Yeast biocontrol agents that were applied to 'Bosc' and 'Anjou' pears in the field up to three weeks prior to harvest were found to survive on the fruit at high population levels on both pear varieties. *Cryptococcus infirmo-miniatu*s, *Cryptococcus laurentii*, and *Rhodotorula glutinis* maintained populations averaging $5 \times 10^6$ cfu/fruit for three weeks. *Candida oleophila* had high initial populations, but the population size quickly declined to levels similar to the total yeast populations on untreated fruit. After a storage period of 2-4 months, fruit that were treated with *C. infirmo-miniatu*s three weeks before harvest showed significantly lower incidence of decay at wounds than did untreated fruit.

Combinations of biocontrol agents with reduced rates of the postharvest fungicides captan and thiabendazole were effective in reducing incidence and severity of blue mold decay caused by *Penicillium expansum* on 'Bosc' pears. Calcium chloride was also effective in combination with some biocontrol agents. Chitosan caused reductions in decay when used alone, but not when combined with most biocontrol agents. The compounds L-asparagine, L-proline, and 2-deoxy-D-glucose were not consistently effective either alone or combined with biocontrol agents.

Storage of 'Bosc' and 'Anjou' pears in atmospheres with carbon dioxide concentrations of 12% or 20% for up to six weeks significantly reduced incidence and severity of gray mold decay caused by *Botrytis cinerea*, but decay was not reduced when the atmospheres was only 3% CO$_2$. In contrast, the 12% or 20% CO$_2$ atmospheres did not have significant effects on decay caused by *P. expansum*. Use of the biocontrol
agents *C. infirmo-miniatus*, *C. laurentii*, or *R. glutinis* led to reductions in decay in all atmospheres, with *C. infirmo-miniatus* being the most consistently effective. The biocontrol products BioSave-110 (EcoScience Corp.) and Aspire (Ecogen Corp.) were less effective than the yeasts *C. laurentii*, *R. glutinis*, and *C. infirmo-miniatus* which were grown in the lab.
Enhancement of Biological Control for Postharvest Diseases of Pear

by

Jesse M. Benbow

A Thesis Submitted
to
Oregon State University

In Partial Fulfillment of
the requirements for the
degree of
Master of Science

Presented June 30, 1998
Commencement June 1999
Master of Science thesis of Jesse M. Benbow presented on June 30, 1998

Approved:

Redacted for Privacy

Major Professor, representing Botany and Plant Pathology

Redacted for Privacy

Chair of Department of Botany and Plant Pathology

Redacted for Privacy

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes the release of my thesis to any reader upon request.

Redacted for Privacy

Jesse M. Benbow, Author
Acknowledgements

I would like to extend sincere thanks to my advisor, Dr. David Sugar for his assistance, guidance, and support during my graduate program. I would also like to thank the other members of my committee: Dr. Ken Johnson of the Botany and Plant Pathology department, Dr. Paul Chen of the Horticulture department, and Dr. Richard Waring of the Forest Science department.

The donation of cultures of the biocontrol agents used in this research by Dr. Robert Spotts and Dr. Rodney Roberts is appreciated. I also thank Kate Powers and Sally Basile for their assistance in this research.
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 1</td>
<td>Introduction</td>
</tr>
<tr>
<td>References</td>
<td>4</td>
</tr>
<tr>
<td>Chapter 2</td>
<td>Fruit Surface Colonization and Biological Control of Postharvest Disease by Preharvest Yeast Applications</td>
</tr>
<tr>
<td>Abstract</td>
<td>6</td>
</tr>
<tr>
<td>Introduction</td>
<td>7</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>9</td>
</tr>
<tr>
<td>Results</td>
<td>12</td>
</tr>
<tr>
<td>Discussion</td>
<td>15</td>
</tr>
<tr>
<td>References</td>
<td>20</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>Enhancement of Biological Control of Postharvest Pear Decay by Combining Antagonistic Microorganisms with Other Compounds</td>
</tr>
<tr>
<td>Abstract</td>
<td>22</td>
</tr>
<tr>
<td>Introduction</td>
<td>23</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>25</td>
</tr>
<tr>
<td>Results</td>
<td>27</td>
</tr>
<tr>
<td>Discussion</td>
<td>34</td>
</tr>
<tr>
<td>References</td>
<td>37</td>
</tr>
<tr>
<td>Chapter 4</td>
<td>High Carbon Dioxide Controlled Atmosphere Storage Combined with Biocontrol Agents to Reduce Postharvest Decay of Pears.</td>
</tr>
<tr>
<td>Abstract</td>
<td>39</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (continued)

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>40</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>42</td>
</tr>
<tr>
<td>Results</td>
<td>44</td>
</tr>
<tr>
<td>Discussion</td>
<td>59</td>
</tr>
<tr>
<td>References</td>
<td>61</td>
</tr>
<tr>
<td>Chapter 5 Summary</td>
<td>64</td>
</tr>
<tr>
<td>References</td>
<td>67</td>
</tr>
<tr>
<td>Bibliography</td>
<td>68</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>(1996) Population per fruit of biocontrol yeast applied to fruit in the field on 'Bosc' (A) and 'Anjou' (B) pears.</td>
<td>13</td>
</tr>
<tr>
<td>2.2</td>
<td>(1997) Population per fruit of biocontrol yeast applied to fruit in the field on 'Bosc' (A) and 'Anjou' (B) pears.</td>
<td>14</td>
</tr>
<tr>
<td>2.3</td>
<td>(1996) Percent of wounds infected by side rots, <em>P. expansum</em>, and <em>B. cinerea</em> on 'Bosc' (A) and 'Anjou' (B) pears treated before harvest with various biocontrol agents.</td>
<td>16</td>
</tr>
<tr>
<td>2.4</td>
<td>(1997) Percent of wounds infected by side rots, <em>P. expansum</em>, and <em>B. cinerea</em> on 'Bosc' (A) and 'Anjou' (B) pears treated before harvest with various biocontrol agents.</td>
<td>17</td>
</tr>
<tr>
<td>3.1</td>
<td>Average lesion diameter caused by <em>P. expansum</em> on pears treated with a combination of various compounds and biocontrol agents: Untreated control (A), BioSave-110 (B), Aspire (C), <em>R. glutinis</em> (D), <em>C. laurentii</em> (E), or <em>C. infirmo-miniatus</em> (F).</td>
<td>28</td>
</tr>
<tr>
<td>3.2</td>
<td>Incidence of decay caused by <em>P. expansum</em> on pears treated with a combination of various compounds and biocontrol agents: Untreated control (A), BioSave-110 (B), Aspire (C), <em>R. glutinis</em> (D), <em>C. laurentii</em> (E), or <em>C. infirmo-miniatus</em> (F).</td>
<td>29</td>
</tr>
<tr>
<td>3.3</td>
<td>Average lesion size (A) and incidence of decay (B) caused by <em>P. expansum</em> on pears treated with a combination of various biocontrol agents and different rates of captan.</td>
<td>31</td>
</tr>
<tr>
<td>3.4</td>
<td>Average lesion size (A) and incidence of decay (B) caused by <em>P. expansum</em> on pears treated with a combination of various biocontrol agents and different rates of chitosan.</td>
<td>32</td>
</tr>
<tr>
<td>3.5</td>
<td>Average lesion size (A) and incidence of decay (B) caused by <em>P. expansum</em> on pears treated with a combination of various biocontrol agents and different rates of calcium chloride.</td>
<td>33</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>4.1</td>
<td>Incidence of decay caused by <em>B. cinerea</em> on 'Anjou' pears treated with (A) no treatment, (B) Aspire, (C) BioSave-110, (D) <em>R. glutinis</em>, (E) <em>C. laurentii</em>, (F) <em>C. infirmo-miniatus</em>, and stored in high-CO₂ atmospheres in 1996.</td>
<td>46</td>
</tr>
<tr>
<td>4.2</td>
<td>Average sizes of lesions caused by <em>B. cinerea</em> on 'Anjou' pears treated with (A) no treatment, (B) Aspire, (C) BioSave-110, (D) <em>R. glutinis</em>, (E) <em>C. laurentii</em>, (F) <em>C. infirmo-miniatus</em>, and stored in high-CO₂ atmospheres in 1996.</td>
<td>47</td>
</tr>
<tr>
<td>4.3</td>
<td>Incidence of decay caused by <em>B. cinerea</em> on 'Bosc' pears treated with (A) no treatment, (B) Aspire, (C) BioSave-110, (D) <em>R. glutinis</em>, (E) <em>C. laurentii</em>, (F) <em>C. infirmo-miniatus</em>, and stored in high-CO₂ atmospheres in 1996.</td>
<td>48</td>
</tr>
<tr>
<td>4.4</td>
<td>Average sizes of lesions caused by <em>B. cinerea</em> on 'Bosc' pears treated with (A) no treatment, (B) Aspire, (C) BioSave-110, (D) <em>R. glutinis</em>, (E) <em>C. laurentii</em>, (F) <em>C. infirmo-miniatus</em>, and stored in high-CO₂ atmospheres in 1996.</td>
<td>49</td>
</tr>
<tr>
<td>4.5</td>
<td>Incidence of decay caused by <em>B. cinerea</em> on 'Anjou' pears treated with (A) no treatment, (B) Aspire, (C) BioSave-110, (D) <em>R. glutinis</em>, (E) <em>C. laurentii</em>, (F) <em>C. infirmo-miniatus</em>, and stored in high-CO₂ atmospheres in 1997.</td>
<td>51</td>
</tr>
<tr>
<td>4.6</td>
<td>Average sizes of lesions caused by <em>B. cinerea</em> on 'Anjou' pears treated with (A) no treatment, (B) Aspire, (C) BioSave-110, (D) <em>R. glutinis</em>, (E) <em>C. laurentii</em>, (F) <em>C. infirmo-miniatus</em>, and stored in high-CO₂ atmospheres in 1997.</td>
<td>53</td>
</tr>
<tr>
<td>4.7</td>
<td>Incidence of decay caused by <em>B. cinerea</em> on 'Bosc' pears treated with (A) no treatment, (B) Aspire, (C) BioSave-110, (D) <em>R. glutinis</em>, (E) <em>C. laurentii</em>, (F) <em>C. infirmo-miniatus</em>, and stored in high-CO₂ atmospheres in 1997.</td>
<td>54</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>4.8</td>
<td>Average sizes of lesions caused by <em>B. cinerea</em> on 'Bosc' pears treated with (A) no treatment, (B) Aspire, (C) BioSave-110, (D) <em>R. glutinis</em>, (E) <em>C. laurentii</em>, (F) <em>C. infirmo-miniatus</em>, and stored in high-CO₂ atmospheres in 1997.</td>
<td>55</td>
</tr>
<tr>
<td>4.9</td>
<td>Incidence of decay caused by <em>P. expansum</em> on 'Bosc' pears treated with (A) no treatment, (B) Aspire, (C) BioSave-110, (D) <em>R. glutinis</em>, (E) <em>C. laurentii</em>, (F) <em>C. infirmo-miniatus</em>, and stored in high-CO₂ atmospheres in 1997.</td>
<td>57</td>
</tr>
<tr>
<td>4.10</td>
<td>Average sizes of lesions caused by <em>P. expansum</em> on 'Bosc' pears treated with (A) no treatment, (B) Aspire, (C) BioSave-110, (D) <em>R. glutinis</em>, (E) <em>C. laurentii</em>, (F) <em>C. infirmo-miniatus</em>, and stored in high-CO₂ atmospheres in 1997.</td>
<td>58</td>
</tr>
</tbody>
</table>
Enhancement of Biological Control for Postharvest Diseases of Pear

Chapter 1

Introduction

Postharvest decays cause serious economic loss to packers and growers of pears (*Pyrus communis* L.) each year. Pears in the Pacific Northwest are typically harvested in a period from mid-August through September, but selling and shipping of the crop continues for another 8-12 months for some varieties. While technological advances in controlled atmosphere (CA) storage have allowed for the maintenance of desirable horticultural qualities (firmness, ripening ability, and flavor) over this long storage period, the pathogens which have been responsible for postharvest decays for decades are still the cause of significant losses. Blue mold and gray mold (caused by *Penicillium expansum* Link and *Botrytis cinerea* Pers.:Fr, respectively) are the two most common and most aggressive postharvest diseases (Pierson *et al.*, 1971). These pathogens cause relatively rapid lesion development on fruit in storage, and occur wherever pears are grown. Side rots (caused by *Phialophora malorum* Kidd. and Beaum., *Cladosporium herbarum* (Pers.:Fr.) Link, or *Alternaria alternata* (Fr.) Keissler) are less aggressive decays that can cause major losses to pears stored for long periods. All of the above are usually associated with wounds in the fruit that occur during harvest, transport, and packaging.

Losses caused by postharvest diseases are controlled primarily by chemical fungicides. Recently, however, there has been increased interest in the use of biological control to manage postharvest diseases. There appear to be several factors involved in this interest in alternative control strategies. First, development of resistance in pathogens to fungicides (Bertrand and Saulie-Carter, 1978; Spotts and Cervantes, 1986) has limited or eliminated the use of several fungicides. Currently there is only one
fungicide, thiabendazole (TBZ), registered for postharvest use on pears in the U. S.
which consistently reduces postharvest decay, but some pathogens (*Mucor piriformis*
Fischer, causal agent of mucor rot, and *P. malorum*) are not controlled by TBZ
(Michailides and Spotts, 1990; Kupferman et al., 1991). Second, TBZ residues on stored
fruit decline over time, decreasing the value of the fungicide for controlling infections
during long-term storage (Palazon, 1982). Third, concerns over pesticide contamination
on food and pesticide levels in the environment have led to the removal of several
fungicides from the market (National Research Council, 1987). Finally, some countries
that import pears from the United States do not permit any residue of TBZ on imported
fruit (Spotts et al, 1992).

Because of these factors and the resulting increase in research into alternative
control strategies, several microorganisms that help to control postharvest rots have been
discovered. The bacterium *Pseudomonas syringae* and the yeast *Candida oleophila*
strain 1-182 were found to provide decay control (Janisiewicz and Marchi, 1992, and
Hofstein et al., 1994, respectively) and have been registered for control of postharvest
rots of pear as BioSave-110 (EcoScience Corp) and Aspire (Ecogen Corp.), respectively. Other microorganisms which have been shown to provide significant
reductions in postharvest decay are the yeasts *Cryptococcus laurentii* strain RR87-108
(Roberts, 1990), *Rhodotorula glutinis* strain HRB6, and *Cryptococcus infirmo-miniatorus*
strain YY6.

Biological control in the postharvest arena is one of the more promising areas of
study in biocontrol, for several reasons. First, while many biological agents have had
difficulty making the transition from the laboratory to the highly variable field
environment, postharvest technology is such that a constant environment is maintained
throughout storage. Thus, an antagonistic microorganism which is found to provide
good control in laboratory trials should also work well in industry, where the
environment is also controlled (Boudreau and Andrews, 1987; Pusey, 1994). Second,
most pathogens cause initial infection at wounds caused during harvest or transport
(Sugar and Spotts, 1993; Spotts et al., 1998). Biological control has a much greater
chance of succeeding when it can be targeted at a specific site or phase of infection,
rather than being targeted to a wide range of possible infection courts, such as in a field application (Wilson and Pusey, 1985). The wounds where most infections originate provide a nutrient source in which the pathogen can germinate and cause an infection. However, the same nutrient source can be used to provide energy for colonization by a beneficial. Third, due to the high value of harvested produce, application of biological control procedures may be more cost-effective than similar procedures in field situations (Wisniewski and Wilson, 1992).

Despite the successes with microbial antagonists, even the maximum results attainable with these agents may not match the efficacy or consistency of synthetic fungicides (Pusey, 1994). Until such time as biological control by microbial antagonists demonstrates the same level and consistency of control as the current fungicides, it may not be widely accepted by fruit growers and packers. Disease control comparable to that attained using chemical fungicides might only be attainable through integration of two or more alternative control methods.

The objective of this thesis research was to identify procedures which may enhance the disease control obtained using biocontrol agents. The thesis is separated into three separate manuscripts. The first manuscript (chapter 2) examines the ability of biocontrol agents applied to the fruit prior to harvest to colonize and prevent decays of wounds created after harvest. In the second manuscript (chapter 3), we tested compounds which have been reported to control pathogen growth or enhance biological control with other antagonists for their compatibility with the biocontrol agents listed previously. Chapter 4 examines the effect of using high carbon dioxide treatments in storage, combined with biological control agents, to reduce disease.
References


Chapter 2

Fruit Surface Colonization and Biological Control of Postharvest Diseases by Preharvest Yeast Applications

Abstract

Biocontrol agents applied to 'Bosc' and 'Anjou' pear fruit in the field three weeks prior to harvest maintained high population levels, and contributed to control of postharvest decay. The yeasts *Cryptococcus infirmo-minutus*, *Cryptococcus laurentii*, *Rhodotorula glutinis*, and *Candida oleophila* were applied individually to fruit as a preharvest spray (yeast concentrations were 1 to 3 x 10^8 colony forming units per ml, approximately 2 ml applied per fruit). Initial population sizes for all four species averaged 5 x 10^6 cfu/fruit. Population sizes of *C. infirmo-minutus*, *C. laurentii*, and *R. glutinis* remained at the same level for the three weeks prior to harvest. The population size of *C. oleophila* declined after one and two weeks, and by the end of the experiment was not significantly different from total yeast populations on untreated fruit. Significant decay control on fruit treated three weeks before harvest was provided only by *C. infirmo-minutus*. 
Introduction

Postharvest spoilage of harvested fruit is a major expense in food production. The fact that the harvested commodity has a much higher value than the produce in the field, due to production and harvesting costs (Wilson and Pusey, 1985), makes the task of finding new and effective postharvest disease control strategies a priority. Historically, fruit growers and packers have relied on chemical control strategies for disease prevention. However, newly developed resistance of microorganisms to fungicides (Spotts and Cervantes, 1986), the loss of chemical fungicides due to bans or withdrawal of postharvest label (National Research Council, 1987), and the resistance of certain markets to pesticide use (Spotts et al., 1992) have all combined to intensify the interest in alternative control strategies.

In pears (Pyrus communis L.), the majority of disease is initiated at wounds which occur during harvest or packing and subsequent infection at the wound by one of several pathogens (Sugar and Spotts, 1993; Spotts et al., 1998). Penicillium expansum Link and Botrytis cinerea Pers.:Fr (causal agents of blue mold and gray mold, respectively) are relatively aggressive, fast-growing pathogens. Phialophora malorum Kidd. and Beaum., Cladosporium herbarum (Pers.:Fr.) Link, and Alternaria alternata (Fr.) Keissler are less aggressive pathogens (causing side rots) that become problems in fruit stored for longer periods (Pierson et al., 1971). P. malorum and A. alternata are not sensitive to the main postharvest fungicide, thiabendazole (TBZ) (Kupferman et al., 1991), while most strains of P. expansum and B. cinerea are sensitive to TBZ.

Several microorganisms have been discovered which are able to colonize wound sites and compete with and reduce pathogen establishment at those sites. Cryptococcus infirmini-miniatus strain YY6 and Rhodotorula glutinis strain HRB6 (Chand-Goyal and Spotts, 1996), and C. laurentii strain RR87-108 (Roberts, 1990) are all yeasts which were isolated from the surfaces of pear or apple fruits. These yeasts were inoculated into wounds on pears and apples after harvest, in combination with pathogens, and shown to provide good control of rots caused by P. expansum, B. cinerea, and P. malorum (Roberts, 1990; Sugar et al., 1994; Chand-Goyal and Spotts, 1996). Candida oleophila strain I-182 has also been shown to be effective in reducing postharvest
decays (Hofstein et al., 1994) and is the active component of the biological fungicide Aspire (Ecogen Corp.), which is currently registered for postharvest use on pears.

While several studies have shown the potential for biocontrol agents to control disease when applied after harvest, few have focused on the practicality of applying the microorganisms to the fruit while it is still in the field, with the purpose of controlling postharvest decays. Sugar and Spotts (unpublished) have found that as the time from wounding and pathogen inoculation to introduction of the biocontrol agent increased, the efficacy of the treatment decreased. This indicates the importance of prompt application of the biocontrol agents.

Leibinger et al. (1997) used mixtures of the yeasts Aureobasidium pullulans strains CF10 and CF40, R. glutinis strain CF35, and the bacterium Bacillus subtilis strains AG704 and HG77 in preharvest applications on apples to control bulls-eye rot (caused by Pezicula malicorticis (H. Jacks.) Nannf.), blue mold, and gray mold. They found that the microbial antagonists were able to colonize the fruit and provided significant reductions in average lesions sizes compared to unsprayed fruit.

An important concern in a preharvest application of biocontrol agents is the ability of the microorganisms to survive at sufficient populations on the fruit's surface once applied. The weather in pear growing regions is generally hot and dry approaching harvest, which may have detrimental effects on yeast populations. They may also be adversely affected by pesticide sprays, or washed off the fruit surface during irrigation or spraying. However, the fact that these yeasts were originally isolated from fruit surfaces (Roberts, 1990; Chand-Goyal and Spotts, 1996a) indicates that they may be tolerant of these conditions. According to Wisniewski and Wilson (1992), yeasts colonize plant surfaces or wounds for long periods under dry conditions and produce extracellular polysaccharides that enhance their survival and restrict pathogen colonization sites. Yeasts can also reduce the flow of germination cues to fungal spores and use available nutrients during rapid population growth, while being minimally impacted by pesticides.

The objectives of this work were (1) to study the survival of the biocontrol agents on fruit when applied prior to harvest, and (2) to determine the ability of biocontrol agents applied to the fruit's surface prior to harvest to control blue mold (P.
expansum), gray mold (B. cinerea), and side rots (P. malorum, C. herbarum, and A. alternata), on wounded 'Bosc' and 'Anjou' pears during cold storage. In this experiment we used both 'Anjou' and 'Bosc' pears in order to present a variety of fruit surfaces, which could impact the survival of microorganisms; 'Anjou' pears have a smooth, waxy surface while 'Bosc' pears have a russeted surface.

Materials and Methods

Culture and preparation of biocontrol agents

The yeasts C. infirmo-miniatus, R. glutinis, and C. laurentii were used in 1996. C. oleophila, isolated from Aspire, was added to the experiment in 1997. Cultures of C. infirmo-miniatus and R. glutinis were supplied by R. A. Spotts of the Mid-Columbia Agricultural Research and Extension Center in Hood River, OR. A culture of C. laurentii was provided by R. G. Roberts of USDA in Wenatchee, WA.

Yeast cultures stored at -20 C were activated by pouring 1 ml of thawed suspension into 75 ml of yeast malt dextrose broth (YMDB: 3 g malt extract, 3 g yeast extract, 5 g peptone, and 10 dextrose per liter of medium). The suspensions were incubated on a shaker for 2 days at room temperature. After incubation, 0.1 ml aliquots of each yeast were spread on petri dishes containing yeast malt dextrose agar (YMDA: YMDB plus 18 g agar per liter) and incubated for 2-3 days at room temperature. After incubation, the colonies were scraped from the plates with a sterile rubber spatula and suspended in sterile distilled water. Suspensions of C. laurentii were then adjusted to a concentration of 2.8-3.3 x 10^8 colony forming units/ml by adjusting to 2% transmittance at 595 nm using a Spectronic 20 spectrophotometer. Suspensions of C. infirmo-miniatus and R. glutinis were adjusted to 1.0-1.5 x 10^8 cfu/ml by adjusting to 2% transmittance at 550 nm, and C. oleophila was adjusted to approximately 1 x 10^8 cfu/ml by adjusting to 2% transmittance at 550 nm and diluting by one-half. Spectrophotometric determinations of yeast concentrations were confirmed by dilution plating.
Fruit

Pear fruit (cv 'Bosc' and 'Anjou') were grown at the Southern Oregon Research and Extension Center in Medford, Oregon. The orchards were managed under a commercial spray program with under-tree sprinkler irrigation. Trees for treatments with each yeast species and untreated controls were selected at random throughout the blocks. Yeast suspensions were applied to the fruit on the tree using a hand held spray bottle, at a rate of approximately 2 ml per fruit. Approximately 30 fruit on each tree received the yeast application. Each treatment was replicated five times in 1996, and four times in 1997, with each tree being one replicate.

The yeast applications for population sampling were made three weeks prior to harvest. Yeast were also applied in the same manner to a separate set of fruit one day prior to harvest for postharvest pathogen inoculation tests.

Population sampling

Three fruit from each replicate were sampled once per week to determine the number of yeast surviving on the fruit surface. The three fruit were combined in beakers containing 900 ml of 0.05 M sterile phosphate buffer (SPB) with 0.006% (v/v) tween 20. The fruit and buffer were stirred for 5 minutes using a magnetic stirrer, and then placed in a sonicating bath for 5 minutes. This method was determined by Chand-Goyal and Spotts (1994) to provide the most efficient removal of microbiotic flora from the surface of pome fruits. A 1 ml sample was removed from each beaker immediately after sonication and 10-fold dilutions were made to 10⁻⁵ in SPB.

Samples of each dilution were then placed on petri plates containing a selective, dilute YMDA medium (1.5 g malt extract, 1.5 g yeast extract, 2.5 g peptone, 5 g dextrose, and 18 g agar per liter of medium, with 100 ppm chloramphenicol, 50 ppm ampicillin, and 2 ppm dichloran). Chloramphenicol and ampicillin were used to inhibit bacterial growth, and dichloran was used to inhibit radial growth of filamentous fungi. The plates were incubated for 2 to 3 days at room temperature. After incubation, population levels were determined using the plate-dilution frequency technique of Harris and Sommers (1968). The four yeast species used in this experiment exhibit distinct colony color and morphology characteristics. Those colony characteristics were
used to distinguish the different species visually. For a given treatment, only yeast species which were applied to that treatment were counted in the population samples.

To determine population levels on the untreated controls, only colonies resembling those of the yeast species used in the treatments were counted.

**Control of postharvest decay**

Ten fruit from each replicate were randomly selected from the harvested samples to be used for the postharvest treatment. Each fruit was wounded five times with the head of a finishing nail (6 mm diameter x 3 mm deep), and were then placed into cold storage at -1°C for the duration of the experiment.

In 1996, only fruit which were sprayed with biocontrol agents one day before harvest were used for postharvest experiments. In 1997, fruit sprayed both three weeks and fruit sprayed one day before harvest were used for the postharvest decay experiments.

Lesions on the fruit were counted and the type of decay determined visually after 2 and 4 months. The types of decay were divided into three categories and recorded as blue mold, gray mold, or side rot.

**Statistical analysis**

Populations were examined for significant differences between treatments using 95% confidence intervals, where non-overlapping confidence intervals indicate a significant difference.

Postharvest decay data were examined by analysis of variance (ANOVA). Fisher's least significant difference procedure was used to indicate significant differences between treatments.
Results

Colonization of fruit surface

1996 results

In 1996, all three species of yeast (C. laurentii, R. glutinis, and C. infirmo-miniatus) maintained populations at high levels throughout the duration of the experiment on both pear varieties (Figure 2.1). There were no significant differences in population between the three treatments, and all three had significantly higher populations than the control. On 'Anjou' pears sprayed with the biocontrol agents, initial populations ranged from $7.5 \times 10^6$ (C. infirmo-miniatus) to $2.9 \times 10^6$ cfu/fruit (C. laurentii). Populations after three weeks were relatively unchanged, ranging from $7.2 \times 10^6$ (C. infirmo-miniatus) to $3.3 \times 10^6$ cfu/fruit (C. laurentii). On treated 'Bosc' pears, initial population levels for the three yeasts ranged from $6.8 \times 10^6$ (C. infirmo-miniatus) to $2.8 \times 10^6$ cfu/fruit (R. glutinis). After three weeks, the populations were from $1.8 \times 10^6$ (R. glutinis) to $8.9 \times 10^5$ cfu/fruit (C. laurentii). Populations of yeast on untreated fruit were $1.1 \times 10^4$ cfu/fruit initially, and $3.0 \times 10^3$ cfu/fruit at the end of the experiment on 'Anjou' pears. On 'Bosc' pears, initial populations averaged $4.6 \times 10^3$ cfu/fruit, ending at $2.3 \times 10^3$ cfu/fruit.

1997 results

In 1997, the three species used in 1996 behaved similarly to the previous year, with yeast populations on treated fruit remaining significantly higher than those on the control fruit throughout the experiment (Figure 2.2). Yeasts applied to 'Anjou' pears had high initial populations and there were no significant differences between the initial population sizes. Initial populations for the four treatments ranged from $2.4 \times 10^7$ (R. glutinis) to $9.1 \times 10^6$ cfu/fruit (C. laurentii). While populations of C. laurentii, C. infirmo-miniatus, and R. glutinis remained high (final populations from $2.9 \times 10^6$ to $9.2 \times 10^5$ cfu/fruit), populations of C. oleophila declined rapidly. After two weeks the
Figure 2.1. (1996) Population per fruit of biocontrol yeast applied to fruit in the field on 'Anjou' (A) and 'Bosc' (B) pears. Error bars represent one-half of the 95% confidence interval. Non-overlapping bars represent significant differences.
Figure 2.2. (1997) Population per fruit of biocontrol yeast applied to fruit in the field on 'Anjou' (A) and 'Bosc' (B) pears. Error bars represent one-half of the 95% confidence interval. Non-overlapping bars represent significant differences.
population of *C. oleophila* on fruit was not significantly different from total yeast population on the untreated fruit, and after three weeks the population was $2.6 \times 10^4$ cfu/fruit. On 'Bosc' pears the yeast populations showed similar patterns. Initial populations for *C. laurentii*, *C. infirmo-miniatus*, *R. glutinis*, and *C. oleophila* were not significantly different, and ranged from $1.3 \times 10^7$ (*C. laurentii*) to $3.2 \times 10^6$ cfu/fruit (*R. glutinis*). Throughout the three week study, populations of *C. laurentii*, *C. infirmo-miniatus*, and *R. glutinis* remained significantly higher than untreated fruit, and final populations ranged from $1.5 \times 10^6$ (*R. glutinis*) to $6.2 \times 10^5$ cfu/fruit (*C. laurentii*). However, as with treatments on 'Anjou' pears, the populations of *C. oleophila* dropped quickly after application, and at the end of the experiment had populations of $8.8 \times 10^3$ cfu/fruit and were not significantly different from the untreated fruit.

**Storage decay**

For 'Bosc' pears in 1996, sprays with the yeasts *C. laurentii* and *C. infirmo-miniatus* one day before harvest resulted in significantly lower levels of decay on the untreated control. There were no significant differences in treatments on 'Anjou' fruit in 1996 (Figure 2.3).

In 1997, 'Bosc' pears sprayed with biocontrol agents one day before harvest all had significantly lower levels of decay than did the unsprayed control fruit. On fruit sprayed three weeks before harvest, only *C. infirmo-miniatus* provided a significant decay inhibition. There were no significant differences in the 'Anjou' pears sprayed one day before harvest. On 'Anjou' fruit sprayed three weeks before harvest, as with 'Bosc', only fruit treated with *C. infirmo-miniatus* had significantly lower incidence of decay than the untreated fruit (Figure 2.4).

**Discussion**

Previous research on biological control for prevention of storage decay has focused mostly on the application of biocontrol agents after harvest. However, the fact that most postharvest decays occur at wounds caused during harvest or packaging of the fruit (Sugar and Spotts, 1993) indicates a need to provide biocontrol agents to the
Figure 2.3. (1996) Percent of wounds infected by side rots, *P. expansum*, and *B. cinerea* on 'Bosc' (A) and 'Anjou' (B) pears treated before harvest with various biocontrol agents. Different letters within graphs represent significant differences according to Fisher's Least Significant Difference procedure (*P*=0.05).
Figure 2.4. (1997) Percent of wounds infected by side rots, *P. expansum*, and *B. cinerea* on 'Bosc' (A) and 'Anjou' (B) pears treated at 1 day or 3 weeks before harvest with various biocontrol agents.

Different letters within sections of graphs represent significant differences according to Fisher's Least Significant Difference procedure (*P*=0.05).
that most postharvest decays occur at wounds caused during harvest or packaging of the fruit (Sugar and Spotts, 1993) indicates a need to provide biocontrol agents to the wounds as soon as possible in order to afford the agents the best chance to out-compete pathogens for nutrients at the wound site and colonize it, thus reducing the risk of decay. If the agent were applied prior to harvest, it could have the ability to immediately colonize wounds that occur during the harvest, thus shortening even further the window of time in which a pathogen may be able to initiate decay.

Results from both 1996 and 1997 show that the yeasts *C. infirmo-miniatus*, *C. laurentii*, and *R. glutinis* are able to survive on the surface of the fruit at high, stable population levels for at least three weeks before harvest under typical hot and dry climate conditions. This rate of survival indicates a good chance for decay control using a preharvest application of the biocontrol agents. Failure of the biocontrol agent *C. oleophila* to maintain a stable population on the fruit surface for a long period of time prior to harvest indicates that it would not be suitable for preharvest applications with the goal of postharvest decay control.

Colonies which appeared to be those of yeast which were used in the treatments made up the large majority of the populations recovered from the untreated fruit. These yeasts may have been transported to the fruit by spray drift, insects, or splashing during rain. They may also have been an original part of the epiphytic community on the fruit, as no samples were taken prior to spray of the treated trees to determine which species were common epiphytes in the orchard. Chand-Goyal and Spotts (1996b) found that pear orchards in the Pacific Northwest typically support epiphytic yeast populations made up of from 3 to 7 morphologically distinct species. *R. glutinis* was the only of the four species used in this experiment that was found in the survey from the Medford area. Typical yeast populations in their study were found to range from $3.5 \times 10^2$ to $7.4 \times 10^3$ cfu per square cm of fruit surface, depending on orchard location, degree of cultivation, and spray practices.

*C. infirmo-miniatus* was the only biocontrol agent which consistently provided significant decay control in this study. This is puzzling, as both *C. laurentii* and *R. glutinis* were recovered from the fruit three weeks after they were applied and had viable
population levels equal to *C. infirmo-miniatus*. It may be that these yeasts become dormant after being exposed to field conditions for long periods, and while able to grow on a relatively rich culture medium, are unable to colonize fruit wounds.

The mechanism by which decay control is provided by the biocontrol agents is not fully understood. However, evidence has shown that the mode of action of several yeast species used as biocontrol agents does not involve antibiosis but rather competition for nutrients at the wounds sites (Chand-Goyal and Spotts, 1996a). This is an important factor if biocontrol agents are to be registered for use on produce, as production of antibiotics by the microorganisms could lead to rejection of the organism as a potential biocontrol due to concerns for human safety (Smilanick, 1994).

Because most decays originate in wounds caused during and immediately after harvest, application of biocontrol agents as close as possible to the time of wounding should provide the agent with the best chance for protecting the fruit. By applying biocontrol agents with the ability to survive on the fruit prior to harvest, it is possible to have active biological control microorganisms on the fruit and ready to colonize wounds which may occur as quickly as possible to exclude decay causing organisms from a critical nutrient base.
References


Chapter 3

Enhancement of Biological Control of Postharvest Pear Decay by Combining Antagonistic Microorganisms with Other Compounds

Abstract

Lab grown yeasts (*Cryptococcus infirmo-miniatus*, *Cryptococcus laurentii*, and *Rhodotorula glutinis*) and the registered biological control products BioSave-110 (EcoScience Corp.) and Aspire (Ecogen Corp.) were applied to wounds on pear fruit in combination with other compounds in an attempt to enhance control of decay caused by *Penicillium expansum*. Captan and thiabendazole fungicides at reduced rates were found to be compatible with most of the biocontrol agents and these combinations provided the best decay control. Other effective compounds were calcium chloride and chitosan. L-asparagine, L-proline, and 2-deoxy D-glucose did not provide significant decay control when used alone or in combination with biocontrol agents.
Introduction

Postharvest pathogens cause major economic losses to fruit producers throughout the world. Because a harvested commodity has a higher value than does the same produce in the field, due to input, harvest, and storage costs (Wilson and Pusey, 1985), there is a great need for effective decay control options. Blue mold, caused by the fungus *Penicillium expansum*, is one of the major decays found in stored apples and pears (Pierson *et al.*, 1971). Fruit growers and packers mainly rely on the use of synthetic fungicides for control of postharvest decays, but the development of fungicide-resistant pathogen strains (Spotts and Cervantes, 1986) and loss of registration for some fungicides have contributed to an increasing interest in alternative control methods.

One of these methods is the use of microorganisms which colonize potential decay sites and exclude decay-causing organisms, either through nutrient competition or by the production of antimicrobial compounds. A bacterium, *Pseudomonas syringae* strain ESC-11 (active component of BioSave-110; EcoScience Corp.), and a yeast, *Candida oleophila* strain I-182 (active component of Aspire; Ecogen Corp.), are two microorganisms which have been found to show biocontrol properties (Janisiewicz and Marchi, 1987 and Hofstein *et al.*, 1994, respectively) and are registered for postharvest use on pears. There are several other microorganisms which have been isolated from the surfaces of apples or pears and have been identified as good candidates for biological control agents. *Cryptococcus laurentii* strain RR87-108 (Roberts, 1990), *C. infirmominutus* strain YY6, and *Rhodotorula glutinis* strain HRB6 (Chand-Goyal and Spotts, 1996) are epiphytic yeasts which, when inoculated with pathogen spores into wounds on fruit, can provide good control of decay.

Despite the successes with microbial antagonists, even the best results attainable with these agents often do not match the efficacy or consistency of synthetic fungicides against some pathogens (Pusey, 1994). Until such time as biological control by microbial antagonists demonstrates the same level and consistency of control as registered fungicides, it may not be widely accepted by fruit growers and packers. Disease control comparable to that attained using chemical fungicides might only be attainable through integration of two or more alternative control methods.
Because of this inconsistency, one strategy to enhance biological control has been to combine the biocontrol agent, when applied after harvest, with compounds which either inhibit the pathogen's growth or enhance the biocontrol agent's growth. Biocontrol agents can be combined with rates as low as three percent of the recommended label rate of thiabendazole (TBZ), and provide control as good as or better than use of the full rate of TBZ alone against TBZ-sensitive pathogens (Sugar et al., 1994; Chand-Goyal and Spotts, 1996). McLaughlin et al. (1990) demonstrated that calcium chloride, when combined with various yeasts, significantly increased the level of disease control over that of either the yeast or CaCl₂ alone. The concentration of yeast needed to gain disease control was also much lower when the yeasts were combined with CaCl₂. Janisiewicz et al. (1992) screened several carbohydrates and nitrogenous compounds for both germination and growth inhibition of pathogens, and growth enhancement of P. syringae. Addition of the amino acids L-asparagine and L-proline caused increased population size of the biocontrol agent and enhanced control. Sugar analogs, including 2-deoxy-D-glucose, have also been shown to inhibit the growth of B. cinerea and P. expansum (El Ghaouth et al., 1995). 2-d-D-glucose was found by Janisiewicz (1994) to enhance the biocontrol activity of P. syringae and the yeast Sporobolomyces roseus. Chitosan, a bi-product of the seafood industry, has been suggested as a fruit coating for increasing fruit storage life (El Ghaouth et al., 1992; Wilson et al., 1994). Chitosan was found to decrease fruit decay in two ways. First, it was shown to inhibit the growth of various fungal pathogens, including B. cinerea, Alternaria alternata, and Rhizopus stolonifer, by causing cellular leakage of amino acids and proteins, and morphological changes in the hyphae. Second, chitosan has been shown to induce defense responses in plants to fungal pathogens (El Ghaouth, 1994).

The objectives of these experiments were to determine if compounds which had been previously reported to enhance biological control were compatible with some more recently discovered biocontrol candidates, and determine if they could enhance the ability of new candidates and currently registered biocontrol products to reduce storage decay of pears.
Materials and Methods

**Pathogen culture and inoculation**

The postharvest pathogen *P. expansum* was grown on potato dextrose agar (PDA) plates, and propagated by transferring agar disks to new plates. Plates of the sporulating fungus were flooded with sterile distilled water and scraped with a sterilized glass rod to remove conidia. The spore washes were then placed in a sonicating bath to break up conidial chains. The concentration was counted by hemacytometer, and applied to the wounds on fruit at the rate of $10^3$ conidia/ml.

**Culture and preparation of biocontrol agents**

Two commercially available biocontrol agents were used. BioSave-110 (*P. syringae*; EcoScience Corp.) and Aspire (*C. oleophila*; Ecogen Corp.) were applied at the rate indicated on the label (1.6 g/liter and 3.0 g/liter, respectively). The yeasts *C. infirmo-miniatus* and *R. glutinis* were obtained from R. A. Spotts of the Mid-Columbia Agricultural Research and Extension Center in Hood River, OR. *C. laurentii* was obtained from R. G. Roberts of USDA in Wenatchee, WA. These yeasts were stored at -20 C in YMDB (yeast malt dextrose broth: 3 g malt extract, 3 g yeast extract, 5 g peptone, and 10 dextrose per liter of medium). The cultures were activated by pouring 1 ml of thawed suspension into 75 ml of YMDB. The suspensions were then incubated on a shaker for 2 days at room temperature. After incubation, 0.1 ml aliquots were spread on petri plates containing yeast malt dextrose agar (YMDA: YMDB plus 18 g bactoagar per liter) and incubated for 2-3 days at room temperature. The cells were then scraped from the plates with a sterile rubber spatula and suspended in sterile distilled water. Cell concentrations were then adjusted to $3 \times 10^8$ (*C. laurentii*) and $1 \times 10^8$ (*C. infirmo-miniatus* and *R. glutinis*) cfu per milliliter (2% transmittance at 595 and 540 nm, respectively) using a Spectronic 20 spectrophotometer. Spectrophotometric determinations of yeast concentrations were confirmed by dilution plating.
**Treatments-1996 experiments**

L-asparagine (Sigma Chemical Co.) was added to each yeast suspension at a concentration of 80 mM. L-proline (Sigma Chemical Co.) also was used at 80 mM. 2-d-D-glucose (Sigma Chemical Co.) was used at a concentration of 1% (w/v). Calcium chloride (J. T. Baker Chemical Co.) was prepared at a concentration of 2% (w/v). Crab shell chitosan (Sigma) was ground and dissolved in 0.25 N HCl. After the chitosan was dissolved, the pH of the solution was adjusted to 5.6 with 1 N NaOH, and the final concentration of the chitosan was adjusted to 2% (w/v). The chitosan solution was then autoclaved (15 minutes at 120 C) to sterilize. Captan fungicide (Captan 50-WP; Micro Flo Co.) was used at the label rate (3.0 g/liter), and TBZ (Mertect 340F; Merck & Co.) was used at 100 ppm (label rate is 570 ppm).

Biocontrol organisms, pathogen spores, and chemical treatments were mixed in test tubes at the appropriate concentrations. Each biocontrol agent was combined with each additive, with the exception of BioSave-110 and chitosan, which would not mix into a uniform suspension.

**Treatments-1997 experiments**

Materials which showed effective decay control in 1996 were evaluated in greater detail in 1997 experiments, using various concentrations of the compounds.

Chitosan was prepared as described above, and added at 2% (w/v), 1%, or 0.5%. CaCl₂ was also used at 2% (w/v), 1%, or 0.5%. Captan was added at either 100% of label rate, 50%, or 25%. The asparagine, proline, and glucose treatments were not used in 1997. All combinations of biocontrol strains and chemical treatments were used except BioSave-110 and chitosan.

**Fruit**

Pear fruit (cv. 'Bosc') were grown under a commercial spray program at the Southern Oregon Research and Extension Center in Medford, OR. Fruit were harvested at normal maturity in mid-September and stored in air at -1 C until the experiments were performed. The fruit were surface sterilized by soaking for five minutes in a 0.14%
solution of sodium hypochlorite, rinsed with tap water, sprayed with 70% ethanol, and allowed to dry. Each fruit received five wounds (6 mm diameter x 3 mm deep) made with the sterile head of a finishing nail. Each Wound was inoculated by micropipette with 40 ul of a mixed suspension of biocontrol agent, pathogen, and chemical treatment. In 1996 experiments, ten fruit received each treatment, with each fruit being one replicate. In 1997, each treatment had five replicates of ten fruit each. The fruit were placed on fiberboard trays and wrapped in perforated polyethylene bags. Trays of inoculated fruit from various treatments were placed randomly into boxes and were stored in air at -1 C for approximately eight weeks, at which time infected wounds were counted and lesion diameters were measured with Vernier calipers.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) for treatment differences. Fisher's least significant difference test was used to determine significant differences in treatment means.

Multiple regression analysis was used to determine the significance of biocontrol treatments and the effects of different rates of chemical treatments in reducing average lesion sizes, and also to determine significant interactions between biocontrols and chemical treatments.

Results

1996

In 1996 tests, treatment with captan or TBZ (100 ppm), with or without biocontrol agents, consistently resulted in smaller average lesion sizes (Figure 3.1) and lower incidence of decay (Figure 3.2) than treatments with biocontrols alone. L-asparagine, L-proline, and 2-d-D-glucose all failed to provide greater protection against decay when combined with biocontrol agents, as compared to use of biocontrol agents alone. Treatment with CaCl2 resulted in lower incidence and severity of decay when combined with C. laurentii. Fruit treated with chitosan had smaller average lesion size
Figure 3.1. Average lesion diameter caused by *P. expansum* on pears treated with a combination of various compounds and biocontrol agents: Untreated control (A), BioSave-110 (B), Aspire (C), *R. glutinis* (D), *C. laurentii* (E), or *C. infirmo-miniatus* (F). Different letters within a graph represent significant differences according to Fisher's LSD procedure ($P=0.05$)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lesion size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>a</td>
</tr>
<tr>
<td>Asparagine</td>
<td>a</td>
</tr>
<tr>
<td>Proline</td>
<td>a</td>
</tr>
<tr>
<td>2-d-D Glucose</td>
<td>a</td>
</tr>
<tr>
<td>CaCl2</td>
<td>a</td>
</tr>
<tr>
<td>Chitosan</td>
<td>b</td>
</tr>
<tr>
<td>Captan</td>
<td>c</td>
</tr>
<tr>
<td>TBZ</td>
<td>bc</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lesion size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>a</td>
</tr>
<tr>
<td>Asparagine</td>
<td>a</td>
</tr>
<tr>
<td>Proline</td>
<td>a</td>
</tr>
<tr>
<td>2-d-D Glucose</td>
<td>ab</td>
</tr>
<tr>
<td>CaCl2</td>
<td>a</td>
</tr>
<tr>
<td>Chitosan</td>
<td>bc</td>
</tr>
<tr>
<td>Captan</td>
<td>c</td>
</tr>
<tr>
<td>TBZ</td>
<td>c</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lesion size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>a</td>
</tr>
<tr>
<td>Asparagine</td>
<td>a</td>
</tr>
<tr>
<td>Proline</td>
<td>ab</td>
</tr>
<tr>
<td>2-d-D Glucose</td>
<td>ab</td>
</tr>
<tr>
<td>CaCl2</td>
<td>b</td>
</tr>
<tr>
<td>Chitosan</td>
<td>b</td>
</tr>
<tr>
<td>Captan</td>
<td>b</td>
</tr>
<tr>
<td>TBZ</td>
<td>b</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lesion size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>a</td>
</tr>
<tr>
<td>Asparagine</td>
<td>a</td>
</tr>
<tr>
<td>Proline</td>
<td>a</td>
</tr>
<tr>
<td>2-d-D Glucose</td>
<td>a</td>
</tr>
<tr>
<td>CaCl2</td>
<td>a</td>
</tr>
<tr>
<td>Chitosan</td>
<td>a</td>
</tr>
<tr>
<td>Captan</td>
<td>a</td>
</tr>
<tr>
<td>TBZ</td>
<td>a</td>
</tr>
</tbody>
</table>
Figure 3.2. Incidence of decay caused by *P. expansum* on pears treated with a combination of various compounds and biocontrol agents: Untreated control (A), BioSave-110 (B), Aspire (C), *R. glutinis* (D), *C. laurentii* (E), or *C. infirmo-miniatu*s (F). Different letters within a graph represent significant differences according to Fisher's LSD procedure (*P*=0.05).
and lower incidence of decay when chitosan was combined with Aspire or *C. laurentii*, and when used alone, as compared to biocontrol only or no treatment.

**1997**

Fruit inoculated with all rates of captan generally had lower levels of decay than those that received biocontrol alone or no control (Figure 3.3). The exception was those fruit inoculated with a combination of captan and *C. laurentii*, which had significantly greater average lesion sizes and incidence of decay than fruit inoculated with *C. laurentii* only. Only the 100% rate of captan with *C. laurentii* did not contribute to significantly larger and more frequent lesions than the treatment with the biocontrol only. According to multiple regression analysis, the use of biocontrol agents did not have a significant effect on lesion size when combined with captan (*P*=0.3899), but the effect of captan was significant (*P*=0.0010). There was no significant interaction between biocontrol and captan (*P*=0.0976) relating to decay severity. Multiple regression analysis indicates that when combined with captan, biocontrol agents caused a slight increase in decay incidence (*P*=0.0437).

Chitosan did not have significant effects on average lesion size or decay incidence when combined with most biocontrol agents (*P*=0.4934 and 0.0753, respectively). Use of biocontrol was significant for both lesion size and incidence (*P*=0.0069 and 0.0096, respectively), and there was no significant interaction between biocontrol and chitosan (*P*=0.6094 and 0.0941). Chitosan only contributed to smaller average lesion sizes and lower decay incidence when used alone or combined with Aspire (Figure 3.4). When it was combined with other yeasts, lesion sizes and decay incidence in several cases were significantly greater than when the yeast was used alone.

Use of CaCl₂, both alone and combined with biocontrol agents, generally resulted in smaller average lesion sizes and lower decay incidence (Figure 3.5). Rates of 0.5%, 1% and 2% CaCl₂ (w/v) were all effective and average lesion sizes were not significantly different between the three rates. Both biocontrol agents and CaCl₂ were significant factors in reducing lesion sizes (*P*=0.0114 and 0.0491, respectively) and incidence (*P*=0.0261 and 0.0468, respectively). There was not a significant interaction between
Figure 3.3. Average lesion size (A) and Incidence of decay (B) caused by *P. expansum* on 'Bosc' pears treated with a combination of various biocontrol agents and different rates of captan.

Different letters within a biocontrol treatment represent significant differences according to Fisher's Least Significant Difference procedure (*P*=0.05).
Figure 3.4. Average lesion size (A) and incidence of decay (B) caused by *P. expansum* on 'Bosc' pears treated with a combination of biocontrol agents and different concentrations of chitosan. Different letters within a biocontrol treatment represent significant differences according to Fisher's Least Significant Difference procedure (*P* = 0.05).
Figure 3.5. Average lesion size (A) and incidence of decay (B) caused by *P. expansum* on 'Bosc' pears treated with a combination of biocontrol agents and different concentrations of calcium chloride. Different letters within a biocontrol treatment represent significant differences according to Fisher's Least Significant Difference procedure (*P*=0.05).
biocontrol agents and CaCl₂ ($P=0.0618$ and 0.9405 for severity and incidence, respectively).

**Discussion**

Interest in the use of biocontrol agents has increased greatly in recent years. In spite of the advances made in biological control there is still a need to make biological control more consistently effective if it is to be accepted as a commercial disease control strategy. One way to do this is to combine biological control agents with other compounds which may enhance the ability of the beneficial microorganisms to control disease causing organisms.

Several compounds which have been reported to enhance the biological control provided by several different microorganisms did not appear to have a beneficial effect when used in combination with biocontrol agents in these tests. L-asparagine and L-proline were both reported by Janisiewicz *et al.* (1992) to significantly increase the population levels of the microbial antagonist *P. syringae*, and contribute to enhance biological control. However, when these two amino acids were used in our experiments in 1996, either alone or in combination with biocontrol agents, the result was either larger lesion sizes or no significant difference from controls. There may be several reasons for this difference in results. First, *P. syringae* is a bacterium, and most likely has much different nutritional requirements than the yeasts used in this experiment. The strain of *P. syringae* used in this experiment was also different from the strain used by Janisiewicz *et al.* (1992). Second, while both experiments used the pathogen *P. expansum*, different strains of the pathogen may have different nutritional requirements, and the strain used in this experiment may have been more efficient at using L-asparagine and L-proline, thus giving it an advantage. Third, Janisiewicz *et al.* (1992) used Golden Delicious apples as the medium for the experiment. Apples and pears, while similar, may provide a growth medium such that a compound which provides nutritional enhancement on one commodity may have no effect or a negative effect on biocontrol agents applied to the other.
2-deoxy-D-glucose is another additive that has been previously reported to be fungicidal (El Ghaouth et al., 1995) and to enhance biological control (Janisiewicz, 1994). In these experiments, however, it had no effect on either lesion size or decay incidence, when applied alone or with biological controls. In both previous reports, however, experiments were done on apple, and the pears used in this experiment may have had an impact on the effectiveness of some compounds.

Chitosan was also identified in previous work as a potential agent for disease control, and is reported to work both by fungal inhibition and by induced host resistance (El Ghaouth, 1994). In those experiments, chitosan was applied as a coating, and not inoculated directly into the wounds as reported here. When we used chitosan alone, it did significantly reduce average lesion sizes and decay incidence. However, when combined with biocontrol agents, the results were usually not significantly different from those obtained with untreated fruit. This indicates that chitosan may have a negative effect on the biocontrol agents, and when used in combination with them, neither are able to inhibit decay.

Captan was generally effective at reducing both incidence and severity of decay, when used alone and in combination with most biocontrol agents in these experiments. Although registered, captan is not widely used as a postharvest fungicide for pears, and may present an alternative to the more commonly used thiabendazole. Captan was not compatible with the biocontrol agent C. laurentii, however, and its effectiveness when combined with biocontrol agents should be studied further.

Fruit treated with a combination of CaCl₂, at several rates, and biocontrol agents had significantly lower severity and incidence of decay than untreated fruit. Other studies have shown that CaCl₂ applied during the growing season can also be a significant factor in the reduction of postharvest decay (Sugar et al., 1994). It appears that the mechanism of CaCl₂ function may be different when applied at different times, as CaCl₂ had inconsistent effects on decay when it was applied without an accompanying biocontrol agent. Conway (1982) found that dipping of harvested apples in CaCl₂ concentrations as high as 8% did not reduce decay. McLaughlin et al. (1990) speculated
that there may be an interaction between CaCl$_2$ and biocontrol agents in the wound site. While there was some evidence of interaction between the biocontrol agents and CaCl$_2$ in this study, it was not statistically significant.

The use of pathogen-inhibiting or biocontrol-enhancing compounds in combination with biological control practices appears to be a promising method for increasing the efficacy of biocontrol agents to more commercially acceptable and more consistent levels. Several compounds have been identified which are able to enhance the performance of biological controls. However, it appears to be important that these compounds be tested with specific microorganisms and crops before they are combined in commercial formulations.
References


Chapter 4

High Carbon Dioxide Controlled Atmosphere Storage Combined with Biocontrol Agents to Reduce Postharvest Decay of Pear

Abstract

Lab grown yeasts (Cryptococcus laurentii, C. infirmo-miniatius, and Rhodotorula glutinis) and the registered biocontrol products BioSave-110 and Aspire were applied to 'Bosc' and 'Anjou' pears by linespray and stored for 0, 2, 4, or 6 weeks in atmospheres of 2% O₂/3% CO₂, 5% O₂/12% CO₂, or 5% O₂/20% CO₂ before transfer to normal controlled atmosphere (2% O₂/<0.5% CO₂). Exposure to high CO₂ atmospheres caused significant reductions in gray mold caused by Botrytis cinerea, with further decay inhibition provided by biocontrol agents. High CO₂ treatments alone had no significant effect on blue mold decay caused by Penicillium expansum. There was no indication of CO₂ damage to the treated fruit.
Introduction

Postharvest spoilage of harvested fruit is a major expense in food production. Most fruit growers and packers rely on chemical control for disease prevention. However, resistance of microorganisms to fungicides (Spotts and Cervantes, 1986), the loss of chemical fungicides due to bans or withdrawal of postharvest usage (National Research Council, 1987), and the resistance of some markets to pesticide use (Spotts et al., 1992) have all combined to intensity interest in alternative control strategies.

In pears (*Pyrus communis* L.), the majority of postharvest disease is initiated at wounds which occur during harvest and packing, and subsequent infection at the wound by one of several pathogens (Sugar and Spotts, 1993; Spotts et al., 1998). *Botrytis cinerea* Pers.:Fr., causal agent of gray mold, and *Penicillium expansum* Link, causal agent of blue mold, are most destructive pathogens of stored pears (Pierson et al., 1971).

Controlled atmosphere (CA) storage has been used for many years to increase the storage life of fruit. At low oxygen levels, the fruit's respiration rate is greatly reduced. As a result, the fruit remain "fresh" much longer, enabling the marketing period to be extended by several months. Allen and Claypool (1948) found that Bartlett pears stored in CA (1% O_2/<1% CO_2) retained market quality 78% longer than fruit stored in an ambient atmosphere at the same temperature. Hansen (1957) showed the benefit of low oxygen environments for the preservation of dessert quality of 'Anjou' pears. While it has also been shown that increased CO_2 levels have a beneficial effect on fruit quality, very high CO_2 levels can cause internal and external fruit damage (Hansen and Mellenthin, 1962; Lau and Looney, 1978). Hansen and Mellenthin (1962) demonstrated that CO_2 damage was related to length of exposure, CO_2 concentration, O_2 concentration, and fruit condition. Further research has shown that short term exposure to high CO_2 levels followed by CA storage or ambient air storage may not damage the fruit and often has a positive effect on the fruit condition (Couey and Wright, 1977; Ke et al., 1990). Wang and Mellenthin (1975) found that short term exposure of 'Anjou' pears to high CO_2 levels increased the fruit's storage life by reducing respiration and ethylene production, and caused increased fruit firmness and better ability to ripen after storage. Pears could be treated for 2 or 4 weeks with 12%
CO₂ before transfer to conventional cold storage in air without showing any signs of injury, but treatment of high CO₂ for 6 weeks did cause injury. Fruit exposed to 26% CO₂ for 2 weeks also developed CO₂ injury.

In addition to its positive effects on fruit quality, increased CO₂ levels have been shown to have a negative effect on the growth of some pathogens. High CO₂ atmospheres can inhibit growth and sporulation of fungi in vitro, including *B. cinerea* and *P. expansum* (Littlefield *et al.*, 1966; Wells and Uota, 1970). Sitton and Patterson (1992) showed that carbon dioxide levels of 2.8%-12% significantly reduced lesion development by *B. cinerea* and other pathogens on 'McIntosh', 'Red Delicious', and 'Golden Delicious' apples. High CO₂ CA has also been shown to reduce storage decays in blueberries (Ceponis and Cappellini, 1985), muskmelons (Stewart, 1979), and strawberries (Couey and Wells, 1970).

Several non-pathogenic microorganisms have been discovered which are able to colonize fruit wounds and compete with and exclude pathogens from those sites. *Cryptococcus infirmo-miniatus* strain YY6 and *Rhodotorula glutinis* strain HRB6 (Chand-Goyal and Spotts, 1996), and *Cryptococcus laurentii* RR87-108 (Roberts, 1990) are all yeasts which were isolated from the surfaces of pear or apple fruits. These yeasts each have provided good control of rots caused by *B. cinerea* and *P. expansum* when inoculated into wounds on pear and apple after harvest, in combination with pathogens (Roberts, 1990; Sugar *et al.*, 1994; Chand-Goyal and Spotts, 1996). Two products which are currently registered for postharvest decay control in pear are BioSave-110 (EcoScience Corp.) which contains the bacterium *Pseudomonas syringae* strain ESC-11, and Aspire (Ecogen Corp.), which contains the yeast *Candida oleophila* strain I-182. Both organisms have been shown to provide decay control in fruit (Janisiewicz and Marchi, 1992, and Hofstein *et al.*, 1994).

The purpose of this work was to evaluate the control of blue mold and gray mold provided by the combination of short term high CO₂ storage regimes and various biocontrol agents, both currently registered and those being developed.
Materials and Methods

**Culture and preparation of biocontrol agents**

The commercial products BioSave-110 and were stored according to package recommendations and were used at the label rates (1.6 g/liter and 3.0 g/liter, respectively). Cultures of *Rhodotorula glutinis* and *Cryptococcus infirmo-miniatus* were provided by R. A. Spotts of the Mid-Columbia Agricultural Research and Extension Center in Hood River, OR. A culture of *Cryptococcus laurentii* was provided by R. G. Roberts of USDA in Wenatchee, WA. The cultures were stored at -20°C in yeast malt dextrose broth (YMDB: 3 g malt extract, 3 g yeast extract, 5 g peptone, and 10 dextrose per liter of medium) until use. The yeasts were activated by dispensing 1 ml of thawed suspension into 75 ml of sterile YMDB in a 250 ml flask. After two days of incubation at room temperature on a shaker, 0.1 ml aliquots of yeast suspension were placed on petri plates containing yeast malt dextrose agar (YMDA: YMDB with 18 g/liter bacto-agar). These plates were incubated for two days at room temperature. The yeast colonies were removed by scraping the plates with a sterile rubber spatula and the yeast were suspended in sterile distilled water. *C. laurentii* suspensions were adjusted to 2% transmittance at 595 nm using a spectrophotometer to give a concentration of 2.8-3.3 x 10^8 cfu per ml. Suspensions of *C. infirmo-miniatus* and *R. glutinis* were adjusted to 2% transmittance at 550 nm to give a concentration of 1.0-1.5 x 10^8 cfu per ml. Spectrophotometric determinations of yeast concentrations were confirmed by dilution plating.

**Pathogen culture and inoculation**

Spore suspensions of *B. cinerea* and *P. expansum* (originally isolated from lesions on pears in Medford, OR) were made from sporulating 2-4 week old cultures of the fungi growing on potato dextrose agar. In 1996, *B. cinerea* was the only pathogen used in the experiments. In 1997, both *B. cinerea* and *P. expansum* were used. Sterile distilled water was added to each plate and the spores were brushed loose using a sterile glass rod. The spore suspension was then placed in a sonicating bath to break spore chains, and the spores were counted using a hemacytometer. An appropriate amount of
the concentrated spore suspension was added to the biocontrol agent suspensions to give a final pathogen spore concentration of $1 \times 10^4$ spores per ml.

**Fruit**

Pear fruit (cv. 'Bosc' and 'Anjou') grown at the Southern Oregon Research and Extension Center in Medford, Oregon were harvested at commercial maturity in mid-September in 1996 and 1997. The fruit were stored in air at -1 C in cardboard boxes lined with perforated polyethylene liners until use. Healthy-appearing fruit were selected for the experiments. The pears were surface-sterilized in a solution of 0.14% sodium hypochlorite for 5 minutes and rinsed with tap water. Each fruit then received five wounds (6 mm diameter x 3 mm deep) with the sterile head of a finishing nail, and was inoculated with a mixture of pathogen and biocontrol agent by linesprayer. Control fruit received linesprays of pathogen spore suspensions only. After inoculation, the fruit were placed in perforated polyethylene bags in cardboard boxes and put into CA storage. The fruit inoculated with *B. cinerea* were evaluated after 10 weeks of storage for decay incidence (percent of wounds decayed) and decay severity (average lesion diameter measured by vernier calipers). Fruit inoculated with *P. expansum* were similarly evaluated after 16 weeks of storage. To evaluate for CO$_2$ damage, fruit were ripened at room temperature for 5-7 days. After ripening, they were sliced perpendicular to the stem/calyx axis and visually inspected for internal browning and pitting.

**Storage atmospheres**

Low-oxygen atmospheres were created in sealed rooms at the Southern Oregon Research and Extension Center using a Prism nitrogen generator (Permea Inc., St. Louis MO). Bottled carbon dioxide was used to increase the CO$_2$ levels of the storage rooms. Storage atmospheres were monitored with a Servomex 1450B3 O$_2$/CO$_2$ analyzer (Servomex Company, Inc., Norwood MA) and a Nova portable CO$_2$ and O$_2$ analyzer (Nova Analytical Systems, Inc., Niagara Falls, NY). All rooms were kept at a temperature of -1 C ( +/- 0.5 C).
In 1996, fruit were stored in high CO$_2$ atmospheres of 5% O$_2$/12% CO$_2$ or 2% O$_2$/3% CO$_2$, or normal CA with 2% O$_2$/<0.5% CO$_2$. Carbon dioxide levels in normal CA were maintained below 0.5% by periodic purging of the storage room with 2% O$_2$/98% N$_2$. High CO$_2$-treated fruit were held in the high CO$_2$ atmospheres for 0, 2, 4, or 6 weeks before being moved to normal CA for the remainder of the storage period.

In 1997, the treatment of 2% O$_2$/3% CO$_2$ was dropped, and a treatment of 5% O$_2$/20% CO$_2$ was added. Treatment timings and other storage atmospheres remained the same as 1996.

**Statistical analysis**

The data were analyzed using Statgraphics 2.0 for regression analysis, analysis of variance (ANOVA) and Fisher's least significant difference procedure. Effects of individual biocontrol treatments in terms of percent reduction of decay were determined by dividing the treatment effect estimate from multiple regression analysis by the decay constant for that treatment.

**Results**

According to multiple regression analysis, interaction terms between the storage atmospheres and biocontrol agents were not significant (data not shown). There was also no fruit damage which could be attributed to CO$_2$ levels in this experiments (data not shown).

**1996 results**

3% CO$_2$

Storage of pears for up to six weeks in 2% O$_2$/3% CO$_2$ did not cause significant reductions in either decay incidence or severity for non-biocontrol treated 'Anjou' (P=1.0 and 0.998, respectively) or 'Bosc' pears (P=1.0 and 0.280, respectively).

On 'Anjou' pears stored in 3% CO$_2$, treatment with *C. laurentii*, *R. glutinis*, or *C. infirmo-miniatus* caused significant reductions in gray mold incidence (58%, 38%, and 37%, respectively, less average decay than untreated fruit) while treatment with either
BioSave-110 or Aspire did not significantly reduce decay incidence (Figure 4.1). All biocontrol treatments on 'Anjou' pears stored in 3% CO₂ caused significant reductions in disease severity (Figure 4.2). Average lesion diameters were reduced by 45% (C. *infirmo-miniatus*), 37% (BioSave-110), 35% (C. *laurentii*), 31% (R. glutinis), and 23% (Aspire) as compared to the untreated control (Figure 4.2). Storage in 3% CO₂ for up to six weeks generally did not enhance biological control of gray mold decay. The only significant decay control enhancement from the 3% CO₂ storage was on fruit treated with C. *laurentii*, which had significantly lower decay severity than did fruit treated with only the yeast (*P*=0.020).

On 'Bosc' pears, all biocontrol treatments except Aspire caused significant reductions in decay incidence. Decay incidence was reduced by 62% (R. glutinis), 56% (C. *infirmo-miniatus*), 26% (BioSave-110), and 24% (C. *laurentii*) (Figure 4.3). Average lesion diameters were reduced by 82% (C. *infirmo-miniatus*), 80% (R. glutinis), 53% (BioSave-110), and 32% (C. *laurentii*), compared to the untreated control (Figure 4.4). As with 'Anjou' pears, storage of 'Bosc' pears in 3% CO₂ did not enhance the decay control provided by the biocontrol agents. Only fruit treated with C. *laurentii* showed enhanced control of decay incidence and severity when stored in 3% CO₂ (*P*=0.042 and 0.003, respectively).

**12% CO₂**

Storage of pears in an atmosphere of 5% O₂/12% CO₂ caused significant reductions in postharvest decay caused by B. cinerea. Regression analysis showed a significant effect of 12% CO₂ on decay incidence and severity for non-biocontrol treated fruit of both 'Anjou' (*P*=0.015 and *P*<0.001, respectively) and 'Bosc' (*P*<0.001 for both incidence and severity) varieties. On non-biocontrol treated 'Anjou' pears, decay incidence was reduced by an average of 8% per week stored in 12% CO₂, and decay severity was reduced by 11% per week. On 'Bosc' pears, decay incidence was reduced by 14% per week, while decay severity was reduced by 20% per week.

For 'Anjou' pears, C. *laurentii* (60.0% less decay), C. *infirmo-miniatus* (46%), and R. glutinis (34%) all contributed to lower decay incidence than on untreated 'Anjou'
Figure 4.1. Incidence of decay caused by *B. cinerea* on 'Anjou' pears treated with (A) no biocontrol agent, (B) Aspire, (C) BioSave-110, (D) *R. glutinis*, (E) *C. laurentii*, or (F) *C. infirmo-minaitus*, and stored in high-CO₂ atmospheres in 1996.
Figure 4.2. Average sizes of lesions caused by *B. cinerea* on 'Anjou' pears treated with (A) no biocontrol agent, (B) Aspire, (C) BioSave-110, (D) *R. glutinis*, (E) *C. laurentii*, or (F) *C. infirmo-minaitus*, and stored in high-CO₂ atmospheres in 1996.
Figure 4.3. Incidence of decay caused by *B. cinerea* on 'Bosc' pears treated with (A) no biocontrol agent, (B) Aspire, (C) BioSave-110, (D) *R. glutinis*, (E) *C. laurentii*, or (F) *C. infirmo-minitus*, and stored in high-CO$_2$ atmospheres in 1996.
Figure 4.4. Average sizes of lesions caused by *B. cinerea* on 'Bosc' pears treated with (A) no biocontrol agent, (B) Aspire, (C) BioSave-110, (D) *R. glutinis*, (E) *C. laurentii*, or (F) *C. infirmo-minaitus*, and stored in high-CO$_2$ atmospheres in 1996.
pears. Aspire and BioSave-110 did not affect decay incidence. Average lesion diameters were reduced by treatment with C. *infirmo-miniatus* (52%) and C. *laurentii* (36%). BioSave-110, *R. glutinis*, and Aspire did not have significant effects on lesion diameter for 'Anjou' pears stored in 12% CO₂.

Storage in 12% CO₂ enhanced biological control of disease severity on 'Anjou' pears treated *C. laurentii* (*P*=0.008), and *C. infirmo-miniatus* (*P*=0.004). Biological control of decay incidence on 'Anjou' pears was not enhanced by storage in 12% CO₂.

'Bosc' pears treated with the biocontrol agents *C. infirmo-miniatus* and *R. glutinis* had a 46% and 39% lower incidence of decay, respectively, than did untreated fruit. The same treatments resulted in 58% and 40% reductions in average lesion sizes on 'Bosc' fruit stored in 12% CO₂. Treatment with *C. laurentii*, BioSave-110, or Aspire did not significantly affect either incidence or severity.

Biological control of gray mold incidence provided by *R. glutinis* and *C. infirmo-miniatus* on 'Bosc' pears was enhanced by storage in 12% CO₂ (*P*<0.001 for both). Control of decay severity by the same two yeasts was also enhanced by 12% CO₂ storage (*P*=0.003 and <0.001, respectively).

1997 results—*B. cinerea*

12% CO₂

Treatment in 1997 with 5% O₂/12% CO₂ also resulted in a significant decrease in gray mold on both varieties of pear. Decay incidence and decay severity on non-biocontrol treated 'Anjou' pears were reduced by 8% and 11% per week stored in 12% CO₂, respectively (*P*<0.001 for both). Incidence and severity of decay on 'Bosc' pears were also significantly reduced by 12% CO₂ storage (*P*<0.001 for both), with average decreases of 10% and 12% per week, respectively.

'Anjou' pears treated with biocontrol agents and stored in 12% CO₂ had reduced gray mold decay incidences of 77% (*C. laurentii*), 71% (*R. glutinis*), 71% (*BioSave-110*), and 52% (*C. infirmo-miniatus*) (Figure 4.5). Lesion diameter was reduced on average by 78% (*C. laurentii*), 61% (*R. glutinis*), 58% (*BioSave-110*), and 39% (*C.
Figure 4.5. Incidence of decay caused by *B. cinerea* on 'Anjou' pears treated with (A) no biocontrol agent, (B) Aspire, (C) BioSave-110, (D) *R. glutinis*, (E) *C. laurentii*, or (F) *C. infirmo-minaitus*, and stored in high-CO₂ atmospheres in 1997.
infirmo-miniatus) in this atmosphere (Figure 4.6). Aspire had no significant effect on either lesion diameter or decay incidence.

Biocontrol of decay incidence in 'Anjou' by C. infirmo-miniatus was enhanced by storage in 12% CO₂ (P<0.001). Control of decay incidence by BioSave-110, R. glutinis, and C. laurentii was not significantly enhanced. Control of average lesion diameter by R. glutinis (P=0.005), C. laurentii (P=0.022) and C. infirmo-miniatus (P<0.001) was enhanced by 12% CO₂, while control by BioSave-110 was not.

Treatment of 'Bosc' pears with C. laurentii or C. infirmo-miniatus in 12% CO₂ resulted in a reduction in gray mold incidence of 38% and 29% respectively (Figure 4.7). These yeasts reduced average decay severity by 32% and 23% respectively (Figure 4.8). BioSave-110, Aspire, and R. glutinis did not have significant effects on either decay incidence or severity in 'Bosc' pears stored in 12% CO₂ in 1997. Control of both disease incidence and severity by C. laurentii (P<0.001 for both) and C. infirmo-miniatus (P<0.001 for both) were enhanced in 12% CO₂.

20% CO₂

Exposure to atmospheres of 20% CO₂/5% O₂ caused significant reductions in incidence of decay caused by B. cinerea. Storage in 20% CO₂ resulted in significant decreases in both decay incidence and severity on non-biocontrol treated 'Anjou' pears (P<0.001 for both). Decay incidence on 'Anjou' pears was reduced by an average of 18% per week, and decay severity was reduced by 21% per week. Non-biocontrol treated 'Bosc' pears also had significantly lower incidence and decay severity when stored in 20% CO₂ (P<0.001 for both). Decay incidence was reduced by 16% per week, and severity was reduced by an average of 18% per week stored in 20% CO₂. All treatments on both pear varieties had zero decay incidence when stored in 20% CO₂ for six weeks.

'Anjou' pears stored in 20% CO₂ also had significantly reduced decay incidence when treated with any of the five biocontrol agents. Gray mold incidence was reduced by an average of 83% (C. laurentii), 74% (R. glutinis), 73% (BioSave-110), 56% (C. infirmo-miniatus), and 24% (Aspire). Disease severity was reduced by 73% (C. laurentii), 61% (R. glutinis), 50% (BioSave-110), and 47% (C. infirmo-miniatus).
Figure 4.6. Average sizes of lesions caused by B. cinerea on 'Anjou' pears treated with (A) no biocontrol agent, (B) Aspire, (C) BioSave-110, (D) R. glutinis, (E) C. laurentii, or (F) C. infirmo-minaitus, and stored in high-CO$_2$ atmospheres in 1997.
Figure 4.7. Incidence of decay caused by *B. cinerea* on 'Bosc' pears treated with (A) no biocontrol agent, (B) Aspire, (C) BioSave-110, (D) *R. glutinis*, (E) *C. laurentii*, or (F) *C. infirmo-minaitus*, and stored in high-CO$_2$ atmospheres in 1997.
Figure 4.8. Average sizes of lesions caused by *B. cinerea* on 'Bosc' pears treated with (A) no biocontrol agent, (B) Aspire, (C) BioSave-110, (D) *R. glutinis*, (E) *C. laurentii*, or (F) *C. infirmo-minaitus*, and stored in high-CO₂ atmospheres in 1997.
Aspire was the only treatment which did not provide a significant reduction in lesion size. Biological control of both decay incidence and severity by BioSave-110 ($P=0.0008$), *R. glutinis* ($P<0.001$), *C. laurentii* ($P<0.001$), and *C. infirmo-miniatus* ($P<0.001$) was enhanced by storage in 20% CO₂. Control of decay incidence by Aspire also was enhanced ($P<0.001$).

Decay incidence of 'Bosc' pears was significantly reduced by treatment with *C. laurentii* (43%) and *C. infirmo-miniatus* (30%). Disease severity was also significantly lower with the same treatments (35% and 27% respectively). Treatment with *R. glutinis*, BioSave-110, or Aspire did not cause significant reductions in either incidence or severity. Biological control of both decay incidence and severity by *C. laurentii* ($P<0.001$ for both) and *C. infirmo-miniatus* ($P<0.001$ for both) was enhanced by 20% CO₂ storage.

1997 results—*P. expansum*

12% CO₂

Incidence and severity of blue mold decay caused by *P. expansum* in 'Bosc' pears were not significantly affected by storage in 12% CO₂/ 5% O₂ alone ($P=0.683$ and 0.182, respectively). The biocontrol agents *C. infirmo-miniatus* and *C. laurentii* reduced the incidence of decay by an average of 34% and 26%, respectively in this atmosphere (Figure 4.9). All other treatments did not have a significant effect on decay incidence (Figure 4.9), and only *C. infirmo-miniatus* reduced the average lesion size significantly (19%) (Figure 4.10). Biological control of blue mold decay was not enhanced by storage in 12% CO₂.

20% CO₂

Storage of 'Bosc' pears in 20% CO₂ for six weeks (Figure 4.10) did not have a significant effect on decay incidence or severity of blue mold decay ($P=0.0741$ and 0.0784, respectively). Treatment with *C. infirmo-miniatus* and *C. laurentii* significantly reduced decay incidence (28% and 24%, respectively). BioSave-110, *R. glutinis*, and Aspire did not cause significant reductions in decay incidence. The only biocontrol
Figure 4.9. Incidence of decay caused by *P. expansum* on 'Bosc' pears treated with (A) no biocontrol agent, (B) Aspire, (C) BioSave-110, (D) *R. glutinis*, (E) *C. laurentii*, or (F) *C. infirmo-minaitus*, and stored in high-CO₂ atmospheres in 1997.
Figure 4.10. Average sizes of lesions caused by *P. expansum* on 'Bosc' pears treated with (A) no biocontrol agent, (B) Aspire, (C) BioSave-110, (D) *R. glutinis*, (E) *C. laurentii*, or (F) *C. infirmo-minaitus*, and stored in high-CO$_2$ atmospheres in 1997.
agent which reduced decay severity was *C. infirmo-miniatus*, which reduced average lesion diameters by 17%. Biological control of blue mold decay was not enhanced by storage in 20% CO₂.

**Discussion**

The combination of biocontrol agents with other disease control strategies shows good potential for enhancing the consistency and efficacy of alternative postharvest disease control. The use of short term, high carbon dioxide storage appears to be compatible with the use of biocontrol agents and provides additional decay control.

Drake (1994) found that storage of 'Anjou' pears for nine months in 3% CO₂ resulted in less stem-end decay. In our experiments, however, exposures of pathogen-inoculated fruit to storage atmospheres of 3% CO₂ for six weeks did not have any significant effect on incidence of decay or severity of decay. Higher CO₂ atmospheres of 12% and 20% both caused significant reductions in decay caused by *B. cinerea*, on both 'Bosc' and 'Anjou' pears. Several other studies have also indicated that high CO₂ could inhibit fungal growth and could be practical method for reducing decay in stored produce (Wells and Uota, 1970; Stewart, 1979; Reyes, 1988; Spalding and Reeder, 1975).

While decay caused by *B. cinerea* was inhibited by high CO₂ levels, there was no apparent effect on another major pathogen, *P. expansum*. Although Littlefield et al. (1966) showed an inhibitory effect of CO₂ on *P. expansum* grown in vitro, and Sitton and Patterson (1992) also showed a decrease in decay caused by *P. expansum* on apples in high CO₂ atmospheres, in both cases the inhibition of *P. expansum* was the least of all of the fungi tested in the respective experiments, while *Botrytis* sp. ranked as one of the most affected.

There were no apparent synergistic interactions between biocontrol agents and storage atmospheres, but several biocontrol agent and atmosphere combinations produced additive effects to reduce gray mold incidence and severity to very low levels. *C. infirmo-miniatus* was the most consistently effective biocontrol agent, followed by *C. laurentii* and *R. glutinis*. These organisms have been previously reported to be effective in controlling postharvest decays of pears (Chand-Goyal and Spotts, 1996; Sugar et al.,
1994), and the use of high CO\textsubscript{2} treatments did not appear to reduce their efficacy. BioSave-110 provided some decay control, while Aspire gave significant decay control in very few of the experiments. Some of these differences may be due to inactivation of commercial biocontrol agents during formulation and storage time (Whitesides et al., 1994), selective pressures in commercial production plants leading to loss of important characteristics (Pusey, 1994), or higher activity levels of "fresh" lab grown yeast. However, we have found that Aspire and \textit{C. oleophila} cultured from Aspire and grown on YMDA were of similar effectiveness as biocontrol agents when used at comparable concentrations (Benbow and Sugar, unpublished).

The fact that there was no fruit damage which could be attributed to CO\textsubscript{2} levels in these experiments is contrary to data reported by Wang and Mellenthin (1975) which showed that 'Anjou' pears stored for 6 weeks in 12\% CO\textsubscript{2} were damaged. Factors which may have been responsible for this discrepancy include fruit maturity, tree vigor, O\textsubscript{2} concentration, length of storage before and after high CO\textsubscript{2} exposure, and storage atmosphere before and after high CO\textsubscript{2} storage (Hansen and Mellenthin, 1962; Watkins \textit{et al.}, 1997). This variability in thresholds for CO\textsubscript{2} damage supports a conservative approach to high CO\textsubscript{2} treatments in commercial fruit storage. The potential for high CO\textsubscript{2} storage to contribute significantly to postharvest disease control makes the determination of more precise CO\textsubscript{2} injury threshold levels an important step in developing alternative decay control strategies for commercial use.
References


Chapter 5

Summary

The combining of biological control agents with other disease control strategies is an important option in the continuing search for alternative disease control measures. The conclusions from this study suggest several directions in which further research could lead to more widespread commercial use of biological control in the postharvest arena.

The first section of this thesis demonstrates that the yeasts *C. infirmo-miniatus*, *R. glutinis*, and *C. laurentii* are all able to survive at high population levels on fruit in the field for up to three weeks prior to harvest. Additionally, the fruit surface does not appear to play an important role in the survival, as the yeast maintained similar populations on both 'Bosc' and 'Anjou' pears, which have heavily russeted and smooth, waxy surfaces, respectively. Significant levels of decay control when the yeast was applied three weeks before harvest was only provided by *C. infirmo-miniatus*. However, the yeasts *C. laurentii* and *R. glutinis* retained viable populations on the fruit surface that were approximately equal to those of *C. infirmo-miniatus*. These yeasts grew quickly when placed on a nutrient medium, and the reasons for their failure to provide decay control is unknown. The yeast *C. oleophila* (active ingredient of Aspire) did not survive at high levels on the fruit surface when applied in the field, and did not provide decay control when fruit was harvested three weeks after yeast application.

The second section of this thesis discussed the postharvest use of biocontrol agents in combination with other compounds. The two compounds which provided the greatest enhancement of biological control were fungicides which are currently registered for postharvest use on pears. Calcium chloride, which has been previously suggested as a compound which may enhance the control provided by biological agents (Conway, 1982; McLaughlin et al., 1990), also enhanced decay control when combined with biocontrol agents. Chitosan, which has been suggested as a fruit coating with decay
control qualities (El Ghaouth et al., 1992) provided decay control when used alone, but appeared to inhibit most of the biocontrol agents when they were used in combination, resulting in more decay than when either treatment was used alone. Some nutritional enhancements which have been suggested as biocontrol enhancers (asparagine and proline; Janisiewicz et al., 1992; 2-deoxy-D-glucose; Janisiewicz, 1994) were not effective when used with the biocontrol agents in these experiments.

As discussed in the third part of the thesis, short-term storage of pears in high CO₂ atmospheres was highly effective in controlling decay caused by B. cinerea. Combination of high CO₂ treatments with some biocontrol treatments reduced decay to very low levels while using shorter exposures to CO₂ than was necessary to get the same levels of control without biocontrol agents. This is an important development, as the main objection to using increased CO₂ levels in storage is the risk of fruit injury. By combining the two treatments, decay could be reduced to very low incidence and severity, while remaining below levels of CO₂ exposure which have been previously reported to cause fruit damage (Wang and Mellenthin, 1975), without the use of fungicides. Combination of treatments, which can enhance the consistency and efficacy of alternative decay control strategies, may provide the necessary confidence in these measure to make them more commercially acceptable as options for commercial use.

The biocontrol agents which provided the most consistent decay control in these studies, while in the commercialization process, have not yet been registered for use on food products. Commercial biocontrol products which were used in these experiments (BioSave-110 and Aspire) were not as consistent or as effective in their decay control qualities. There may be several reasons for the difference in biocontrol agents. As part of a "newer generation" of postharvest biocontrol agents, C. infirmo-miniatus, C. laurentii, and R. glutinis may simply have better inherent biological control qualities than those organisms which were discovered and developed earlier. Other reasons, however, may include inactivation of commercial biocontrol agents during formulation and storage time (Whitesides et al., 1994), selective pressures in commercial production plants leading to loss of important characteristics (Pusey, 1994), or higher activity levels of "fresh" lab grown yeast. In order for biological control to become a widely used
practice, problems like these will need to be solved. However, studies such as this, which seek to enhance the levels of decay control through combination of treatments, are an important step in the continuing development of biocontrol practices.
References


Bibliography


