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Potential for Hypobaric Storage as a Phytosanitary Treatment: Mortality of *Rhagoletis pomonella* (Diptera: Tephritidae) in Apples and Effects on Fruit Quality

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ABSTRACT The efficacy of low-oxygen atmospheres using low pressure, referred to as hypobaric conditions, to kill egg and third-instar *Rhagoletis pomonella* (Walsh) in apples was investigated. Infested apples were exposed to 3.33 and 6.67 kPa in glass jars at 25 and 30°C for times ranging from 3 to 120 h. Probit analyses and lethal dose ratio tests were performed to determine differences in lethal time values. Eggs were more tolerant of low pressure compared with third-instar *R. pomonella*. Mortality of eggs and larvae increased with increase in time of exposure to low pressure and temperature. Lower pressures increased percent mortality of eggs, but these values were not significantly different at the pressures tested in this investigation. The LT_{99} for *R. pomonella* eggs at 3.33 kPa was 105.98 and 51.46 h, respectively, at 25 and 30°C, which was a significant effect of the higher temperature on egg mortality. Investigation into consumer acceptance of low-pressure-treated apples was done with 'Red Delicious' and 'Golden Delicious'. Apples exposed to 3.33 kPa at 25 and 30°C for 3 and 5 d were stored at 1°C for 2 wk and presented to a sensory panel for evaluation. The panelists rated treated apples with untreated controls for external and internal appearance and taste. Golden Delicious apples were unaffected for all three sensory factors across both temperatures and exposure times. Although taste was unaffected for Red Delicious, the internal and external appearances deteriorated. Use of low pressure for disinfestation and preservation of apples is a potential nonchemical alternative to chemical fumigants such as methyl bromide and phosphine.

KEY WORDS apple maggot, Diptera: Tephritidae, controlled atmosphere, low pressure, low oxygen

Phytosanitary treatments are used to disinfest commodities of invasive species so that the commodities may be traded across quarantine boundaries. Commercially used treatments include lethal heat and cold, fumigants, and ionizing irradiation (Heather and Hallman 2008). Modified and controlled atmospheres in the form of low oxygen and/or high carbon dioxide relative to ambient conditions have been researched as phytosanitary treatments, although they have not been used commercially, save for one successful pilot shipment of asparagus from New Zealand to Japan in 1990 (Carpenter and Potter 1994, Heather and Hallman 2008).

Low pressure, or hypobaric storage, achieves a controlled atmosphere by removing air from a relatively air-tight space or structure containing a commodity and thus reducing oxygen concentration to insecticidal levels. Published research into the use of low pressure for disinfestation of stored products infested by stored-product pests dates back to >85 yr (Back and Cotton 1925, Bare 1948, Calderon et al. 1966, Calderon and Navarro 1968, Navarro and Calderon 1979, Friedlander and Navarro 1983, Mbata and Phillips 2001, Johnson and Zettler 2009, Johnson 2010). Low oxygen concentrations cause the insect mortality rather than any physical effect of the low pressure or dehydration resulting from low moisture (Navarro and Calderon 1979, Friedlander and Navarro 1983).

Low pressure is used to cool lettuce after harvest, and the effect of low pressure on pests of lettuce has been studied. Aharoni et al. (1986) found that 2.66 kPa for 52 h at 2°C killed 100% of the aphid *Myzus persicae* (Sulzer) on lettuce. Liu (2003) used a combination of vacuum-induced low pressure (5.0 kPa) plus injections every 30 min of nitrogen or carbon dioxide to attempt control of two aphids and the leafminer *Liriomyza langei* Frick (Diptera: Agromyzidae) on lettuce leaves in petri dishes and plastic bags. Davenport et al. (2006) controlled the tephritid *Anastrepha suspensa* (Loew) eggs in petri dishes at 13°C with 2.00 and 2.67 kPa in 9 and 11 d, respectively. Young larvae under the same conditions were killed in 8 d. The articles by

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Aharoni et al., Liu, and, Davenport et al. are the extent of studies on the effect of hypobaric storage on insects of fresh commodities of which we are aware.

Tephritid fruit flies comprise the most important group of quarantine pests for which phytosanitary treatments are used. The apple maggot *Rhagoletis pomonella* (Walsh) is an insect native to North America that is a primary pest of cultivated apples, especially in the northeastern United States and southeastern Canada. It is found in eastern North Dakota and southern Manitoba to Nova Scotia, southward to eastern Texas to central Florida, occurring over the entire middle and eastern region of the United States. In 1981, it became established in Oregon, from where it spread to California, Washington, Utah, and Colorado, and eventually Nebraska in 1991 (White and Elson–Harris 1994). *R. pomonella* is a quarantine pest for apples exported from the United States and Canada, which may require a phytosanitary treatment.

The objective of this study was to evaluate hypobaric storage as a controlled atmosphere for low oxygen as a phytosanitary treatment for fresh commodities using *R. pomonella* and apples as the model experimental subjects.

Materials and Methods

Infestation of Apples. *R. pomonella* were collected as immatures in apples from orchards near Biglerville, PA, 2 yr before this research was conducted and reared in the laboratory at U.S. Department of Agriculture–Agriculture Research Services (USDA–ARS) Weslaco, TX, on organically grown 'Red Delicious' apples from Washington State. About a dozen experimental Red Delicious apples were placed in plastic-screened cages (28 cm^3) at 27 ± 0.5°C, 65 ± 5% relative humidity (RH), and a photoperiod of 14:10 (L:D) h for 8 h with ≈100 adult *R. pomonella* (sex ratio ≈50:50). Egg-infested apples were immediately shipped overnight to Stillwater, OK, for low-pressure treatment, whereas those for third-instar tests were held for 11 d before being shipped. Groups of apples were infested in Weslaco and shipped to Stillwater for several months over the course of this study.

Low-Pressure Treatment of Apples. Infested Red Delicious apples were placed in 3.8-liter glass jars (25 by 15 cm with neck diameter of 11.4 cm) on a 15 mm-thick layer of white sand. The screw-on metal lid of the glass jar was modified by drilling a hole (8 mm diameter) in the center and welding a brass tubing (6 mm diameter) with a 90 degree elbow. The brass tubing passed through a 'T' joint and ended into the inlet of a ball-valve (6 mm diameter). A dial-type pressure gauge (Fisher, Hampton, NH) that had been previously calibrated to a mercury column manometer was mounted on the T joint with a threaded coupling. A threaded coupling was fitted at the outlet of the ball-valve to clamp a flexible Tygon vacuum hose (5 mm internal diameter and 1.5 mm thick, Tygon, Paris, France). The flexible Tygon vacuum hose was attached to a vacuum pump (Duo-Seal, Chicago, IL) to obtain the desired pressure in the glass jars. All the joints and couplings were sealed air-tight to avoid any loss of pressure. The modified metal lid was screwed on to the glass jar with vacuum lubricant to avoid any pressure leak. The vacuum pump evacuated the air from the glass jars to 3.33 or 6.67 kPa (equivalent to 25 mm and 50 mmHg, respectively), pressures chosen for their ease of measurement and insecticidal low oxygen concentrations of 1–2%, whereupon the ball-valve was closed and vacuum hose disconnected from the vacuum pump. The glass jar was then transferred to temperature-controlled chambers maintained at 25 or 30°C for various exposure times. Any treatment jar that showed an increase in pressure on its pressure gauge during the course of an exposure treatment was eliminated from the study. Plastic containers (22 by 18 by 11 cm) with a layer of white sand on the bottom (15 mm thick) were used to store the individually treated apples to allow for emergence of nonfeeding third instars. The plastic containers were ventilated at the top by cutting a round hole (18 cm diameter) in the plastic lid and gluing a fine-mesh nylon screen over the hole to allow ventilation and prevent contamination. Two additional holes (9 cm diameter) were cut on diametrically opposite walls of the container, over which a fine-mesh nylon screen was glued, for ventilation.

Experimental Protocol for Low-Pressure Treatment. The experimental tests for mortality of egg and third-instar *R. pomonella* were conducted at low pressures of 3.33 and 6.67 kPa at 25 and 30°C. The pressures and temperatures tested in this study were those known to be effective with stored-product insects (Mbata and Phillips 2001) so that the potential for hypobaric disinfestations of tephritids from fresh fruit could be demonstrated. Commercial application of low pressure on fresh fruit would likely be at lower temperatures for longer periods of time, in accord with industry practices. For egg mortality, tests were initiated on the infested apples within 24 h of infestation by *R. pomonella* adults. Three or four apples were placed inside 3.8-liter glass jars, each jar considered a replicate, on a bed of white sand and evacuated to the predetermined pressure of 3.33 or 6.67 kPa. The evacuated glass jars were placed in a temperature-controlled chamber set at 25 or 30°C. Six or more exposure times (3–120 h) were done for each combination of pressure and temperature. Controls consisted on *R. pomonella*-infested apples in glass jars at ambient pressure and room temperature (≈25°C) for all the treatments. The glass jars under treatment were taken out of the temperature-controlled chamber at the end of the exposure period, vented to ambient pressure by opening the ball-valve on the lid assembly, and the apples from each jar were removed and placed on a bed of white sand in a plastic container at 25°C to allow for continued insect development. Three replicates were done at each pressure and temperature for the same exposure times. The period for complete emergence of larvae from *R. pomonella*-infested apples in controls at 25°C ranged from 3 to 4 wk; therefore, a consistent period of 4 wk after treatment was observed for all treatments to count the larvae or puparia. At the

end of 4 wk, the live emerged larvae or puparia were counted and the apples were dissected to count any live and dead larvae within. The number of larvae and puparia emerging from the controls was also counted. The numbers of treated eggs of *R. pomonella* were estimated from the number of larvae and puparia emerging from the controls from the same batch for a given trial.

For larval mortality, the batch of *R. pomonella*-infested apples was placed in a temperature-controlled chamber for 11 d and the trials commenced on the 12th day as explained previously. By this time, most of the insects were late third instars, but they had not begun to make exit holes in the apple peel. Research into the mortality of stored-product insects has shown the egg stage to be generally more tolerant of low pressures compared with nondiapausing larvae and therefore the exposure times for larvae were kept lower compared with egg mortality trials. The apple maggot diapauses in the pupal stage, which is not present in the fruit. For each combination of pressure and temperature, there were five or more exposure times between 3 and 96 h. The procedure for evacuation and venting of glass jars and placement of apples in plastic containers was similar to that of egg mortality determination. The mortality count of larvae was taken after a recovery period of 4 wk after treatment by dissecting the apples and counting the live and dead larvae. Three replicates were done at each pressure and temperature for the same exposure times.

Experimental Protocol for Sensory Quality of Apples. We conducted limited sensory tests with a small panel to obtain preliminary consumer preference in terms of external and internal appearance and taste on noninfested Red Delicious and Golden Delicious apples procured from a local market and for which we knew nothing about previous handling, treatment, or storage. An untrained human panel consisting of randomly selected consumers was asked to rate the treated and control apples for the three factors. The estimated exposure times for the LT_{99} for eggs (see results in Table 4) ranged from 52 h (at 3.33 kPa, 30°C) to 106 h (at 3.33 kPa, 25°C). In addition, the LT_{99} for eggs at 3.33 and 6.67 kPa was not significantly different (Table 4). In commercial practice, we assume that a pressure of 6.67 kPa would be easier to achieve than 3.33 kPa. Therefore, the experiment for sensory acceptance of apples was designed for a treatment pressure of 6.67 kPa and exposure times of 3 and 5 d (72 and 120 h) for the apple cultivars of Red Delicious and Golden Delicious. Experimental trials were conducted separately for each cultivar–temperature combination.

Batches of apples of both cultivars procured from a local market were divided into controls and experimental trials. An experimental trial was commenced by placing apples in a vacuum desiccator (30 cm diameter and 20 cm height) evacuated to 6.67 kPa and exposed to a constant temperature (25 or 30°C) for a period of 3 and 5 d. A replicate for a cultivar trial, at predetermined temperature and pressure, consisted of three apples in a vacuum desiccator for each exposure time. In all there were three replicates for each of the two exposure times. Hence, in a trial with one cultivar and temperature, there were six vacuum desiccators under low-pressure (6.67 kPa) treatment. For each predetermined cultivar and temperature combination, the trial began with the 5 d exposure period and was followed after 48 h by the 3 d exposure under the same environmental conditions. This allowed pulling out the apples from both exposure times on the same day. At the end of the treatments, the apples were removed from the vacuum desiccator and stored at 1°C for 2 wk in paper bags. Nontreated controls were also stored for 2 wk in paper bags in cold storage at 1°C after prechilling exposure to ambient temperature and pressure, until evaluation of all treatment and control groups at the same time.

At the end of 2 wk, the apples were taken out and kept at room temperature (25°C) for 6 h before sensory testing by panelists. Four sensory tests were conducted with panelists for each combination of two cultivars and temperatures. For each variety and temperature there were three replicates, hence nine panelists were presented with three apples with randomized combination of control and 3- and 5-d apples. The panelists were unaware of the treatment identity of the apples.

The panelists evaluated the apples for external and internal appearance and taste. For external appearance, the panelists held the apples and considered color of the apples, absence of damage, firmness, and general freshness; internal appearance was evaluated by cutting open the apple and looking for color and visible internal damage. Cut apples were evaluated in terms of odor, color, and perceived internal damage (e.g., brown, black, or rotting areas) and acceptability of flavor and eating after taking a bite and eating part of the apple. Evaluation for each of the three criteria was done by rating on a numerical scale of 1–5, with 5 being best for all three criteria.

Data Analyses. Data for the effect of low pressure on *R. pomonella* mortality were analyzed using the PROC PROBIT routine of SAS (SAS Institute 2000). For the effect of low pressure, the data were analyzed separately for each stage of *R. pomonella* within each temperature and pressure. The initial number of eggs laid by adult *R. pomonella* flies on the apples was unknown and was assumed to be at least equal to the highest number of larvae emerging from the same batch of apples exposed to ovipositing females in that group designated as controls. The number of *R. pomonella* eggs killed was the difference between the number of emerging larvae from the controls and those emerging from treated apples in a given oviposition group. Estimated exposure times (h) to achieve 50, 90, and 99% mortality were obtained using the lethal dose ratio test of Robertson and Preisler (1992) among all life stages treated at two temperature and pressures and within combination of temperature and pressure for a life stage. Data analyses for sensory tests by human panelists were performed using the routine PROC MIXED of SAS (SAS Institute 2000).

Table 1. Sample sizes and slopes from probit analyses of mortality data from eggs (E) and third instars (L) of *R. pomonella* exposed to low pressures at 25 and 30°C vs time of exposure in hours

Pressure (kPa)	Life stage	Temperature			
		25°C		30°C	
		n^a	Slope (SE)^b	n^a	Slope (SE)^b
3.33	Eggs	3,531	0.031 (0.001)	1,544	0.074 (0.003)
	Larvae	1,776	0.293 (0.004)	1,472	0.161 (0.010)
6.67	Eggs	1,616	0.037 (0.002)	1,224	0.073 (0.004)
	Larvae	1,984	0.048 (0.002)	1,049	0.108 (0.006)

[a] The value for *n* is an estimate of the number of eggs or third instars treated based on emergence of subsequent stage from untreated controls.

[b] χ^2 analyses determined that all slopes reported for probit regressions were highly significantly different for zero ($P < 0.001$).

Results

Low Pressure Treatment of Infested Apples. The sample sizes and slopes from probit analyses for eggs and larvae of *R. pomonella* at two pressures and temperature are given in Table 1. The LT_{50}, LT_{90}, and LT_{99} levels for eggs and larvae of *R. pomonella* at two temperatures and low pressures resulting from exposure times ranging from 3 to 120 h are presented in Tables 2-4. Slope values in Table 1 for both stages indicate the differences in tolerance among the life stages. The slope values for the eggs of *R. pomonella* are lower than those for larvae in all the treatments.

The estimated times to kill eggs and larvae decreased with the higher temperature (Tables 2–4) and increased with the higher pressure in most cases, except for larvae held at 3.33 kPa, which took longer to die at the higher temperature. The eggs were more tolerant compared with larvae in all cases at both levels of pressure (Tables 2–4). Complete mortality of eggs was achieved in 106 and 98 h at 25°C and 3.33 and 6.67 kPa, respectively. At 30°C, 100% mortality of eggs was achieved in approximately half the time compared with 25°C, demonstrating the effect of higher temperature to achieve rapid mortality. Third-instar *R. pomonella* were more susceptible to low-pressure regimens compared with eggs at both temperatures and required significantly lower exposure times at all levels of mortality (Tables 2–4). The probit analyses at LT_{90} and LT_{99} also indicate that the exposure times for mortality of eggs at 3.33 and 6.67 kPa are not significantly different (Tables 3 and 4).

Table 3. Exposure time (h) and fiducial limits (with 95% CI) required to obtain 90% mortality (LT_{90}) of eggs and larvae of *R. pomonella* at two temperatures and pressures

Pressure (kPa)	Life stage	Exposure time (h)	
		25°C	30°C
3.33	Eggs	71.74Aa (67.86–76.19)	37.41Ba (35.46–39.65)
	Larvae	6.59Ac (6.32–6.86)	14.48Bc (13.29–15.96)
6.67	Eggs	69.34Aa (65.53–73.73)	38.25Ba (35.27–41.95)
	Larvae	41.99Ab (39.25–45.25)	23.73Bb (21.80–26.12)

Values in the same column with different lowercase letters are significantly different ($P < 0.05$) and values in the same row with different uppercase letters are significantly different ($P < 0.05$); lethal dose ratio test.

Sensory Evaluation of Low-Pressure-Treated Apples. Cultivar and temperature had significant ($P < 0.05$) effect on sensory criteria of apples (Table 5). Golden Delicious apples for all three sensory parameters were not significantly different across all exposure time–temperature combinations. However, in the case of Red Delicious, we observed that internal and external appearance was significantly affected (Table 5). At 25°C, for both internal and external appearance, 5-d-treated Red Delicious apples were rated higher compared with 3-d-treated apples. Whereas, at 30°C, the 5-d-treated apples were rated the lowest quality in appearance and controls were the highest. Taste of Golden Delicious and Red Delicious was not affected by treatment or temperature.

Discussion

This research demonstrates that hypobaric storage to achieve an insecticidal low oxygen atmosphere shows promise as a phytosanitary treatment against cryptically feeding quarantine pests of fresh commodities such as *R. pomonella.* An overall objective of this research was to use apples and *R. pomonella* as models to test the possibility of successfully using the hypobaric technology on a cryptically infesting pest in a fruit without unacceptably reducing fruit quality. Our aim was not to develop a specific treatment for apples against apple maggot; thus, we do not discuss current phytosanitary treatments used for apples nor propose

Table 2. Exposure time (h) and fiducial limits (with 95% CI) required to obtain 50% mortality (LT_{50}) of eggs and third instars of *R. pomonella* at two temperatures and pressures

Pressure (kPa)	Life stage	Exposure time (h)	
		25°C	30°C
3.33	Eggs	29.74Ab (28.06–31.54)	20.18Ba (18.91–21.47)
	Larvae	2.22Ad (1.90–2.53)	6.53Bc (5.88–7.22)
6.67	Eggs	34.73Aa (32.48–37.06)	20.79Ba (19.11–22.66)
	Larvae	15.47Ac (14.06–16.89)	11.85Bb (10.82–12.99)

Values in the same column with different lowercase letters are significantly different ($P < 0.05$) and values in the same row with different uppercase letters are significantly different ($P < 0.05$); lethal dose ratio test.

Table 4. Exposure time (h) and fiducial limits (with 95% CI) required to obtain 99% mortality (LT_{99}) of eggs and larvae of *R. pomonella* at two temperatures and pressures

Pressure (kPa)	Life stage	Exposure time (h)	
		25°C	30°C
3.33	Eggs	105.98Aa (99.89–113.01)	51.46Ba (48.54–54.90)
	Larvae	10.16Ac (9.88–10.43)	20.97Bc (19.12–23.30)
6.67	Eggs	97.55Aa (91.78–104.32)	52.48Ba (48.08–58.04)
	Larvae	63.62Ab (59.14–69.00)	33.41Bb (30.53–37.04)

Values in the same column with different lowercase letters are significantly different ($P < 0.05$) and values in the same row with different uppercase letters are significantly different ($P < 0.05$); lethal dose ratio test.

Table 5. Comparison of rating scores for two cultivars of apples exposed to 6.67 kPa at 25 and 30°C for 3 and 5 d with untreated controls

Treatment	'Golden Delicious'		'Red Delicious'	
	25°C	30°C	25°C	30°C
Rating: external appearance				
Controls	3.67a (±0.33)	3.89a (±0.26)	3.56ab (±0.41)	3.44b (±0.29)
3 d	3.56a (±0.34)	3.67a (±0.29)	2.89a (±0.42)	2.33a (±0.5)
5 d	3.67a (±0.33)	3.00a (±0.37)	4.00b (±0.33)	1.89a (±0.35)
Rating: internal appearance				
Controls	4.11a (±0.26)	4.33a (±0.24)	3.56a (±0.41)	3.44b (±0.24)
3 d	4.00a (±0.33)	3.89a (±0.26)	3.22a (±0.40)	2.22a (±0.55)
5 d	4.22a (±0.22)	3.67a (±0.17)	4.56b (±0.18)	2.00a (±0.41)
Rating: taste				
Controls	3.89a (±0.26)	3.89a (±0.31)	3.44a (±0.47)	3.11a (±0.26)
3 d	3.22a (±0.49)	3.56a (±0.29)	3.22a (±0.32)	2.89a (±0.35)
5 d	3.44a (±0.47)	3.78a (±0.22)	4.11a (±0.35)	2.89a (±0.48)

Means within the same column with the different letters are significantly different at $P < 0.05$ using least significant difference in LSMEANS statement with a SLICE option.

The numerical rating on a scale of 1–5 refers to 1 as worst and graduating to 5 as the best for the three sensory factors.

Untreated control apples were kept at ambient room conditions until low-pressure exposures were finished on treatment apples, then all apples were stored at 1°C for 2 wk before evaluation.

how hypobaric storage might be commercially applied for apples. These details will require more applied research focused on specific pest–fruit combinations to develop commercial applications. *R. pomonella* eggs and larvae, as with stored-product insects (Mbata and Phillips 2001), generally showed decreased exposure time needed for acceptable mortality when the temperature was increased, except in the case of fly larvae at 3.33 kPa, which required more time at high temperature and may represent a true biological phenomenon, or may require further research to explain. Hypobaric storage has been shown to extend the shelf life of many fresh commodities (Burg 2004, Thompson 2010). Its use as a phytosanitary treatment would be unique in that phytosanitary treatments such as fumigation or heat usually may shorten the shelf life of fresh commodities, and almost never lengthen them (Heather and Hallman 2008).

Hypobaric storage is often done in practice at low temperatures, and the effect of hypobaric storage at low temperatures should be examined. Heather and Hallman (2008) point out that in some studies combining cold with low-oxygen storage, the cold by itself seemed to be providing essentially all of the insect mortality, and when cold temperatures were not low enough to be fully lethal to insects, the cold seemed to be reducing efficacy of the low oxygen. The latter observation may be logical in that cold reduces metabolism of poikilotherms, making them less susceptible to deficiencies or excesses of atmospheric components. If treatments could not be efficaciously done at low temperatures, they should be developed so the commodity is increased to ambient temperatures for hypobaric treatment until efficacy was achieved, and then the commodities could be returned to the desired low temperature for storage and transportation.

The lower consumer acceptance rating for 3- and 5-d-treated apples of Red Delicious at 30°C is perhaps an indication of detrimental effect of higher temperature on appearance of apples. Interestingly, for taste criteria, the three treatments were not significantly different (Table 5). External appearance is one of the important factors for marketability of apples and therefore low-pressure treatment at 30°C may not be the proper choice for the Red Delicious. Although we recognize that our sensory study was limited in scope and not conducted according to rigorous standards of that field (Watts et al. 1989),the results suggest that a cultivar's response to low pressure varies at different temperatures because the Golden Delicious was unaffected compared with Red Delicious. In practice, a temperature of 25°C is reasonable and achievable in many localities. Overall, the research reported here should encourage additional work on hypobaric storage for phytosanitary treatments of a number of commercially important fresh commodities and their actionable quarantine insect pests.

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