

# Efficacy of Delayed Atmospheric Modification in a Heat/Modified Atmosphere Phytosanitary Treatment

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**ABSTRACT** The combination of heat and low levels of oxygen increases mortality to insects infesting fruit compared with either heat or low oxygen alone. This combination treatment shows promise to disinfest commodities of quarantine pests. Heated air/modified atmosphere treatments employ the modified atmosphere (e.g., low oxygen) during the entire treatment interval. There is a positive relationship between temperature and efficacy of heat/modified atmosphere treatments. Efficacy of delaying atmospheric modification in a heat/modified atmosphere treatment was studied with the Mexican fruit fly, *Anastrepha ludens* (Loew) (Diptera: Tephritidae), a quarantine pest of citrus and other fruit in Mexico, Central America, and southern Texas. Larvae were subjected to heat/low oxygen treatments in vitro as well as in grapefruit, *Citrus paradisi* Macfayden. The relationship between time delay of the modified atmosphere and estimated time required to kill 99% of Mexican fruit fly third instars was not linear, which would indicate an additive relationship, but followed a sigmoid relationship. When infested grapefruit were heated with 47°C air in three atmospheric regimes: 1) air; 2) N<sub>2</sub> at 99 kPa plus O<sub>2</sub> at 1 kPa; or 3) air for 55 min then N<sub>2</sub> at 99 kPa plus O<sub>2</sub> at 1 kPa for the remainder of the treatment, estimated 99% prevention of pupariation was 157, 127, and 141 min, respectively.

**KEY WORDS** *Anastrepha ludens*, quarantine, commodity treatment, controlled atmosphere, disinfestation

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The combination of heat with low levels of oxygen and/or raised levels of carbon dioxide (modified atmospheres) has been shown to provide considerably increased levels of mortality to insects infesting fruit compared with either heat or modified atmosphere alone or sequentially (Neven et al. 2009). This combination treatment is promising as a phytosanitary measure to disinfest commodities of quarantine pests. Recently, the USDA approved heated air/modified atmosphere phytosanitary treatments against *Cydia pomonella* (L.) and *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae) on four fruit (Neven and Rehfield-Ray 2006, Neven et al. 2006, APHIS 2009).

Heat/modified atmosphere treatments may be the most complicated to apply of all approved commercial treatments (Heather and Hallman 2008), leading to a greater risk of treatment failure or rejection compared with simpler treatments when one of the components of the treatment strays outside of the accepted limits of the treatment protocol control parameters. For example, the heat/modified atmosphere treatments approved by APHIS (2009) specify relative humidity, O<sub>2</sub> and CO<sub>2</sub> concentrations, air speed, heating rate, final temperature, temperature reached at the center of fruit after 2.5 h, holding time at that center temperature, and total treatment time. Steps to simplify these

treatments without risking efficacy would be beneficial.

Heated air/modified atmosphere treatments typically employ the modified atmosphere during the entire time that the treatment is conducted. There is a marked positive relationship between temperature and efficacy of heat/modified atmosphere treatments (Heather and Hallman 2008, Neven et al. 2009). Therefore, during a heat/modified atmosphere treatment the greatest synergistic effect should occur later in the treatment as temperatures increase. Delaying the modification of the atmosphere in the treatment chamber until later in the treatment may save some cost of producing the atmosphere during the entire treatment as well as lower the risk that the modified, controlled atmospheres may not be maintained within the specified limits for the duration of the treatment.

Shellie et al. (1997) considered the third instar the most heat-resistant stage and found heated modified atmosphere (1% O<sub>2</sub>) to be more effective than heated air in killing Mexican fruit fly, *Anastrepha ludens* (Loew), third instars placed inside grapefruit, *Citrus paradisi* Macfayden.

The objective of this research was to determine whether delaying atmospheric modification in a heat/modified atmosphere phytosanitary treatment would reduce efficacy in a nonlinear fashion. This research was conducted with the Mexican fruit fly a quarantine

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pest of citrus and other fruit in extreme southern Texas and much of Mexico south to Costa Rica.

### Materials and Methods

**Source of Mexican Fruit Fly.** Mexican fruit flies were from the USDA-APHIS Mexican Fruit Fly Rearing Facility at Mission, TX, and originated with flies collected from mangoes in Morelos, Mexico (Moreno et al. 1991). The strain, continuously cultured since 1953, is used in Mexican fruit fly sterile release programs in Texas, California, and Mexico.

**Heating Block Tests with Modified Atmosphere.** A heating system (WSU Heating Block System, model HB02.7, Pullman, WA) developed by Ikediala et al. (2000) was used to heat third instar Mexican fruit flies. It consisted of two aluminum blocks (25.4 cm<sup>2</sup>), with a bottom and top thickness of 2.5 and 2.0 cm, respectively. The blocks fit together, leaving a space 21.4 by 22.1 cm in width by 3 mm in depth where the insects are placed. Electric heating pads are attached to the back of each plate. Type T (copper-constantan) thermocouples (Omega Engineering, Stamford, CT) inserted through holes near the center of each block monitor temperatures. The thermocouples were calibrated in ice water and checked at operating temperatures with an electronic thermometer (model 4021, Control Company, Friendswood, TX) calibrated with an ASTM-certified glass mercury thermometer (model 1003-FC, Ertco, Sparta, NJ). Heating rate, set-point temperature, and exposure time were controlled by a customized Visual Basic program and PID controllers (Omega Engineering) via a solid state relay. The system was found to vary  $\leq 0.1^{\circ}\text{C}$  compared with the certified thermometer.

An ultralow oxygen environment was achieved in the heating block by passing gaseous nitrogen from a cylinder through a flowmeter (model EW-32466-14, Cole-Palmer Instrument Co., Vernon Hills, IL) at the rate of 2.3 liters/min. The humidity was raised by passing the nitrogen through 1.9 liters of reverse osmosis-treated water in a 4-liter plastic container before entering the block. Gas passes through a channel in the block to be heated to operating temperature before entering the insect treatment space. A rubber seal between the two halves of the heating block helps maintain the modified atmosphere within the treatment space.

One hundred diet-reared Mexican fruit fly third instars with 50 ml of diet were placed in the heating block at a starting temperature of 25.0°C. Air was flowing through the heating block at 2.3 liters/min. The heating rate was set at 0.3°C/min to simulate the heating rate of citrus fruit subjected to heated air treatments and heating was stabilized when the operating temperature reached 44°C, which is similar to the temperatures reached in the center of citrus fruit during heated air treatments (Shellie et al. 1997). When the temperature reached 30, 32, 34, 36, 38, 40, 41, 42, 43, or 44°C, airflow was stopped and 100% nitrogen gas began flowing through the heating block. Five other treatments consisted of all N<sub>2</sub>; all air; and

air until 10, 30, or 50 min after the operating temperature reached 44°C followed by N<sub>2</sub> thereafter. The insects were removed from the heating block at various holding times at 44°C with the goal of achieving a series of quantal responses for probit analysis (PROC PROBIT, SAS 9.1, SAS Institute, Cary, NC).

The objective of heat and modified atmosphere phytosanitary treatments is generally acute mortality (Heather and Hallman 2008), i.e., the insects should be dead soon after the treatment is terminated for it to be considered successful. However, larvae often seem dead (do not move) soon after treatment only to become mobile or pupariate later. Because the moment of larval mortality is difficult to ascertain in a simple manner, efficacy was determined by prevention of pupariation in any form, a more discrete and easier to verify measurement than larval mortality at some point in time after treatment. Of course, prevention of pupariation may not be the measure of efficacy ultimately used to determine efficacy of a phytosanitary treatment. Indeed, inspectors of imported fruit treated with heat or modified atmospheres would not wait to determine prevention of pupariation of any apparently live tephritid larvae found but would consider the shipment in violation of quarantine (Heather and Hallman 2008). But for the objective of this research (relative comparisons of heat/modified atmosphere treatments) prevention of pupariation suffices. After treatment larvae were placed in 200-ml plastic containers with 50 ml of vermiculite as a pupariation medium and subsequently observed for pupariation. Control cohorts were placed directly in the vermiculite. If any larvae in the treatments pupariated, treatment time was increased by 5–20 min (depending on the pupariation rate observed) until a total of six replicates (600 third instars) were treated with no pupariation.

O<sub>2</sub> and CO<sub>2</sub> levels were determined periodically with a headspace gas analyzer (CheckMate II, PBI Dansensor, Ringsted, Denmark) by inserting a sampling needle into a hole in the heating block. A zirconia sensor measures O<sub>2</sub> levels and an infrared sensor is used for CO<sub>2</sub>. The hole in the block was plugged with a small tuft of cotton on a wire to keep larvae from escaping and reduce gas flow from outside the heating block. The analyzer was calibrated by the manufacturer at the beginning of this research, which lasted 9 mo in total.

Data from each treatment were analyzed with PROC PROBIT (SAS 9.1) using the normal probability density function with log 10 of dose tested with Pearson goodness-of-fit. The relationship between the time delay before applying N<sub>2</sub> and the total time to reach the ED<sub>99</sub> for all treatments combined was analyzed with nonlinear models (Prism 4, GraphPad Software Inc., San Diego, CA).

**Scale-Up Testing With a Modified Atmosphere/Heat Fruit Treatment System.** Tests were conducted on the effect of delaying onset of low oxygen in a heat/modified atmosphere treatment against Mexican fruit fly third instars infesting grapefruit. A device (Controlled Atmosphere Temperature Treatment

**Table 1.** Failure of Mexican fruit fly pupariation at various holding times at 44°C when atmosphere was switched from air to N<sub>2</sub> at different temperatures during heating of third instars from 25 to 44°C in a heating block system

Temp at switch to N <sub>2</sub> (°C)	Holding time at 44°C (min): failure of pupariation at that time (%)					
25	25: 95.0	30: 97.5	35: 99.33	40: 99.08	45: 100	
30	35: 94.3	40: 98.3	45: 99.8	50: 100		
32	35: 91.0	40: 96.3	45: 99.2	50: 99.3	55: 100	
34	35: 95.1	40: 98.2	45: 99.2	50: 99.0	55: 99.8	60: 100
36	40: 94.5	45: 96.8	50: 98.2	55: 99.2	60: 100	
38	30: 73.0	45: 96.0	55: 98.0	60: 99.7	65: 100	
40	40: 94.7	55: 97.0	60: 99.3	65: 99.7	70: 99.8	75: 100
41	50: 97.5	55: 99.3	60: 99.0	65: 99.5	70: 99.3	75: 100
42	55: 95.7	60: 99.5	65: 99.5	70: 99.5	75: 99.8	80: 100
43	50: 91.0	55: 96.3	65: 99.7	75: 99.7	80: 100	
44	50: 65	70: 99.0	75: 99.5	80: 99.7	85: 100	
44 + 10 min <sup>a</sup>	75: 93.0	80: 98.5	85: 99.5	90: 99.0	95: 100	
44 + 30 min <sup>a</sup>	70: 76.0	75: 96.5	80: 95.5	85: 97.5	90: 99.6	95: 100
44 + 50 min <sup>a</sup>	95: 97.0	100: 97.0	105: 99.8	110: 99.5	115: 100	
All air <sup>b</sup>	110: 97.3	115: 98.3	130: 99.3	135: 98.5	145: 98.3	150: 100

Heat-up period (rate, 0.3°C/min) lasted ≈63.3 min. Numbers of third instars treated at each data point vary from 100 to 600 and increased as the level of control increased.

<sup>a</sup> 44°C + 10, 30, or 50 min means that the switch was made 10, 30, or 50 min, respectively, after the temp reached the holding temperature of 44°C.

<sup>b</sup> All air had a seventh data point: 100 min with 91.5%.

Chamber [CATTC], Techni-Systems, Chelan, WA) capable of controlling temperature, humidity, and atmospheric components in a treatment space (0.6 by 0.4 by 1.2 m) that can hold >100 grapefruit was used (Neven and Mitcham 1996). The machine and software were updated and calibrated by the manufacturer 1 mo before this study was initiated in the same fashion as the machine used by Neven and Rehfield-Ray (2006) and Neven et al. (2006), and this part of the research lasted 2 mo.

The CATTC uses 3-mm thick stainless steel-sheathed thermistors to record temperature under the surface and at the center of several fruit. Because of their thickness, it is hypothesized that the thermistors might transmit heat from the air and surface of the fruit in the same way that aluminum or stainless steel nails are claimed to accelerate the baking of potatoes, thus resulting in higher temperature readings in the center of the fruit than really occur. To check that possibility #36

gauge (0.13-mm-diameter wire) thermocouples were inserted into two fruit at each run near the center where probes were inserted and the difference between readings from both instruments 50 min after initiation of the tests was compared with a *t*-test.

Approximately 35 'Rio Red' grapefruit harvested near Weslaco, TX, were placed in a screen cage (1.2 by 0.8 by 0.5 m) with ≈20,000 Mexican fruit fly adults for 1–2 h. Infested fruit were held at 24 ± 0.5°C and 65% RH, until late third instars had formed, 15–20 d after infestation.

In total, 56 grapefruit were placed in two bins in the CATTC; 24–28 of the fruit were infested with Mexican fruit fly third instars. The three treatments were as follows: 1) air during the entire time, 2) 1 kPa O<sub>2</sub> plus 99 kPa N<sub>2</sub> the entire time, and 3) air until 55 min into the treatment upon which the atmosphere was switched to 1 kPa O<sub>2</sub> plus 99 kPa N<sub>2</sub> for the duration of the treatment. Treatments were done at four dif-

**Table 2.** Probit analyses of failure of pupariation of third-instar Mexican fruit fly reared in diet and treated in vitro in air until a certain temperature was reached and then N<sub>2</sub> thereafter

Temp at switch to N <sub>2</sub> (°C)	No. larvae tested	χ <sup>2</sup>	df	Slope ± SE	ED <sub>90</sub> in min (95% FL) <sup>a</sup>	ED <sub>99</sub> in min (95% FL) <sup>a</sup>
25	2400	0.21	3	4.29 ± 0.99	20.8 (13.2–24.8)	36.4 (33.3–40.5)
30	1800	0.67	2	11.9 ± 2.22	33.2 (30.6–34.7)	40.6 (39.1–43.3)
32	1900	0.52	3	9.33 ± 1.60	34.8 (31.6–36.8)	45.1 (43.2–48.1)
34	2700	0.79	4	6.41 ± 1.20	30.1 (25.4–32.8)	43.8 (41.6–47.7)
36	2500	0.38	3	6.79 ± 1.12	36.6 (32.6–39.0)	52.1 (49.6–56.5)
38	1600	0.26	3	6.73 ± 0.68	38.1 (35.9–40.2)	54.5 (51.1–59.4)
40	2200	0.33	4	5.22 ± 0.84	35.1 (29.4–39.0)	55.7 (51.6–61.8)
41	1800	0.41	4	5.21 ± 1.76	36.1 (13.9–44.3)	57.4 (50.3–63.6)
42	2200	0.28	4	9.95 ± 2.30	48.5 (40.8–52.2)	61.8 (59.2–66.0)
43	1600	0.46	3	9.94 ± 1.96	49.0 (44.3–51.6)	62.4 (59.3–68.2)
44	1500	0.77	3	12.67 ± 1.13	58.8 (57.1–60.9)	71.1 (67.8–75.9)
44 + 10 min	1200	0.18	3	17.53 ± 4.46	72.5 (65.3–75.5)	83.1 (80.5–88.3)
44 + 30 min	1800	0.01	4	16.17 ± 3.4	74.0 (66.9–77.9)	85.8 (81.0–100.9)
44 + 50 min	2300	0.07	3	15.5 ± 5.2	88.2 (79.7–92.2)	103.0 (100.5–106.6)
All air	2600	0.002	5	6.13 ± 2.10	90.4 (12.5–105.3)	133.9 (118.9–376.8)

<sup>a</sup> ED, effective dose (dose in minutes that prevented pupariation); 90th and 99th percentiles.

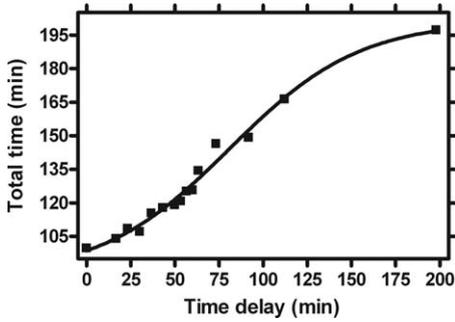


Fig. 1. Estimated effective dose to prevent 99% pupariation (ED<sub>99</sub>) of Mexican fruit fly third instars in a heating block system when the relationship between time delay before applying N<sub>2</sub> and the total time to reach the ED<sub>99</sub> is analyzed with a four-parameter logistic model. Heating rate is 0.3°C/min.

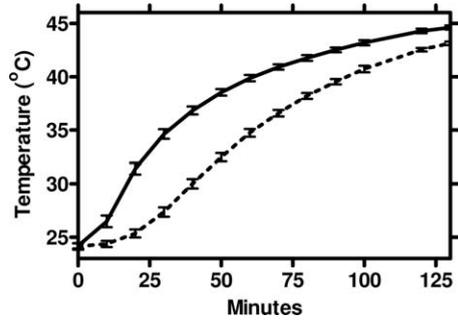


Fig. 2. Mean temperatures at grapefruit centers (dotted line) and 1–2 mm under the peel (solid line) during heated air treatment at 47°C in a controlled atmosphere temperature treatment chamber.

ferent times to generate a series of quantal responses for probit analysis. The treatment of air followed by 1 kPa O<sub>2</sub> represented an intermediate treatment to determine whether the tendency observed in the in vitro testing would be manifested in a semicommercial situation. Other than the atmosphere, the remaining components of the treatments except total treatment time (90–160 min) were the same throughout. The heating rate was 2°C/min until the temperature reached 47°C, where it was held stable. Relative humidity was near 100%, and air velocity was 2.8–3.0 m/s.

In total, 12 thermistors were placed in the three largest fruit (mass, 441.4 ± 10.7 g; polar diameter, 89.3 ± 5.9 mm) in both bins. One thermistor per fruit was placed 1–2 mm under the peel and another at the center.

Immediately after treatment, the fruit were removed and placed individually in 2-liter plastic containers (model 64S, Sweetheart Plastics, Wilmington, MA) with screened lids at 24 ± 0.5°C and 65% RH for ≈24 h after which the fruit were opened and larvae removed, counted, and placed in 200-ml plastic containers with 50 ml of vermiculite. Survival was scored as larvae that pupariated regardless of the shape of the puparia. Data were analyzed with PROC PROBIT (SAS 9.1) using the normal probability density function with log 10 of dose tested with Pearson goodness-of-fit.

**Results and Discussion**

**Heating Block Tests With Modified Atmosphere.** O<sub>2</sub> concentrations fell in a logarithmic manner, reaching 1% of ambient O<sub>2</sub> levels after 10 min. O<sub>2</sub> concentrations continued to fall and were between 0.0 and 0.07 kPa at the end of all treatments involving N<sub>2</sub>. CO<sub>2</sub> readings were usually 0.1 kPa but occasionally were as high as 0.8 kPa.

Percentage of failure of pupariation versus holding time at 44°C for each treatment is presented in Table 1. The holding time to achieve 100% failure of pupariation was inversely proportional to the time delay in applying N<sub>2</sub>. One-hundred percent failure of pupariation ranged from 45 min when N<sub>2</sub> was applied during

the entire treatment to 150 min when only air was used.

All of the 15 treatment atmospheres except two (purging with N<sub>2</sub> 30 min after the temperature reached 44°C and air during the entire treatment time) fit the probit model (Table 2). Delaying the application of N<sub>2</sub> almost 17 min until the temperature reached 30°C increased the estimated holding time to reach the ED<sub>99</sub> compared with N<sub>2</sub> during the entire treatment by 4 min, showing that even a slight delay in the application of N<sub>2</sub> when temperatures were not yet lethal was reflected in increased time required for control of Mexican fruit fly. However, even when application of N<sub>2</sub> was delayed until 50 min after the temperature reached 44°C the estimated holding time to reach the ED<sub>99</sub> compared with ambient air and heat was reduced from 134 to 103 min, showing that application of N<sub>2</sub> relatively late during a heat treatment may still reduce treatment times appreciably.

The relationship between the time delay before applying N<sub>2</sub> and the total time to reach the ED<sub>99</sub> fit the four-parameter logistic model  $Y = 201.4 / (1 + 10^{(81.1 - X)0.0118})$ , where Y is the total treatment time (minutes) to prevent 99% pupariation, and X is the logarithm of the time delay

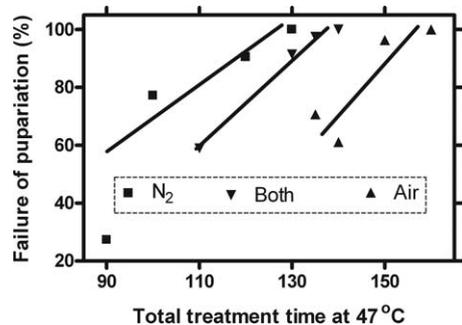


Fig. 3. Failure of Mexican fruit fly pupariation versus total treatment time when infested grapefruit treated at an air temperature of 47°C under three atmospheric regimes in a controlled atmosphere temperature treatment chamber: air, air during the entire treatment; N<sub>2</sub>, 1 kPa O<sub>2</sub> plus 99 kPa N<sub>2</sub> the entire time; and both, air for the initial 55 min then 1 kPa O<sub>2</sub> plus 99 kPa N<sub>2</sub> for the duration.

**Table 3. Probit analyses of failure of pupariation of Mexican fruit fly treated with heated air (47°C) as third instars infesting grapefruit**

Treatment atmosphere	No. larvae tested	$\chi^2$	Slope $\pm$ SE <sup>a</sup>	ED <sub>90</sub> in min (95% FL) <sup>b</sup>	ED <sub>99</sub> in min (95% FL) <sup>b</sup>
Air	2592	0.001	33.55 $\pm$ 3.12	147.3 (145.9–149.1)	158.2 (155.3–162.5)
1 kPa O <sub>2</sub> + 99 kPa N <sub>2</sub>	1237	0.001	17.57 $\pm$ 1.15	111.4 (109.4–113.9)	127.7 (123.7–133.0)
Air then 1 kPa O <sub>2</sub> + 99 kPa N <sub>2</sub> <sup>c</sup>	591	0.23	18.81 $\pm$ 1.82	125.3 (122.9–128.6)	142.5 (137.4–150.0)

<sup>a</sup> df = 2.

<sup>b</sup> ED, effective dose (dose in minutes that prevented pupariation); 90th and 99th percentiles.

<sup>c</sup> Held in air for the first 55 min then 1 kPa O<sub>2</sub> + 99 kPa N<sub>2</sub> for the remainder.

(minutes) before replacing air with N<sub>2</sub> ( $R^2 = 0.99$ ); deviation from the model is not significant according to a runs test ( $P = 0.70$ ) (Fig. 1).

A linear relationship between the time delay in applying N<sub>2</sub> and the total time required to prevent pupariation would be expected if the effect of the modified atmosphere on prevention of pupariation depended entirely on the amount of time under modified atmosphere. Because these data showed close fit to a nonlinear model suggests that delaying onset of the modified atmosphere in a heat/modified atmosphere treatment would not increase the required treatment time as much as might be expected by an additive relationship, at least during the first half of the treatment. But because the treatment using all air did not fit the model, the value of its ED<sub>99</sub> (which would form the upper anchor of any line defining an additive relationship between the delay in applying N<sub>2</sub> and the total time required to prevent pupariation) is tenuous.

**Scale-Up Testing With a Modified Atmosphere/Heat Fruit Treatment System.** The mean increase in temperature registered by the 3-mm-thick thermistors compared with the thin thermocouples 50 min after the treatments were begun was a statistically insignificant 0.086°C ( $t = 0.26$ ,  $df = 13$ ,  $P = 0.80$ ).

Temperature readings at grapefruit centers and under the peel are presented in Fig. 2. Temperature increased in a sigmoid fashion with center temperatures lagging behind subsurface temperatures as much as 8°C. The O<sub>2</sub> concentration declined in a logarithmic fashion as it did in the in vitro tests reaching the target concentration of 1 kPa after 20 min.

The relationship between failure of Mexican fruit fly pupariation and total treatment time when infested grapefruit were treated at an air temperature of 47°C under three atmospheric regimes is presented in Fig. 3. Data for heating in air and heating in 1 kPa O<sub>2</sub> plus 99 kPa nitrogen did not fit probit analysis (Table 3). However, results are presented to compare them with the treatment of air followed by 1 kPa O<sub>2</sub> plus 99 kPa nitrogen 55 min into the treatment, which did fit probit analysis. The ED<sub>99</sub> value for prevention of Mexican fruit fly pupariation was 157, 127, and 141 min, respectively, for heating in air, 1 kPa O<sub>2</sub> plus 99 kPa nitrogen, and air followed by 1 kPa O<sub>2</sub> plus 99 kPa nitrogen 55 min into the treatment. Those results together with the raw data (Fig. 3) showing a increase in prevention of pupariation with increased exposure to N<sub>2</sub> indicate that the delayed application of modified atmosphere provided an intermediate level of control as observed in the in vitro tests.

This research demonstrates that delaying onset of the modified atmosphere in a heat/modified atmosphere phytosanitary treatment against Mexican fruit fly is compensated by less than expected increases in total treatment time, indicating that the presence of the modified atmosphere early in the treatment when the load is not yet hot is not as critical as might be expected by an additive relationship between treatment time and time under modified atmosphere. The atmosphere inside tephritid-infested fruit may be low in oxygen, at least until the time when the larvae bore holes in the peel in preparation for exiting (Hallman et al. 1994). Hence, the effect of treatment under modified atmospheres may not be as pronounced as it could be for other insects, such as larvae of Lepidoptera, which are typically exposed to ambient atmospheres while infesting fruit (Neven and Rehfield-Ray 2006, Neven et al. 2006). Therefore, delay of modified atmosphere may have a greater benefit against pests entirely exposed to ambient atmospheres than it does for tephritids.

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