polyoxin D zinc salts. Non-inoculated and inoculated untreated control groups were included. Each rose was dipped for 15 s into the treatment solution, moving the head of the rose gently to improve contact of the solution with the multiple layers of flower petals. The roses were then randomly placed in a humid chamber (0.55-m length × 0.12-m width × 0.12-m height) covered with clear, heavy-duty, polyethylene plastic. Ten trays were placed in the bottom of each humid chamber. Each tray contained 1 L water to provide hydration to the roses and to humidify the chamber. Each tray held PVC structure (a 35-cm length × 20-cm width × 22-cm height) with plastic mesh netting to hold the stems upright. The roses were held for 24 h in the open humid chambers to allow the treatment solutions to dry. Then the roses were inoculated with a 1×10^5 spores/mL suspension using the mist sprayer. After inoculation, the humid chamber was closed to let the humidity increase. A 96%-100% relative humidity was provided in the humid chamber as measured with a psychrometer (RH300, Extech Instruments, Nashua, NH, USA). Data were collected at day 3, 5 and 7 after inoculation by visually rating the flowers based on a total area infected using a 0 to 8 botrytis severity scale (Calidonio, Chapter 1). This experiment was performed three times.

**Statistical analysis.** Data analysis was performed using JMP pro version 16.0.0 (SAS Institute Inc., Cary, NC, USA). Treatment effects were assessed using analysis of
variance (ANOVA) and Fisher’s LSD student’s T-test was used to compare means between treatments at p<0.05. For rose, the cumulative effect of the bioproducts over time was analyzed using the area under the disease progression curve (AUDPC) calculated with the data collected 3, 5 and 7 d after inoculation using the calculation described by Bennett et al., (2020).

Synergistic or antagonistic effects of the combinations were calculated using the formula $E= \frac{If + IN - IfIN}{100}$ from Colby (Colby, 1967). Where (If) is the observed percentage of control provided by one of the single products and (IN) is the observed percentage of control provided by the other single products used for the combination. (E) is the expected percentage control by the combination of the two products. When the observed control is higher than the expected the combination presents a synergistic effect, when the observed control presents a lower result than the expected control is referred to the antagonistic effect described by Peng et al. 2014. (Peng et al 20014)

**RESULTS AND DISCUSSION**

The application of the selected biorational products and their combinations to petunia flowers showed a reduction on *B. cinerea* damage compared to the inoculated control (Fig. 3.1 & 3.2). Calcium, natamycin, and *P. chlororaphis* reduced botrytis blight by 56%, 62% and 68% respectively in comparison to the inoculated control and was equivalent in efficacy to the non-inoculated control. Calcium, natamycin, and *P. chlororaphis* performed better than captan, but they did not perform as well as fludioxonil + pydiflumetofen. All combinations performed better than the individual
products, except for natamycin + *P. chlororaphis*, which performed as well as *P. chlororaphis* alone. The combination of natamycin + *P. chlororaphis*, calcium + *P. chlororaphis* and calcium + natamycin reduced botrytis blight by 77%, 79% and 91% respectively. However, the combination of natamycin + *P. chlororaphis*, calcium + *P. chlororaphis* showed an antagonistic effect presenting an observable control lower than the expected control. The combination of calcium + natamycin showed a synergistic effect, the observable control was higher than the expected. Calcium + natamycin performed as well as fludioxonil + pydiflumetofen. The combination of calcium chloride + natamycin + *P. chlororaphis* reduced botrytis blight by 90%, performing equally to fludioxonil + pydiflumetofen. However, the efficacy of the mixture was not better than calcium + natamycin. (Fig. 3.2).

In cut rose flowers, all biorational combinations reduced disease severity in comparison to the inoculated control (Fig. 3.3 and Fig. 3.4). The combinations of acibenzolar-S-methyl + calcium, acibenzolar-S-methyl + polyoxin D zinc salts, calcium + natamycin, calcium + polyoxin D zinc salts and natamycin + polyoxin D zinc salts showed synergistic effects all these combinations presented higher results in the observable control than the expected except for acibenzolar-S-methyl + natamycin that showed an antagonistic effect, meaning that the observable control was lower than the expected control. Acibenzolar-S-methyl, calcium, natamycin and polyoxin D zinc salts performed better than the inoculated control, and showed reduction of botrytis blight of 49%, 33%, 40% and 61%, respectively. All combinations performed significantly better than the products alone except for acibenzolar-S-methyl + natamycin, which performed
as well as acibenzolar-S-methyl and natamycin alone. Acibenzolar-S-methyl + polyoxin D zinc salt and calcium + polyoxin D zinc salts performed similarly to fludioxonil + pydiflumetofen (Fig. 3.4).

The spray applications of calcium + *P. chlororaphis*, calcium + natamycin, natamycin + *P. chlororaphis*, and calcium + natamycin + *P. chlororaphis* reduced botrytis blight on petunia flowers. The combinations of calcium + *P. chlororaphis* and calcium + natamycin performed better than the products by themselves. Studies have reported that the combination of different microorganisms can improve their efficacy due to their different modes of action (Sylla et al. 2015; Shtienberg and Elad 1997). Disease severity was reduced by 80% to 99% when *Pichia guilermondii*, a yeast, and *Bacillus mycoides*, a bacterium, were used in combination, while they suppressed only 74% of the disease when applied alone (Guetsky et al. 2001).

*P. chlororaphis* is a bacterium, that works against pathogens through antibiosis, cell wall degradation and host resistance (Chin-A-woeng et al. 1998; Han et al. 2006). Zivion (natamycin) is a product obtained from the natural fermentation of *Streptomyces natalientes* (Oostendorp 1981). Natamycin has antifungal properties and binds the ergosterol of fungi, limiting the growth of the pathogen (Aparicio et al. 2016). ON-Gard Calcium (calcium) help increase nutrient uptake and is compatible with most chemicals and biological products, making it a good product for combinations (Bioworks 2019). This compatibility could be related to calcium helping to strengthen the plant cells while other fungicides can act by directly inhibiting or killing the fungi. Calcium (Ca) creates bonds with the pectic material located in the middle lamella, which serves to strengthen
the cell wall. This strengthening process increases the resistance of the tissue to fungal penetration (Gislerød 1997).

Actigard (acibenzolar-S-methyl) is a product recognized for inducing plant defense mechanisms against diseases. (Lawton et al. 1996). Affirm has worked by reducing sporulation on botrytis, the active ingredient of this product is polyoxin D zinc salt. (Hausbeck et al. 2019). These modes of actions used in the combination with Calcium and natamycin could be the reason for the great effect reducing \textit{B. cinerea} in cut rose flowers. The use of calcium in combination with natamycin was found to reduce \textit{B. cinerea} in cut rose flowers having a better effect than when the products were used alone (Muñoz 2022).

These results demonstrated that the use of biorational products offers an alternative to manage botrytis blight in ornamental flowers. The combination of calcium, \textit{P. chlororaphis} and natamycin showed promising results for managing botrytis in petunia flowers, while the combination of acibenzolar-S-methyl, polyoxin D zinc salts. Calcium and natamycin showed promising results for managing botrytis on cut rose flowers.
Figure 3.1. The effect of biorational products and their combinations on botrytis blight of petunia flowers 72 h after inoculation.
Figure 3.2. Botrytis blight severity on detached petunia flowers treated with biorational products and their combinations 24 h prior to inoculation. Captan and Miravis Prime (fludioxonil + pydiflumetofen) were used as fungicide controls. An inoculated and non-inoculated control was included. Data show results 72 h after inoculation. Lettering indicates significant differences between the treatments using Fisher’s least significant difference test (α = 0.05). Error bars represent ±1 SE. Average data from three independent replications using 10 flowers per treatment per replication.
Figure 3.3. Effect of protective dips with biorationals and their combinations on botrytis blight of cut rose flowers 7 days after inoculation.
Figure 3.4 Botrytis blight severity rating on cut rose flowers dipped with biorational and their combinations. Fludioxonil + pydiflumetofen served as fungicide control. Botrytis blight severity was expressed as the area under the disease progression curve (AUDPC) which included the severity ratings at 3, 5, and 7 days after inoculation with a spore suspension of $10^5$ spores/mL. Lettering indicates significant differences between the treatments using Fisher’s least significant difference test ($\alpha = 0.05$). Error bars represent $\pm 1$ SE. Average data from 3 independent replications using six rose flowers per treatment per replication.
REFERENCES


Muñoz M. 2022. Unveiling the potential of calcium and natamycin for botrytis blight management on cut rose flowers (PhD Diss). Clemson University. Clemson, South Carolina, United States.


