FINAL PROJECT REPORT

Project Title: Ozone in apple storage: microbial safety and decay management

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Cooperators: Allan Brothers. Inc., Stemilt Growers LLC.,

Total Project Request: **Year 1:** $104,707 **Year 2:** 108,515 **Year 3:** **no request**

Other funding sources: None

WTFRC Collaborative expenses

Item	2016	2017	2018
Wages	5,000	5,000	
Benefits	2,000	2,000	
Total	7,000	7,000	**0**

Budget 1: Meijun Zhu

Organization Name: WSU-Pullman **Contract Administrator:** Katy Roberts
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Item	2016	2017	2018[1]
Salaries[1]	$22,000	$22,880	
Benefits	$10,193	$10,601	
Wages[2]	$27,565	$28,667	
Benefits	$5,623	$5,848	
Supplies[3]	$25,326	$26,219	
Travel[4]	$2,000	$2,300	
Miscellaneous[5]	$5,000	$5,000	
Total	$97,707	$101,515	**0**

[1]**Footnotes:** No cost extension for 2018

RECAP OF ORIGINAL OBJECTIVES

1. Examine fate of *Listeria* and natural microorganisms on apple fruit surfaces when stored in refrigerated air, controlled atmosphere in the presence or absence of continuous low doses of ozone.
2. Examine the effect of ozone in the storage environment on final fruit quality.
3. Evaluate the efficacy of continuous low doses of ozone on postharvest pathogens

SIGNIFICANT FINDINGS

1. *Listeria monocytogenes* is a tough foodborne pathogen. A limited reduction of *L. monocytogenes* on apple surfaces occurred during 12 weeks of refrigerated storage either at 1°C/33°F, 4°C/39.2°F or 10°C/50°F. There was about ~1 Log reduction when stored at room temperature (22 °C / 71-72 °F) at ambient relative humidity (RH, 40-50%).
2. We determined a ~3-Log reduction of *Listeria* on Fuji apples after 30 weeks of cold storage under a commercial RA and CA storage environment. Continuously low dose ozone gas application (87.0 ± 38.8 ppb) in CA storage generated an additional 2-Log reduction.

   Additional 2-week storage under RA beyond their respective initial storage treatments had little influence on *L. innocua* survival.
3. Natural bacterial counts of Fuji apples stored at CA/RA remained stable throughout storage. Continuous low dose ozone gradually resulted in about 1-Log reduction after 30-week storage.
4. Indigenous yeasts/molds (Y/M) count of un-inoculated Fuji apples stored in RA remained relatively stable during first 12 weeks of storage. By the end of the 30-week storage, the Y/M count of RA stored apples increased about one log. The Y/M count of Fuji apples in CA room remained relatively stable over 30 weeks of storage. There is about 0.6-Log reduction of Y/M count in Fuji apples of CA with ozone storage during first 12 weeks. Nevertheless, the inhibitory effect of ozone was compromised with prolonged storage time.
5. Continuous low doze ozone gas used at this study had no negative influence on apple visual quality, including both external and internal disorders during 6-month CA cold storage.
6. Ozone at 60-80 ppm did not reduce the incidence of blue mold, gray mold and bull's eye rot and reduced severity (lesion diameters) very slightly (10 to 20%) on wounded and artificially inoculated Fuji and Granny Smith after 4 months of storage.
7. A negative impact (increased decay) on Mucor infection was seen in the 2017-18 season in fruit inoculated with Mucor at harvest.
8. The efficacy of ozone at 60-80 ppb was slightly higher (4 to 90% reduction) on the density of spores of *Penicillium expansum* and *Botrytis cinerea* on non-wounded fruit but was not effective against *Neofabraea perennans.*
9. Ozone at 60-80 ppb reduced nesting of gray mold (Botrytis) by 60 and 40%, respectively, compared to a non-ozone treatment.
10. Ozone at 60-80 ppb did not reduce residue levels of TBZ, pyrimethanil or fludioxonil on the surface of the fruit after 5 months of storage and did not reduce the efficacy of the fungicides against *P. expansum* (Blue mold) after 8 months of storage.
11. Data show that cold storage with continuously low dose ozone can be an additional hurdle for controlling *Listeria* on apple fruits; however, it can not completely eliminate *Listeria*.
12. A systematic/hurdle approach is needed to ensure apple microbial safety.

RESULTS AND DISCUSSION

Objective 1

1. Fate of *Listeria monocytogenes* on fresh apples of selected varieties during storage at different temperatures

Currently, there is barely any information availbale on how easily *L. monocytogenes* survives on fresh apples under different storage conditions. Thus, we first did a short-term storage study with *L. monocytogenes* established on fresh apples of selected varieties (Fuji, Granny Smith) under different storage temperatures. In the study, we include both high and low inoculation level of *Listeria* on fresh apples and chose the following storage temperatures per stated reasons.

- 1 °C (33 °F, a typical cold storage temperature).
- 4 °C (36 to 38 °F, a temperature commonly used for Honeycrisp long-term storage).
- 10 °C (50 °F, a temperature condition often used for Honeycrisp in preparation for storage).
- 22 °C (72°F, mimic situation of consumer purchased apples which are put on their kitchen count before consumption, though unlikely in commercial scenario).

During two weeks of short-term storage, *L. monocyotogenes* level on organic Granny Smith apples stored at 1, 4, and 10 °C stayed stable, though there was ~0.3 Log CFU/apple after 1-day storage (Figure 1A). More *L. monocyotogenes* reduction was observed when organic Granny Smith apples were stored at 22°C; there was ~1.0 Log CFU/apple reduction after 14-day storage (Figure 1A). Similar survival patterns of *L. monocytogenes* on conventional Granny Smith apples (Figure 1B) and Fuji apples (Figure 1C) were observed during the 14-day storage.

We further examined fate of *L. monocytogenes* on fresh apples during 12-week cold storage. Very limited die-off of *L. monocytogenes* was observed on fresh Fuji and Granny Smith apples (Fig. 2) during the 12-week of cold storage, whether apples were inoculated with high level (Fig. 2AC) or low level (Fig. 2BD) of *L. monocytogenes*. There

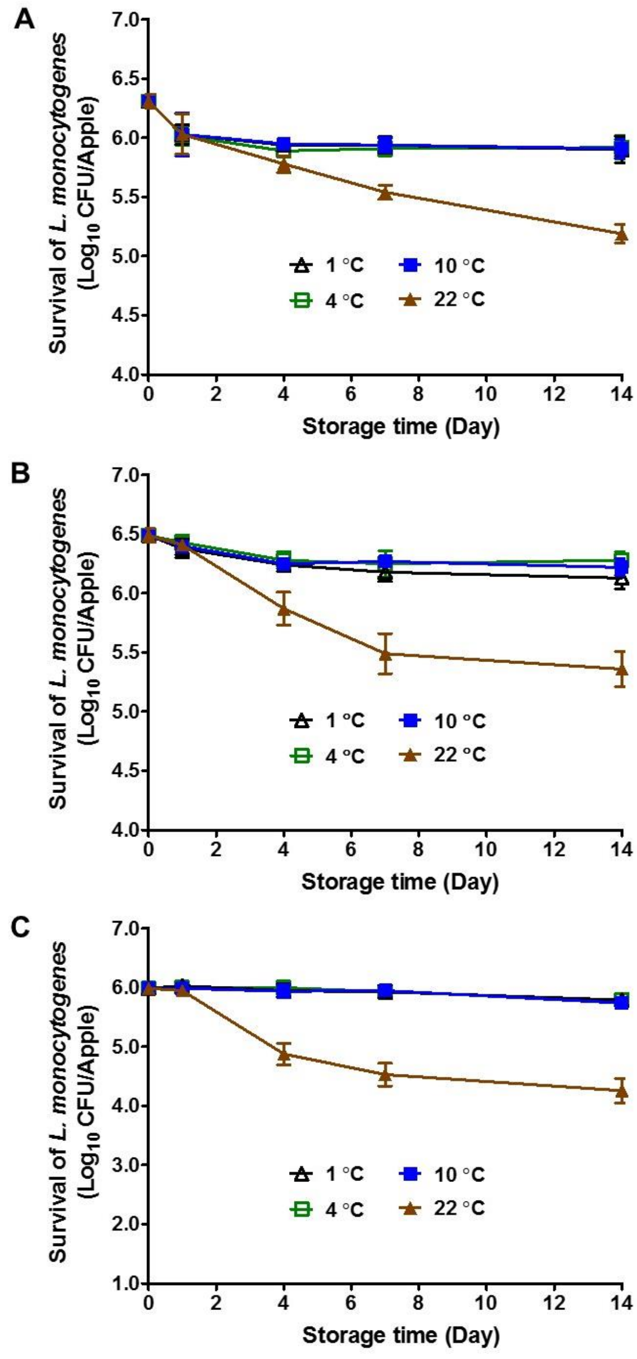

Fig. 1. Fate of *Listeria monocytogenes* on fresh apples during short-term storage under different temperatures when inoculated at 1×10^6 CFU/apple. A. Organic Granny Smith; B. Conventional Granny Smith; C. Conventional Fuji. Mean ± SEM, n=12.

was no significant difference in survival of *L. monocytogenes* among the three storage temperatures (Fig. 2).

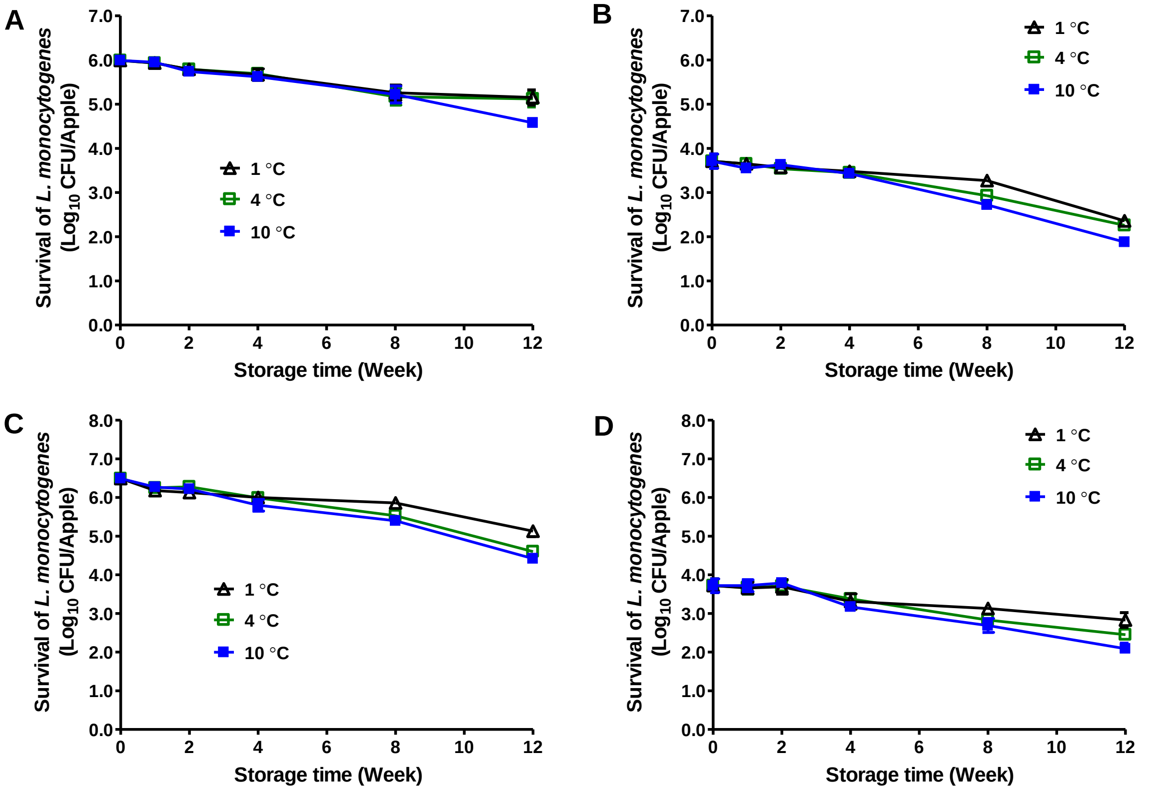

Fig. 2. Survival of *Listeria monocytogenes* on Fuji (AB) and Granny Smith apples (CD) during 3-month cold storage. AC. Apples were inoculated at ~1×10^6 CFU/apple; BD. Apples were inoculated at 1×10^4 CFU/apple. Mean ± SEM, n=12

2. Fate of *L. innocua* established on fresh apples during under different cold storage at a commercial packing facility

We further conducted an 18-week cold storage experiment in a typical commercial apple facility using *L. innocua* inoculated apples in which Fuji apples were inoculated and established to 7.05 ± 0.02 Log_{10} CFU/apple before being subjected to RA, CA, and CA with 50 ozone gas. During the 6-week storage, *L. innocua* populations on apples under RA and CA storage decreased by ~2.0 Log_{10} CFU/apple (Fig. 1B). Supplementation of CA with continuous gaseous ozone at a concentration of 50.0 ± 28.5 ppb resulted in an additional ~1.7 Log_{10} CFU/apple reduction after 6 weeks (Fig. 3). Upon further 12-week storage, a gradual reduction of *L. innocua* on apples under all three storage conditions was observed. RA,

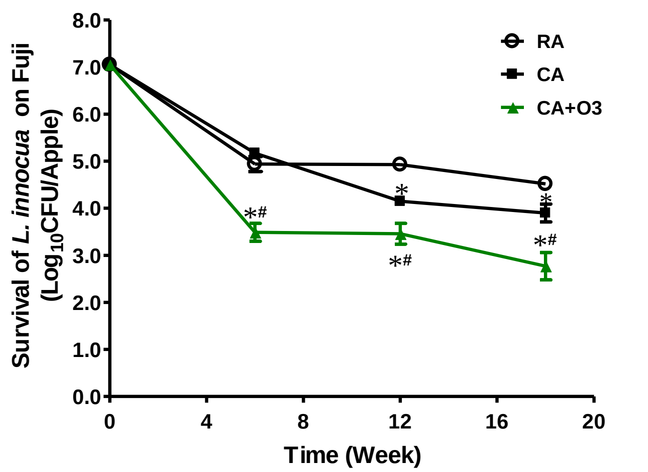

Fig. 3. Survival of *Listeria* on Fuji apple under 18-week commercial cold storages. RA: refrigerated atmosphere (33°F); CA: controlled atmosphere (33°F, 2% O2, 1% CO2); CA + O3: CA with ~50.0 ppb ozone. Mean ± SEM, n=40. In survival curve: *significant difference from RA (P < 0.05), #significant difference from CA (P < 0.05).

CA, and CA with 50 ppb ozone storage for 18 weeks reduced *L. innocua* on Fuji apples by 2.5, 3.1, and 4.2 Log_{10} CFU/apple, respectively (Fig. 3).

To confirm the promising antimicrobial effect of low-dose continuous ozone application in CA storage, we next conducted a 30 -week cold storage experiment in a typical commercial apple facility using *L. innocua* inoculated apples in which Fuji apples were inoculated and established to 6.20 ± 0.06 Log_{10} CFU/apple before being subjected to RA, CA, and CA with 87 ± 39 ppb ozone gas. A rapid reduction of 1.4-1.8 Log_{10} CFU/apple was observed within the first 3 weeks in all three storage conditions (Fig. 4). In the following 15-week storage, *L. innocua* on Fuji apples under RA and CA storage behaved similarly with about a 2.6 Log reduction. Interestingly, by the end of the 30-week storage, bacterial count on apples in RA decreased by ~0.9 Log_{10} CFU/apple compared with 18-week, while that in CA storage remained the same (Fig. 3). For CA storage with 87 ppb ozone gas, *L. innocua* population was reduced by ~5.0 Log after 24-week storage and remained at the similar level till 30-week. To mimic packing facility practice, apples in CA and CA with ozone storage were moved to RA by the end of the 30-week storage, at which time *L. innocua* was enumerated. Additional 2-week storage under RA beyond their respective initial storage treatments had little influence on *L. innocua* survival (Fig. 3).

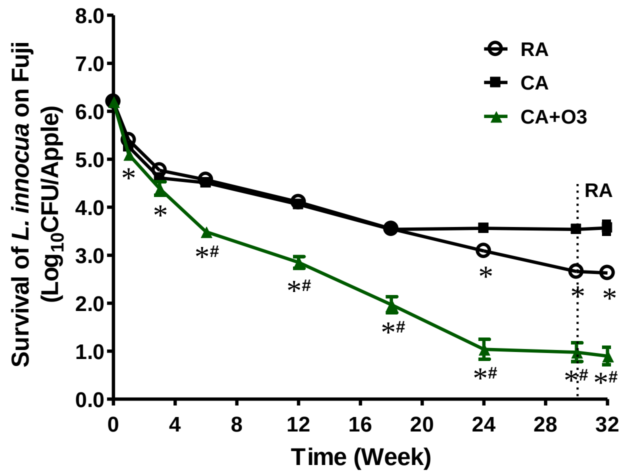

Fig. 4. Survival of *Listeria* on Fuji apple under commercial cold storages. RA: refrigerated atmosphere (33°F); CA: controlled atmosphere (33°F, 2% O2, 1% CO2); CA + O3: CA with ~87 ppb ozone. Mean ± SEM, n=40.

3. Natural microbial reduction on apple fruit surfaces under different cold storage conditions

Another set of Fuji apple fruits (non-waxed and non-inoculation) was subjected to different storage conditions (RA, CA and CA with 87 ± 39 ppb ozone), total plate count (TPC) and yeasts/molds (Y/M) count were evaluated as described above. The initial TPC and Y/M count were 3.44 ± 0.07 and 4.77 ± 0.07 Log_{10} CFU/apple, respectively (Fig. 5). TPC remained stable on Fuji apples in RA and CA storages throughout storage (Fig. 5A). Continuous application of ozone at low doses gradually achieved ~1.0 Log_{10} CFU/apple reduction of TPC after 30-week storage (Fig. 4A). At

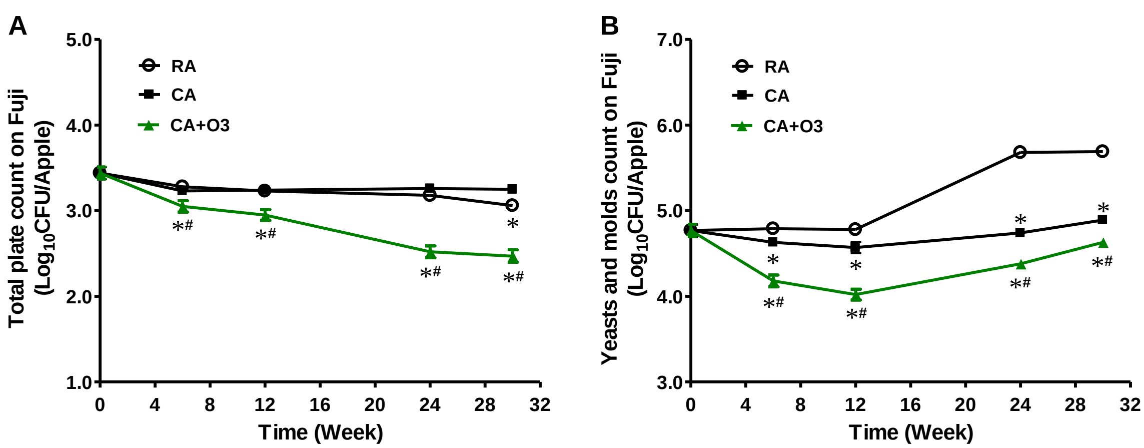

Fig. 5. Fuji apple decay during cold storages. A. TPC. B. Y/M count. RA: refrigerated atmosphere (33°F); CA: controlled atmosphere (33°F, 2% O2, 1% CO2); CA + O3: CA with ~87 ppb ozone. Mean ± SEM, n=40.

12 weeks, Y/M count in RA and CA storage remained relatively stable, while the Y/M count in CA + O_3 was reduced by ~ 0.6 Log_{10} CFU/apple (Fig. 5B). Nevertheless, the inhibitory effect of CA ± O_3 was compromised with prolonged storage time. By the end of the 30-week storage, the Y/M count of apples in CA and CA ± O_3 reached 4.89 ± 0.05 and 4.63 ± 0.04 Log_{10} CFU/apple, respectively (Fig. 5B). Y/M count on apples under RA storage stayed at the same level within the first 12-week storage, increased by ~1.0 Log_{10} CFU/apple from the 12^{th} to the 24^{th} week of storage, then remained constant in the last 6 weeks of storage (Fig. 5B).

Objective 2

1. Examine the effect of ozone in the storage environment on final fruit quality

Quality parameters of apple fruits under different storage treatments were assessed both at harvest and after storage. Firmness decreased after storage for all apples, and no difference was found between CA and CA + O_3 storage. Apples subjected to RA storage had a significantly lower firmness than those in CA with or without gaseous ozone storage. Compared to harvest levels, TSS levels did not change in apples post-6-month storage in RA, CA, or CA + O_3. TA after storage decreased to between one third to two thirds of the TA at harvest. TA reduction in apples was significantly mitigated by CA storage, while addition of ozone had no impact on TA (Table 1).

Table 1. Fuji apples quality parameters at harvest and after 6 months of cold storage and ripen at RT for 7 days.

Treatment	Weight (g)	Diameter (in)	Firmness (lbs)	TSS (% Brix)	TA (% malic acid)
At harvest					
	204 ± 10.1	3.0 ± 0.1	15 ± 0.2	12.7 ± 0.3	0.307 ± 0.01
After 6 months storage					
RA	188 ± 4.6^{a}	2.97 ± 0.03^{a}	10.0 ± 0.4^{b}	12.4 ± 0.8^{a}	0.173 ± 0.01^{b}
CA	183 ± 3.3^{a}	2.92 ± 0.03^{b}	13.8 ± 0.3^{a}	12.5 ± 0.5^{a}	0.232 ± 0.02^{a}
CA + O_3	186 ± 4.3^{a}	2.95 ± 0.02^{ab}	13.7 ± 0.4^{a}	12.4 ± 0.8^{a}	0.220 ± 0.02^{a}

Means within a column with no common letter differ significantly ($P < 0.05$). TSS: total soluble solids; TA: titratable acidity; Mean ± SEM, n=40.

The incidence of external and internal disorders was visually evaluated at the end of each storage treatment. Overall, the parameters evaluated for either external disorders or internal disorders were not significantly different among apples stored under RA, CA, or CA + O_3 (Table 2 & 3). No ozone

Table 2. External disorders analysis for Fuji apples after 6 months of cold storage and ripen at RT for 1 and 7 days.

Treatment	External disorders (%)						
	Ozone burn	Superficial scald	Lenticel decay	Visible decay	Sunburn	Russet	CO_2 damage
1 day at RT							
RA	0^{a}	15 ± 2.2^{a}	0^{a}	1 ± 0.4^{a}	23 ± 4.4^{a}	5 ± 2.8^{a}	0^{a}
CA	0^{a}	5 ± 5.3^{a}	0^{a}	1 ± 0.9^{a}	20 ± 2.3^{a}	5 ± 1.3^{a}	0^{a}
CA + O_3	0^{a}	1 ± 0.4^{a}	0^{a}	1 ± 0.4^{a}	22 ± 2.9^{a}	7 ± 2.3^{a}	0^{a}
7 days at RT							
RA	0^{a}	16 ± 6.2^{a}	0^{a}	1 ± 4.3^{a}	22 ± 2.1^{a}	5 ± 2.1^{a}	0^{a}
CA	0^{a}	4 ± 2.5^{a}	0^{a}	0^{a}	16 ± 3.8^{a}	7 ± 2.4^{a}	0^{a}
CA + O_3	0^{a}	1 ± 0.4^{a}	0^{a}	0^{a}	23 ± 1.9^{a}	8 ± 1.9^{a}	0^{a}

Means within a column with no common letter differ significantly ($P < 0.05$). Mean ± SEM, n=200.

burn, lenticel breakdown, decay, or CO_2 damage was found in any apples subjected to 6-month of low-dose ozone gas storage (Table 2). No watercore, internal browning, or cavity was observed in apples stored under CA or CA + O_3. A small number of apples under RA stored were found to have watercore and internal browning, but the incidence rate was not significantly different from those fruit kept under CA storage (Table 3).

Table 3. Internal disorders analysis for Fuji apples at harvest and after 6 months of cold storage and ripen at RT for 7 days.

Treatment	Internal disorders (%)		
	Watercore	Internal browning	Cavity
At harvest			
	0^a	0^a	0^a
After 6 months of cold storage			
RA	3 ± 0.4^a	10 ± 1.0^a	0^a
CA	0^a	0^a	0^a
CA + O_3	0^a	0^a	0^a

Means within a column with no common letter differ significantly ($P < 0.05$). Mean ± SEM, n=200.

<u>Objective 3</u>

1. Efficacy of ozone on artificially wounded and inoculated fruit and non-wounded fruit

After 60 days of storage, incidence of blue mold, gray mold and bull's eye rot was reduced by 52, 45 and 60%. After 90 days, respective reduction decreased to 18, 12 and 20%, respectively (Fig. 6A). On non-wounded fruit, ozone reduced spore loads of blue mold, gray mold and bull's eye by 78, 62 and 4%, respectively after 90 days of storage. After 180 days, reduction of blue mold spores decreased to 58% but remained steady for the two other pathogens (Fig. 6B).

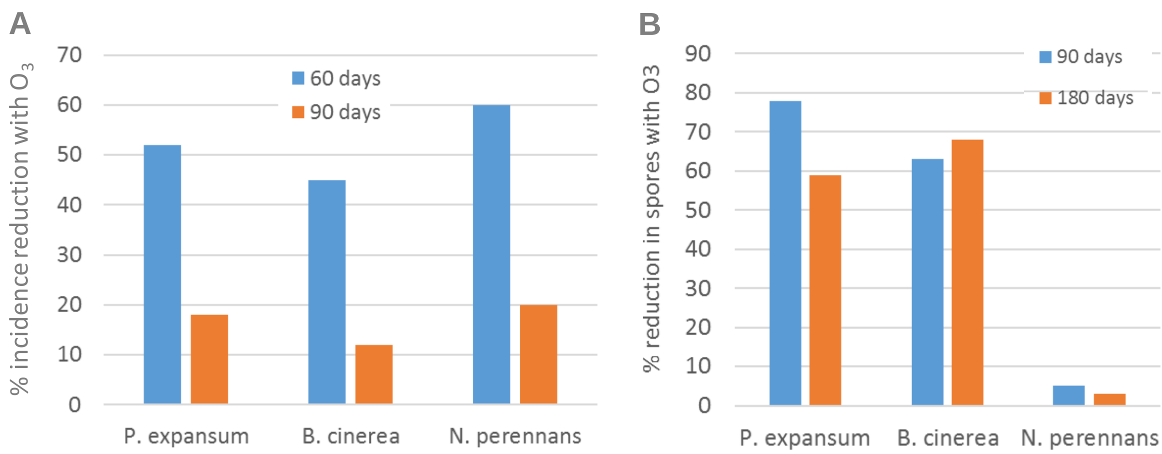

Fig. 6. Reduction of incidence of blue mold, gray mold and bulls eye rot on wounded fruit (A, wounded fruit) and of spore load on non-wounded fruit (B, surface inoculation) treated with ozone relative to non-ozonated fruit.

2. Efficacy of ozone in reducing nesting of Botrytis cinerea (gray mold)

Ozone at 60 ppb reduced nesting of gray mold (*Botrytis*) from 50% in the CA room without ozone to 20% on Fuji apples after 6 months of storage, versus 33% when ozone was applied at 80 ppb (Fig. 7).

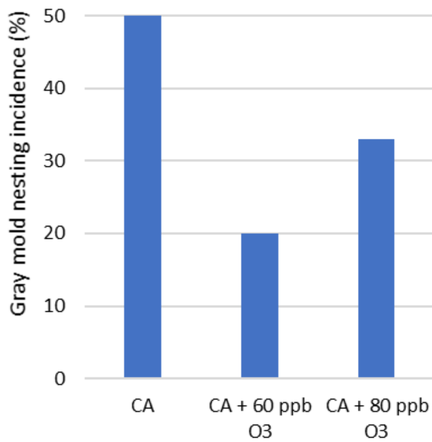

Fig. 7. Efficacy of continuous ozone application in reducing nesting of Botrytis cinerea (gray mold) after 6 months of storage

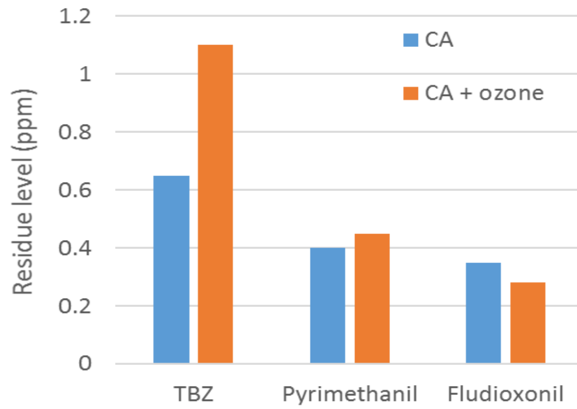

Fig. 8. Residue levels of TBZ, pyrimethanil, and fludioxonil on Fuji fruit stored inn CA and CA + ozone (80 ppb) for 6 months.

3. Interaction of postharvest fungicides with a low dose of ozone on non-wounded fruit

Gaseous ozone at 60-80 ppb did not decrease residue levels of TBZ, pyrimethanil or fludioxonil after 6 months of storage (Fig. 8).

EXECUTIVE SUMMARY

The overall goal of the proposed studies was to evaluate the antimicrobial efficacy of low dose continuous gaeous ozone against *L. monocytogenes* contaminated apples, as well as apple fungal pathogens during commercial cold storage, and to further evaluate its impacts on apple fruit quality and its interaction with fungicides over an extended period of storage.

Listeria is a tough foodborne pathogen to eliminate once it has contaminated apple surface. At ambient/low relative humidity (RH), limited reduction of *L. monocytogenes* on apple surfaces occurred during 12 weeks of refrigerated storage either at 1°C/33°F, 4°C/39.2°F or 10°C/50°F. However, a 6-month commercial cold storage (RA and CA) with high RH decreased *L. innocua*, a non-pathogenic surrogate of *L. monocytogenes* count on Fuji apples by 2.5-3.0 Log_{10} CFU/apple. Continuous application of gaseous ozone at ~87 ppb in CA storage facilitated *L. innocua* reduction and resulted in ~5.0 Log_{10} CFU/apple reduction after 30-week storage, and inhibited apple residence microflora. Furthermore, continuous low dose ozone gas used in this study had no negative influence on Fuji apple visual quality, including both external and internal disorders during 6-month CA cold storage. Data collectively show that cold storage with continuous low dose ozone can be an additional hurdle for controlling *Listeria* on apple fruits; however, it can not completely eliminate *Listeria*. A systematic/hurdle approach is needed to ensure apple microbial safety.

Furthermore, ozone at the tested concentration is beneficial in reducing the inoculum load on fruit surfaces and nesting of some pathogens such as *Botrytis* and *Phacidiopycnis*, which resulted in an overall reduction of 15 to 20% in disease incidence. Low dose ozone may also be effective in controlling infections that starts on fresh wounds and therefore should not be viewed as a replacement for fungicides in conventional packinghouses. At the concentrations evaluated in this study ozone is safe to use on fruit treated with fungicides at harvest, as no reduction in residue levels was observed after 6 months of storage.

In summary, continuous low-dose ozone gas has the potential to be applied during long term apple cold storage, as well as other fresh produce industries with similar practices as a supplemental intervention method to ensure fresh produce safety and control decay.