Entomologia Experimentalis et Applicata

DOI: 10.1111/j.1570-7458.2012.01229.x

TECHNICAL NOTE

An improved system to assess insect tolerance to heated controlled atmosphere quarantine treatment

Lisa G. Neven[1]*, Shaojin Wang[2] & Juming Tang[3]

[1]*USDA-ARS, Yakima Agricultural Research Laboratory, Wapato, WA 98951, USA,* [2]*Northwest A&F University, College of Mechanical and Electronic Engineering, Yangling, Shaanxi 712100, China, and* [3]*Biological Systems Engineering, Washington State University, Pullman, WA 99164, USA*

Accepted: 4 January 2012

Key words: Oriental fruit moth, *Grapholita molesta*, Lepidoptera, Tortricidae, commodity treatments, controlled atmosphere temperature treatment system

Introduction

When developing a new postharvest quarantine treatment for fresh fruits and vegetables, certain elements should be included in the experimentation and documentation of the procedure (NAPPO, 2011). First, it is essential to identify the pest(s) of concern and the most tolerant developmental stage of each species that could be in or on the commodity at the time of harvest or export. These tests usually involve the mass rearing thousands of insects, identification of the developmental stages, and treatment of large numbers of each stage over a range of 'doses' (e.g., time and temperature in the case of thermal treatments). The experiments need to be designed so that appropriate statistical analyses can be performed to compare the response of the different stages with one another to identify the most tolerant stage. Following the identification of the most tolerant stage of a species, it may be necessary to compare the relative tolerance of two or more species with one another to identify the most tolerant species to a particular treatment. If these treatments have to be developed using an infested commodity, labor costs involved in pre- and post-treatment assessments as well as the cost of the commodity may make the development of the treatment cost prohibitive for many minor crops. Therefore, it is desirable to develop a model system in which the most tolerant stage of a pest can quickly be assessed without the necessity of infesting the commodity and then examining the treated commodity for survivors.

Several controlled atmosphere/temperature treatment system (CATTS) treatments have been developed to control codling moth and oriental fruit moth in apples, peaches, and nectarines (Neven & Rehfield-Ray, 2006a; Neven et al., 2006; APHIS, 2011), as well as codling moth and Western cherry fruit fly in sweet cherries (Neven, 2005; Neven & Rehfield-Ray, 2006b; APHIS, 2011). The CATTS treatments involve the application of a hot moist forced air under a modified atmosphere consisting of low O_2, ca. 1%, and high CO_2, ca. 15%. The development of these treatments involved extensive in-fruit assessments of dose mortality, determination of the most tolerant stage, the most tolerant species, efficacy tests, and finally, confirmatory tests. These tests took several years to perform and were very costly. Not only was it necessary to rear the insects used in the tests, but also high-quality organic fruit needed to be purchased, infested, treated, and evaluated. Efficacy tests required over 5 000 insects to be treated and controlled. The confirmatory tests required >30 000 insects to be treated and controlled. When final costs were evaluated, we determined that it cost ca. $1 per insect to conduct these tests. It was apparent that a model system was needed to rapidly assess insect tolerance to CATTS that did not involve in-fruit treatments.

We have previously published on the development of a controlled atmosphere/water bath (CA-WB) system for rapidly assessing the most tolerant stage and the most tolerant species to a CATTS treatment (Neven, 2008; Johnson & Neven, 2010, 2011). The CA-WB system involves the use of programmable water baths and a series of test tubes in a treatment container, which allowed for the treatment of up to 30 individual larvae at a time to a heat treatment under a modified atmosphere environment. The atmospheres were modified by using hand-adjusted flow meters connected in series and monitored with an O_2/CO_2 gas analyzer (Neven, 2008). This system is technically effective, but is relatively slow, labor-intensive, and not amenable to treating large numbers of insects at one time. To solve this problem, we decided to design a device that would allow for the treatment of large numbers of insects while minimizing handling and labor.

*Correspondence: Lisa G. Neven, USDA-ARS Yakima Agricultural Research Laboratory, 5230 Konnowac Pass Road, Wapato, WA 98951, USA. E-mail: lisa.neven@ars.usda.gov

A programmable heat block which was capable of heating a small space (20 × 20 × 0.5 cm) over a wide range of heating rates and temperatures was developed at Washington State University (WSU) Biological Systems Engineering Department (Wang et al., 2002). In the research reported herein, we modified the heat block to apply humidified controlled atmospheres using a computerized gas mixing system. The goal of the research was to develop an easy-to-use system to treat insects under a heated controlled atmosphere with higher capacity and improved reproducibility of treatments to facilitate identifying the insect stage most resistant to the treatment. We compared the effectiveness of the improved controlled atmosphere heat block (CA-HB) to the CA-WB system and in-fruit CATTS treatment.

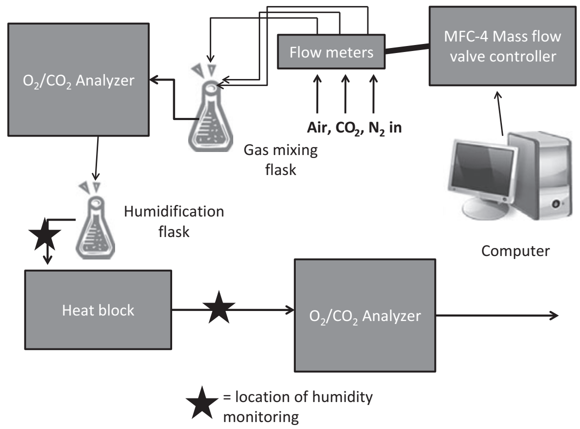

Figure 1 Diagram of controlled atmosphere heat block (CA-HB) system showing location of humidification flasks, humidity monitoring, O_2 and CO_2 monitoring, atmospheric gas mixing system, and heat block with computer controls and monitoring.

Materials and methods

Heat block

A heat block (Wang et al., 2002) was modified to allow the addition of two entry and one exit ports to allow for gas flow to establish a controlled atmosphere. The heat block is controlled and temperature is continually monitored with a notebook computer using the Windows-based WSU-i-Heater program (WSU, 2004). A schematic and description of the heat block may be found in Yin et al. (2006). The block is composed of a top and bottom constructed from aluminum. The bottom contained the insect chamber (20 × 20 × 0.5 cm). To form a tight seal, a rubber gasket was placed in a groove around the outside rim of the bottom plate, which was held in place when the top plate was put into place.

Gas mixing system

Controlled atmospheres were mixed using an MFC-Mass Flow Valve Controller and MFC-Control Software from Sable Systems International (Las Vegas, NV, USA). The flow of individual gases, air (ca. 21% O_2), nitrogen, and carbon dioxide, were controlled through mass flow meters (Side Trak™ Sierra Series Mass Flow Meters; Sierra Instruments, Monterey, CA, USA) (Figure 1). To ensure consistent gas mixing, the three flow meters were plumbed into a 2-l side-arm flask with ¼-inch (ca. 0.6 cm) tubing that ended in standard aquarium air stones. The flask was placed on top of a magnetic stir plate and a stir magnet was set in the flask for continuous mixing of gases.

Levels of oxygen and carbon dioxide were monitored using two O_2/CO_2 analyzers (Techni-Systems, Chelan, WA, USA) with a detection range of 0–100% for O_2 and 0–80% for CO_2 and a sensitivity of ±0.1% for each gas. One analyzer monitored atmospheric gas composition entering the heat block, the other monitored gas composition coming out of the heat block.

To maintain humidity levels in the chamber to those normally experienced by the insects in a treated commodity, the gas stream was humidified by bubbling the gas mixture through a 2-l side-arm flask containing 1 l of deionized water. In addition, a wetted 20 × 20 cm piece of 3-mm filter paper was placed on the bottom of the insect chamber to maintain humidity during the tests. The relative humidity of the gas stream was periodically monitored by inserting a Hobo U12 data logger (Onset®, Poncasset, MA, USA) into a 1-l Mason jar located after the humidification flask or after the heat block to ensure 95–99% r.h. The flow rate through the heat block was 0.47 l min^{-1}. The gas stream entered the heat block top on one side, which was split into two streams that circled the inside of the top of the heat block to heat the air to the block temperature. The air stream then passed through the insect treatment chamber and exited through a port directly underneath the entry port. Two strips of fine wire mesh were placed along the sides of the inside of the bottom plate to prevent larvae from exiting the chamber during treatments. The addition of the gas stream did not alter the temperature in the insect treatment chamber as evidenced by the recorded thermal data during treatments and compared with previous tests (Wang et al., 2002).

Insects

Oriental fruit moth, *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae), was chosen as a model organism against which treatment parameters and system conditions could be tested because its CATTS tolerance in fruit as well as in the CA-WB system was previously published (Neven et al., 2006; Neven, 2008). Oriental fruit moths were reared on a wheat germ-based diet originally developed for codling moth (Toba & Howell, 1991) under conditions previously described in Neven (2008). Insects were reared at 23 ± 2 °C, 50% r.h., and L16:D8 photoperiod. The fourth instars were extracted from artificial diet and treated in the CA heat block under air and 1% O_2/15% CO_2 CA environment, using a 0.4 °C min^{-1} heating rate starting from an initial 23 °C to a final temperature of 44.5 °C at total treatment durations of 0.5, 1, 1.5, and 2 h. Controls were held at 23 °C under normal ambient atmospheric conditions when the treatments were conducted. Untreated controls and treated larvae were then placed directly in 103.5 ml cups with 30 ml artificial diet and held at 23 ± 2 °C, 50% r.h., and L16:D8 photoperiod, for 7 days. Following the 7-day holding period, controls and treatments were examined for larval survival. A minimum of three replications of 25 individuals were treated in a single day. Corrected mortality was calculated using Abbott's equation (Abbott, 1925).

Operation of system

The following steps were required to operate the CA heat block system.

1. Turn on the MFC-4 controller 0.5 h prior to use.
2. Turn on the heat block controller and computer.
3. Set program for heat block program with heat rate, final hold temp, and hold time.
4. Start up the MFC-4 control software.
5. Open the valves from house air (ca. 21% O_2), house nitrogen (ca. 99.5% N_2), and bottled CO_2 gases. No more than 68.95 psi total pressure for all three lines.
6. Enter desired gas levels in the MFC-4 program to those defined for the test.
7. Turn on O_2/CO_2 analyzers.
8. Mist pre-cut 3-mm filter paper with water and place on to heat block bottom.
9. Place pre-counted test insects on to filter paper on heat block bottom.
10. Immediately replace top of heat block unit to make tight seal.
11. Ensure that O_2/CO_2 levels are at set point for test.
12. Begin heat block program and name storage file appropriately.
13. At the end of the test, remove the top of the heat block and immediately remove the filter paper with insects.
14. At the end of the run, stop the CA mixing system and stop the heat block program.

Comparisons with controlled atmosphere/water bath systems and controlled atmosphere/temperature treatment system

Comparisons with previous publications of CATTS (Neven et al., 2006) and CA-WB system (Neven, 2008) on the mortality of fourth instar oriental fruit moths were made to larvae treated in the CA-HB (this study). The CATTS system is a hot forced air system that is capable of applying a heat treatment under a modified atmosphere (Neven & Mitcham, 1996). Treatment conditions consisted of the establishment of a 1% O_2/15% CO_2 modified atmosphere with the dew point set at 2 °C below the lowest fruit surface temperature, and air speed was between 1.2 and 2 m s^{-1} (Neven et al., 2006). The temperature in the chamber was raised from an initial 23 °C to a final chamber temperature of 46 °C at a rate of 24 °C h^{-1}.

The CA-WB system, fully described in Neven (2008), was used to determine the response of fourth instar oriental fruit moth to heat treatments under both normal atmospheric air (RA) and modified atmospheres. Two programmable water baths were operated simultaneously using a heating rate of 24 °C h^{-1} from an initial temperature of 23 °C to a final bath temperature of 45.5 °C. One system operated under normal atmospheric air and the other operated under a modified atmosphere mixture of 1% O_2/15% CO_2 at a flow rate of 1 l min^{-1} (Neven, 2008). Insects from controls and treatments were handled as previously described for the CA-HB.

Results and discussion

Percent corrected mortalities of fourth instar oriental fruit moths after treatment with the CA-HB were compared with those obtained using another 'off fruit' test system, the CA-WB system (Neven, 2008), and an 'in fruit' CATTS treatment using infested nectarines (Neven et al., 2006) (Table 1). Mortality of oriental fruit moth in nectarines was lower for the 1.0 and 1.5 h time points as compared with the CA-HB and CA-WB model systems. This is most likely because the center of the fruit reached target temperatures later than in the model systems. However, the model systems are designed for determining relative tolerance to CATTS treatments, and are not intended to be the final step in the development of an in-fruit treatment. Those types of treatments, efficacy and confirmatory tests, do require treatments of several thousand insects in the selected commodity with no survivors (Follett & Neven, 2006).

Interestingly, fourth instar oriental fruit moth in CATTS-treated nectarines were completely controlled at 2 h. There have been reports in the literature where insect tolerance varied in relation to the substrate in which it was treated (Hallman, 1996; Hansen & Sharp, 2000). Hallman

Table 1 Comparison of mean (± SEM) corrected mortality (%) of fourth instar oriental fruit moth after heat treatments in a controlled atmosphere (CA) heat block (CA-HB) system (this study) to previously published data from a CA water bath (CA-WB; Neven, 2008) system and controlled atmosphere/temperature treatment system (CATTS) treatments in nectarines (Neven et al., 2006)

Time (h)	CA-HB AIR (n = 75)	CA (n = 75)	CA-WB AIR (n = 72)	CA (n = 112)	CATTS Nectarines (n = 180)
0.5	5.6 ± 13.5	0.0 ± 0.0	0.0 ± 0.9	16.7 ± 1.2	18.0 ± 1.4
1.0	18.9 ± 11.6	70.7 ± 15.7	55.6 ± 20.3	98.6 ± 0.9	34.0 ± 13.2
1.5	23.4 ± 8.5	97.3 ± 18.2	79.6 ± 8.6	100 ± 0.0	69.0 ± 0.9
2.0	49.4 ± 6.5	95.7 ± 2.5	57.2 ± 14.7	97.4 ± 2.6	100 ± 0.0

Sample sizes are indicated in parentheses. CA conditions were 1% O_2 and 15% CO_2. AIR indicates normal atmospheric air. Heat treatments using CA-HB and CA-WB systems started at 23 °C and temperature increased at a rate of 0.4 °C min^{-1} to a final temperature of 44.5 °C. CATTS treatments were conducted using a 0.4 °C min^{-1} heating rate from a chamber temperature of 23–46 °C under a 1% O_2/15% CO_2, −2 °C dew point, and air speed of 1 m s^{-1}.

(1996) found that third instars of the caribbean fruit fly were more tolerant to immersion in hot water than in hot grapefruit juice. Hansen & Sharp (2000) found that mortality of third instar caribbean fruit flies from heat treatments in air, water, artificial diet, or fruit pulp blends varies, with treatments in air and water resulting in the highest and lowest survival, respectively. It is possible that oriental fruit moth may be less able to withstand CATTS treatments when surrounded by fruit juices; this is worthy of further investigation.

Mortality results were different at the 1.0 and 1.5 h treatments in the CA-HB and CA-WB test systems. The CA-HB system had more uniform heating, faster CA establishment, less variable CA conditions once established, and higher humidity compared with the CA-WB system.

The heating lag time (difference between set point and actual block temperatures) was about 2 s in the CA-HB system (Wang et al., 2005) as opposed to 5 min in the glass tubes of the CA-WB system (Neven, 2008). The temperature range between set point and actual block temperature were within normal operating parameters previously reported for the heat block, which was 0.2 °C (Wang et al., 2002, 2005, 2009). In studies with the CA-WB system, we observed a 1 °C difference between water bath temperature and the temperature in the test tube where the insects were located (Neven, 2008). The CA-HB provides a more consistent application of the heat treatment, which resulted in more consistent results.

The CA-HB system had better gas controls and air tightness compared with the CA-WB system. The controlled atmosphere in the CA-HB system was established in 5 min or less after the gas flow was established. The CA-WB system took 5–10 min to establish the controlled atmospheres. When the top of the heat block was placed on to the unit, the controlled atmosphere levels were determined to be the same as the levels monitored before the heat block within 30 s. The oxygen levels remained within 0.1% of set point and the CO_2 levels remained within 0.5% of set point throughout the treatment. The MF-4 CA control system is an improvement over the CA-WB system where we observed that O_2 levels varied by 0.2% and CO_2 levels by 1%.

When the relative humidity of the air stream was monitored before injection into the heat block, levels were between 95 and 98% with an average (± SE) of 97.8 ± 0.1%. The relative humidity of the air stream exiting the heat block was on average 99.6 ± 0.02% (range 98–100%). The higher humidity was most likely due to evaporation as the air stream passed over the damp filter paper. The humidity levels in the CA-WB system averaged 72.3 ± 0.21% during the runs under a controlled atmosphere. The CA-WB system did not contain a humidification flask in the air stream line, but relied on wetted sponges to provide humidification. The addition of the humidification flask into the air stream greatly improved the ability to maintain high humidity during the treatments in the CA-HB system. It is essential to maintain high humidity levels when using a model system to monitor responses to heat and controlled atmospheres in internally feeding insects of fresh fruits as the humidity in the fruit is 100%, and insects have been documented to reduce frequency and duration of spiracle opening in response to desiccating environments (Mill, 1985; Chown & Davis, 2003; Gibbs et al., 2003). A reduction in spiracle opening would reduce respiration and metabolism, which in turn would mask the effects of the combination heat and CA treatment (Neven, 2003).

In conclusion, the CA-HB system with the computer programmable ability of both the heating unit and the atmospheres was a great improvement over the CA-WB system. This is a model system, which will be useful in the identification of the most tolerant stage and most tolerant species to a combination heat treatment under controlled atmospheres. This system is not a substitute for in-commodity testing, but reaches test conditions and maintains them more uniformly, saves material costs, and offers improved humidity control relative to the much larger CA-WB system previously used. The heat block system should help researchers quickly identify temperature/duration/CA conditions that might result in 100% insect mortality when insects are tested within the actual commodities of interest.

Acknowledgements

The authors thank Barbara Joos of Sable Systems for helping in the design of the CA control system. We thank Mrs. Michele Watkins and Ms. Anne Kenny Chapman for their technical assistance in conducting these tests. We also thank Dr. Lawrence Lacey and Dr. Robert Hollingsworth for peer reviews that were used to revise this manuscript prior to submission to the journal. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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