# PMRG RESEARCH GUIDELINE:

# GUIDELINES FOR THE DEVELOPMENT OF VAPOR HEAT DISINFESTATION TREATMENTS FOR FRUIT FLY HOST COMMODITIES

Produced by the

Phytosanitary Measures Research Group

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# CONTENTS

# INTRODUCTION

## Scope

[1] This research guideline outlines technical procedures that might be used to develop vapour heat treatments against quarantine fruit fly in host commodities.

## References

**Abbott, W.S.** 1925. A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology*, 18: 265–267.

**APPPC RSPM No.1.** 2004. Guidelines for the development of heat disinfestation treatments of fruit fly host commodities. Bangkok, APPPC, FAO

**ASTM E1847-96** 2013. Standard Practice for statistical Analysis of Toxicity Tests Conducted Under ASTM Guidelines, ASTM International, West Conshohocken, PA, 2013, www.astm.org

**ASTM E563-11** 2016. Standard Practice for Preparation and Use of an Ice-Point Bath as a reference temperature.

**Couey H M, & Chew V** 1986. Confidence limits and sample size in quarantine research. Journal of Economic Entomology 79: pp 887-890

**IPPC.** 1997. International Plant Protection Convention. Rome, IPPC, FAO.

**IPPC.** 2016. IPPC Procedure Manual For Standard Setting. International Plant Protection Convention. Rome, IPPC, FAO.

**ISPM 5**. 2007. Glossary of phytosanitary terms. Rome, IPPC, FAO.

**ISPM 18.** 2003. Guidelines for the use of irradiation as a phytosanitary measure. Rome, IPPC, FAO.

**ISPM 28.** 2007. Phytosanitary treatments for regulated pests. Rome, IPPC, FAO.

**ISPM 42** 2018. Requirements for the use of temperature treatments as phytosanitary measures. Rome, IPPC, FAO.

**Japan MAFF.** Guideline for experiment of development on quarantine vapour-heat treatment. 2014.

**Japan MAFF.** Minimum requirement for the report of cold treatment. 2014.

**JFTA.** 1996. Textbook for vapour heat disinfestation test technicians (revised) (in Japanese). Japan Fumigation Technology Association.

**PTTEG report 2015**. *Report: Expert Consultation on Cold Treatments December, 2013* in Buenos Aires, Argentina 2-6 December, 2013. IPPC, FAO

**WHO**. 2014. Calibration of temperature control and monitoring devices. Technical supplement to WHO Technical Report Series, No.961, 2011. Annex 9: Model guidance for the storage and transport of time and temperature-sensitive pharmaceutical products.

## Definitions

[2] Definitions of phytosanitary terms used in the present standard can be found in ISPM 5 (*Glossary of phytosanitary terms*).

## Background

[3] The Commission on Phytosanitary Measures (CPM), the governing body of the International Plant Protection Convention (IPPC), oversees the work of the IPPC Secretariat, which is tasked to facilitate the setting of international standards for phytosanitary measures (ISPMs).

[4] Phytosanitary treatments adopted by the CPM are published as annexes to ISPM 28 (*Phytosanitary treatments for regulated pests*). The IPPC Secretariat issues a call for new treatments[1], and the Technical Panel on Phytosanitary Treatments (TPPT) is responsible for evaluating data submissions from National Plant Protection Organization (NPPO'S) against the requirements in ISPM 28. The TPPT has faced challenges because many of the submissions do not address all requirements in Section 3 of ISPM 28.

[5] The aim of these guidelines is to provide guidance on the production of research documentation that fulfill the criteria set in ISPM 28. The Phytosanitary Measures Research Group (PMRG) was initially established as the Expert Consultation on Cold Treatments (ECCT) before becoming the Phytosanitary Temperature Treatment Expert Group (PTTEG). The PMRG has agreed to compile research guidelines on a range of treatment technologies and aid the development of expertise and technical cooperation between the contracting parties to the IPPC.

## Outline of Procedures

[6] The development of a vapour heat treatment as phytosanitary treatment (including high temperature forced air treatment[2]) involves a number of distinct steps. For the TPPT to evaluate a submission and the CPM to adopt annexes to ISPM 28, detailed information of experimental facilities and equipment, target pest, regulated article, and the methodology employed should be available. The following steps are provided as a general guideline for the development of a vapour heat phytosanitary treatment.

[1] IPPC: Standard Setting - Calls for treatments: https://www.ippc.int/en/core-activities/standards-setting/calls-treatments/

[2] IPPC Procedure Manual for Standard Setting (Section 10 of 8.8. Technical Panel on Phytosanitary Treatments (TPPT)): High temperature forced air is a variation of vapour heat treatment. [...] The main distinction between vapour heat treatment and high temperature forced air is based on moisture content of the heated air and the consequential heating which results. Vapour heat treatment typically uses air near saturation, which results in condensation of water on the fruit surface until the fruit surface temperature increases to near the air temperature. During high temperature forced air treatment the dew point is typically always kept below the surface temperature of the commodity being heated resulting in no condensation on the fruit surface.

i) Determination of the most heat tolerant life stage of the target fruit fly species on the regulated article (host commodity) (Comparison test for heat tolerance of insects)

ii) Identification of the time-temperature combination that provides a specified efficacy level using the most tolerant stage (exploratory testing).

iii) Demonstration of treatment efficacy of the vapour heat treatment using sufficient numbers of the most tolerant stage to provide 95% confidence that the specified efficacy will be achieved (large scale testing).

*[7]* General information (researchers, test insect, test fruit and treatment facility etc.) should be described as in Part I of this guidance document. In Part II, the details of disinfestation test methods (experimental design) mentioned above (i-iii) are to be described (an example is given using oriental fruit fly, *Bactrocera dorsalis* and melon fly, *Zeugodacus cucurbitae* and a host plant). Evaluation of the effects of the vapor heat treatment on the quality of the regulated article (fruit quality effects) does not constitute part of the treatment development procedure, but due to its importance for successful commercial adoption, some procedural recommendations are provided.

# PART I: GENERAL INFORMATION

## 1. Organization and researchers

*[8]* Information on the laboratory, organization and researchers involved in producing the data should be provided in a document (refer to ISPM 28-3.1 Summary information).

*< Documentation >*
*(1) Lead research agency and location of the laboratory*
*(2) Contact person*
*(3) Research Timeframe*

## 2. Test insects

*[9]* The insects should be reliably identified, preferably by a suitable expert, and voucher specimens should be archived.

*[10]* The laboratory colony should be established with an appropriate founder population (e.g. 100 to 1 000 individuals), preferably collected from a large quantity of field infested host fruit from different areas. The colony should only contain the target species and be held in conditions that ensure peak vigour and limit the accidental introduction of parasites and diseases.

*[11]* The laboratory colony should be regularly replenished with new wild flies or replaced with new founder populations to maintain genetic diversity in the laboratory culture.

*[12]* The health of the colony should be regularly checked by monitoring fecundity, developmental time and developmental success through the various life stages.

*< Documentation >*

*(1) Scientific name of insects*

*(2) Origin of the colony*

*- Location, date of collection of host plant (scientific name and quantity of fruit collected).*

*(3) Rearing method*

*- Temperature, humidity and lighting schedule in the rearing room.*

*- Egg collection method.*

*- Composition of larval medium and adult diet.*

*- Life cycle for each developmental stage and adult insects under rearing conditions.*

*- Hatchability, pupation rate and adult emergence rate.*

*- Dates of colony replenishment or replacement.*

*- Location where voucher specimens have been submitted.*

## 3. Test fruit

*[13]* Fruits to be used in the disinfestation tests should be identified botanically, including the variety or cultivar type and the condition of the fruit. Fruits should be free from non-target and target pest infestation, and any disorders that would cause the fruit to break down prematurely (i.e. potentially impact on treatment efficacy). Fruit should be free of pesticides that may be deleterious to fruit fly survival.

*< Documentation >*

*(1) Scientific name and cultivar of fruit*

*- Weight, shape.*

*- Other characteristics and difference from other cultivars.*

*- Photographs of fruits.*

*(2) Origin*

*- Location and date of harvesting.*

*- maturity at harvest (criteria such as firmness, sugar, acidity, colour, starch index…)*

*- maturity before infestation.*

*(3)Storage conditions after harvest.*

*- Temperature, humidity and duration in cold storage or controlled atmosphere storage, etc.*

## 4. Treatment facilities

*[14]* Information on the vapour heat treatment chambers or facilities used for most heat tolerant life stage, exploratory testing and large scale testing should be reported.

< Documentation >

*(1) Location and organization responsible for operation of the vapour heat chamber.*

*(2) Specifications and dimensions of the chambers and precision of temperature and humidity control in the chambers.*

*(3) Information on the measurement and recording of the temperature and humidity in the chambers (type, no of sensors, resolution, recording intervals),*
*(4) Details for loading configuration of commodities in the chambers including the loading factor*

## 5. Measurement of temperature

### 5.1. Calibration of sensors & temperature recorder

[15] Temperature sensors and recording devices to be used for experiments must be calibrated at the target temperature prior to the start of each trial. Temperature sensors are calibrated by placing them in a water bath set and calibrated at the target temperature. Sensors reading more than ±0.3 from the target temperature should not be used (for more details refer e.g. ASTM E563-11).

[16] An example on how to record calibration readings and calculate a calibration factor is shown below in Table 1.

**Table 1.** Calibration of sensors at 46.0°C, March 29, 2016

| Time* | Sensor Number | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | --- | 12 | 13 | 14 | 15 |
| 9:05 | 46.0 | 46.0 | 46.0 | --- | 46.0 | 46.0 | 46.1 | 46.0 |
| 9:10 | 46.0 | 46.1 | 46.0 | --- | 46.0 | 45.9 | 46.1 | 46.0 |
| 9:15 | 46.0 | 46.1 | 46.0 | -- | 46.0 | 45.9 | 46.1 | 46.0 |
| 9:20 | 46.0 | 46.1 | 46.0 | --- | 46.0 | 45.9 | 46.0 | 46.0 |
| Calibration factor** | 0.0 | -0.1 | 0.0 | | 0.0 | 0.1 | -0.1 | 0.0 |

*Readings: at least 3 times at 3-5 minutes intervals after readings have stabilised.

**Calibration factor = (True temperature: 46.0°C) - (Reading)

### 5.2. Measurement of temperature and relative humidity during treatment

[17] Fruit core temperature, air temperature and humidity in the vapour heat chamber should be measured continuously or at regular intervals (e.g. every 5 minutes) by using temperature sensors and recording devices. Fruit used to measure core temperature should be not be infested.

#### *5.2.2. Fruit core temperature*

[18] Sensors to monitor fruit core temperatures should distributed evenly amongst the infested fruits in the chamber. Sensors are normally placed in the upper, middle and lower layers of fruit and at the center, sides and corners of each layer. Chamber mapping is also recommended to identify any hot or cold spots within the chamber. Temperature should be recorded from the time of loading of infested fruit through to the completion of the treatment. Preferably 8 sensors should be used for the large scale testing and 5 sensors for exploratory and most tolerant stage testing.

### *5.2.3. Record of temperature data*

*[19]* Temperature recordings for air temperature, relative humidity and fruit core temperatures must be recorded from the time fruit is loaded into the treatment chamber until the treatment is completed. It is important to clearly identify when the target temperature is reached and the dwell time starts and finishes (**Example: Table 2** is drawn from a treatment at 46.5°C core temperature with a holding time or dwell time of 30 minutes). If possible temperature recordings should continue during the cool down period. Temperature records for each replication of the confirmatory tests should be provided as they are important for the development of the treatment schedule; for more details refer to section 9.4.

**Table 2** Chamber air and fruit core temperatures during vapour heat treatment, Replicate 1. March 29, 2016

| Time | Dry Bulb | Relative Humidity | Fruit Temperature (°C) | | | | | | Remarks |
|---|---|---|---|---|---|---|---|---|---|
| | | | Sensor Number | | | | | | |
| | | | 4 | 5 | 6 | - | 9 | 10 | |
| 10:00 | 27.0 | 79.2 | 27.9 | 27.8 | 27.3 | | 27.8 | 27.5 | Loading fruit into chamber (=heating starts) |
| 10:05 | 30.6 | 76.5 | 27.9 | 27.8 | 27.3 | -- | 27.8 | 27.5 | |
| 10:10 | 31.1 | 75.5 | 27.9 | 27.8 | 27.3 | -- | 27.8 | 27.5 | |
| -- | -- | -- | -- | -- | -- | --- | -- | -- | |
| 13:25 | 47.6 | 94.5 | 46.4 | 46.4 | 46.3 | -- | 46.4 | 46.3 | |
| 13:30 | 47.6 | 94.8 | 46.5 | 46.5 | 46.3 | -- | 46.5 | 46.4 | |
| 13:34 | 47.6 | 95.0 | 46.5 | 46.5 | 46.4* | -- | 46.5 | 46.4* | Start of dwell time when half the sensors reach 46.5°C |
| 13:35 | 47.6 | 95.2 | 46.5 | 46.5 | 46.4 | -- | 46.5 | 46.4 | |
| 13.40 | 47.5 | 95.3 | 46.6 | 46.5 | 46.5 | -- | 46.6 | 46.5 | |
| 13:45 | 47.5 | 95.4 | 46.6 | 46.6 | 46.5 | -- | 46.6 | 46.5 | |
| 13.50 | 47.6 | 95.2 | 46.7 | 46.6 | 46.5 | -- | 46.6 | 46.5 | |
| 13:55 | 47.6 | 95.3 | 46.7 | 46.7 | 46.5 | -- | 46.7 | 46.5 | |
| 14:00 | 47.5 | 95.5 | 46.8 | 46.7 | 46.6 | -- | 46.7 | 46.6 | |
| 14:04 | 47.5 | 95.5 | 46.8 | 46.8 | 46.6 | -- | 46.8 | 46.6 | Dwell time (30 min) ends. Then cooling begins. |

* Each sensor must be adjusted to account for the calibration factor i.e. sensor 10 has a calibration factor of 0.1°C so a reading of 46.4°C is actually 46.5°C

*< Documentation >*

*(1) Method of calibration of sensors and temperature recording equipment*

*- Procedures and pictures, for example hot water immersion etc., and results of calibration.*

*(2) Measurement of temperatures from the time infested fruit is loaded into the chamber until the treatment is completed (number of sensor used, location of the sensors in the chamber, frequency of recordings…)*

# PART II: GUIDELINES FOR TEST PROCEDURES

## 6. Preparation of infested fruit and investigation of the developmental rate of fruit fly in fruit

### 6.1. Purpose

[20] Before undertaking efficacy trials the development rates of each lifestage need to be accurately determined. Development rates for eggs (> 50% development), immature larvae (1st instar, 2nd instar), mature larvae (3rd instar), puparia and adults are required to determine infestation times and appropriate holding times before mortality assessments can be undertaken. Development rates should be determined in the commodity being studied as development rates do vary between fruit fly species and different hosts. Another important factor influencing development rates is temperature. As such the temperature regime used to store infested fruit for the development trials needs to the same temperature regime used to hold infested fruit before efficacy trials commence.

### 6.2. Methods

[21] In general, there are 2 main methods for obtaining infested fruit; artificial inoculation and simulated natural infestation. In both cases, every effort should be made to simulate natural conditions as far as possible. In some cases laboratory infestation may not be feasible for the pest host combination and the use of field collected infested fruit may be necessary. The degree of ripeness of fruits and population density per fruit should be considered so that these conditions are suitable for the development, health and survival of fruit flies. For example the use of very ripe fruit may result in fruit breaking down before larvae can complete their development or conversely the use of immature fruit may result in poor egg hatch and highly variable development rates. Ideally the maturity of fruit used in these trails should be the same maturity as export quality fruit.

[22] For **artificial inoculation**, using eggs collected by egging device (oviposition time: e.g. 1-2 hours), e.g. 100 eggs per fruit are artificially inoculated into e.g. 60 fruits (= 5 fruits/day x 8 days for larvae observations + 20 fruits for pupae observations) and incubated at rearing temperature (e.g. 27 °C). The number of inoculated eggs per fruit should be adjusted according to the size of fruit.

[23] For **simulated natural infestation**, a specified number of fruit are placed in a cage with gravid females flies (e.g. 2 000 /cage) for certain period (e.g. 30-60 minutes). The number of eggs laid per fruit is difficult to control accurately but strategies to encourage or limit oviposition activity can be employed (e.g. the number of adult flies per cage, exposure times, piercing the fruit or placing fruit in vinyl bags with small holes to limit oviposition sites).

[24] Observations:

- **Larvae:** After egg-inoculation, at least 5 fruits are dissected every day for 8 days to check the life stage present and the number of insects of each developmental stage.
- **Puparia and adult**: Similarly, at least 20 infested fruits are placed in rearing cages on a bed of sand, sawdust or paper as a pupation medium. After 8 days storage the fruit and the pupation medium are examined for the presence of puparia. Adult emergence from recovered puparia is also counted.
- **Eggs:** The tolerance of eggs to thermal treatments does vary depending on the age of the eggs. To undertake embryonic development trials eggs are collected as per the methodology used for artificial infestation for larvae except eggs are usually placed on black filter paper before being placed in the fruit. Counts on the number of unhatched eggs are then made at regular interval until egghatch is completed. Once the number of viable eggs per sample is known the results can be reviewed to determine the time when the majority of eggs have hatched (e.g. more than 50% of the viable eggs in the sample had hatched by 44 hours). This time is then used to calculate the infestation times in subsequent efficacy trials. For example if you want to treat 60% developed eggs you would make egg collections 26.5 hours before the start of the treatment (i.e. 60% of 44 hours equals 26.4 hours or approximately 26 ½ hours).

## 6.3. Test results

*[25]* Test results from artificial inoculation (e.g. 100 eggs/fruit x 5 fruit/day x 8 days observation) can be recorded as follows. **(Example: Table 3)**.

**Table 3** Development of oriental fruit fly and melon fly in the infested fruits (at 27 ℃) Replication 1: December 1, 2015

| Days after egg-collection/ oviposition* | Number of insects observed | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Oriental fruit fly | | | | Melon Fly | | | |
| | Eggs* | 1st Instar | 2nd Instar | 3rd Instar | Eggs* | 1st Instar | 2nd Instar | 3rd Instar |
| 0* | 500 | 0 | 0 | 0 | 500 | 0 | 0 | 0 |
| 1 | 500 | 0 | 0 | 0 | 500 | 0 | 0 | 0 |
| 2 | 408 | 92 | 0 | 0 | 42 | 458 | 0 | 0 |
| 3 | 0 | 106 | 154 | 0 | 0 | 55 | 0 | 0 |
| 4 | 0 | 0 | 360 | 22 | 0 | 0 | 132 | 0 |
| 5 | 0 | 0 | 0 | 393 | 0 | 0 | 348 | 0 |
| 6 | 0 | 0 | 0 | 415 | 0 | 0 | 363 | 0 |
| 7 | 0 | 0 | 0 | 396 | 0 | 0 | 291 | 15 |
| 8 | 0 | 0 | 0 | 340 | 0 | 0 | 183 | 208 |

*"0" means the day of collection and inoculation of eggs. "1" means the next day (24 hours) after egg collection.

## 6.4 Replication

*[26]* These tests should be replicated twice or more unless published information on the on insect development rate in the target fruit is available.

*< Documentation >*

*(1) Purpose*

*(2) Materials and Methods*

*- Provide detailed information of methodology including figures and photographs (artificial inoculation or natural infestation, number of fruit per experimental plot, number of insects per fruit, number of fruit tested per replicate, number of replicates, condition during infestation (duration, temperature, RH), storage conditions of infested fruits (temperature, humidity and duration, etc.), characteristics used to identify the different larval stages, etc.).*

*(3) Results and discussion*

*- Results are used to recommend infestation times for each life stage.*

*- Data on development rates of eggs and larval stages in each replicate should be tabulated.*

*- Data for emergence of mature larvae from fruits, pupation rate and adult emergence rate should also be tabulated for each replicate.*

## 7. Most heat tolerant life stage

### 7.1. Purpose

[27] The most cold tolerant life stage of the fruit flies can be determined by comparing sensitivity of the different life stages that could occur in or on the commodity.

[28] Thereafter, the most tolerant stage should be used in a series of experiments.

### 7.2. Methods

#### *7.2.1 Developmental stage of fruit fly for testing*

[29] When undertaking trials with larvae it is not always possible to choose a time where 100% of the larvae present are the same stage. More often there will be an overlap or stages present (e.g. results from development rates of *B. dorsalis* on day three show there was 106 first instar and 154 second instars present). As such infestation times should be chosen so that the majority of the larvae present are the target life stage.

[30] To confirm that the correct larval stage has been treated extra fruit (sometimes called instar fruit) are normally infested when efficacy trials are being undertaken. At the commencement of the trial the instar fruit are sampled and the percentage of each life stage present determined. If the results from the instar fruit show that the majority of the larvae present are the target lifestage then the results are considered valid. If the target life stage is present but represents less than 50% of the total number of larvae the trial may need to be repeated using different infestation times.

#### *7.2.2 Preparation of fruit infested with fruit fly*

[31] Either simulated natural infestation or artificial inoculation can be used. Fruit infested with each stage should be prepared with the method described in section 6.

[32] The optimal infestation times will be based on the results from the development rate trials.

[33] Although some fruits may be difficult to infest larvae grown on artificial diet should not be used in efficacy trials unless research has demonstrated that these larvae are not easier to kill than those developing in fruit.

### *7.2.3 Treatment conditions*

[34] Target fruit (core) temperature will be selected from the range of temperatures which would be used practically in quarantine treatments without inducing negative impacts on fruit quality. The tolerance of each life stage can be compared by using a range of treatment temperatures (e.g., 42.0, 43.0, 44.0, 45.0, 46.0 and 47.0°C) or treatment times (e.g. 46.0°C for 0, 5, 10, 15, 20, 40 and 60 minutes).

### *7.2.4 Number of test insects and fruits*

[35] For each life stage and each dose being tested there should be a minimum of 200 test insects per replicate. The number of fruit required will depend on infestation rates and the size of the fruit. For large fruit (e.g. mango) you may use 5 fruit with 40 insect per fruit per dose. For smaller fruit you may need to use 100 fruit with 2 insect's per fruit per dose.

### *7.2.5 Replication*

[36] A minimum of three replicates are required and each replicate should be conducted separately (i.e. you cannot infest three times as much fruit and place all the fruit in the same chamber at the same time).

### *7.2.6 Treatment methods*

[37] When placing infested fruit in the treatment chamber fruit are normally grouped together based on the treatment conditions being tested. For example, if a range of treatment times (42.0, 43.0, 44.0, 45.0, 46.0 and 47.0°C) are used to compare the heat tolerance between life stages then the fruit all the eggs and larval stages for the 42°C treatment will be grouped together in the chamber. This makes it easier to remove fruit from the chamber (which must be done very quickly) and more importantly tries to ensure that each life stage at a particular temperature has received similar treatment conditions. While there is always some temperature variations within a treatment chamber, grouping samples together should minimize these variations within each treatment group.

[38] As fruit reach the prescribed temperature/time, test fruits are removed from the treatment chamber, cooled and then stored in constant temperature rooms until mortality assessments are made.

[39] Once again, each replicate must be conducted separately and the loading configuration for each trial must be provided.

### *7.2.7 Mortality*

[40] Assessments of mortality can be classified as either chronic mortality (lack of successful pupation) or acute mortality (inspection for live/dead larvae and eggs).

[41] Assessments of acute mortality in fruit containing third instar larvae are usually undertaken 24 hours after the treatment has concluded. Fruit containing other life stages should be held long enough to allow viable insects to develop into third instars before undertaking dissections. The number of live and dead larvae should be recorded.

### 7.3. Test results

[42] Test results can be recorded as per Table 4.

**Table 4** Heat tolerance test; Mortality of each stage of oriental fruit fly and melon fly in the fruit treated at fruit core temperature of 42-47°C. Replicate 1; January 15, 2016 – Example for artificial inoculation (100 eggs per fruit inoculated)

| Target fruit core temperature | Number of survivors | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Oriental fruit fly | | | | Melon Fly | | | |
| | Eggs* | 1st Instar | 2nd Instar | 3rd Instar | Eggs* | 1st Instar | 2nd Instar | 3rd Instar |
| Control | 325 | 369 | 381 | 391 | 330 | 371 | 385 | 389 |
| 42°C | 301 | 241 | 192 | 280 | 336 | 300 | 203 | 299 |
| 43°C | 239 | 189 | 55 | 96 | 266 | 239 | 71 | 101 |
| 44°C | 170 | 160 | 28 | 22 | 90 | 86 | 32 | 12 |
| 45°C | 103 | 49 | 8 | 13 | 16 | 5 | 2 | 0 |
| 46°C | 24 | 12 | 1 | 0 | 0 | 0 | 0 | 0 |
| 47°C | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

*27 hour old eggs in oriental fruit fly and 21 hour old eggs in melon fly were used.

[43] Analysis to determine the most tolerant stage should be undertaken using corrected mortality data. Several models can be used (probit, logit, complementary log-log, each with and without log transformation) and LT values (e.g. LT99, LT95 and LT90) and associated fiducial limits can be determined. Analysis may show that there is no significant differences between the life stages. In this situation the stage with the highest LT value would be considered as "arithmetically" the most tolerant stage and subsequent trials will be undertaken using this life stage.

### 7.4. Other Information

[44] Another option when investigating the heat tolerance of fruit fly species is to use in vitro testing methodologies. This involves taking eggs and larval stages of different species, dipping them directly into hot water (or an appropriate heat source) and then transferring them to an appropriate rearing medium. Details for this procedure can be found in APPPC RSPM No.1 *Guidelines for the development of heat disinfestation treatments of fruit fly host commodities.* While in vitro testing has been used to accurately determine differences in heat tolerance between fruit fly species it does not reliably determine the most tolerant stage of a species in fruit. As such it is always recommended to undertake most tolerant stage testing in fruit.

*< Documentation >*

*(1) Purpose*

*(2)Materials and methods*

*- Details of methods (preparation of infested fruits (number of insects per fruit, number of test fruits per experimental plot in each replication, number of fruit in control group in each replication), treatment conditions (temperature and dwell time), thermometry (size and weight of fruits for temperature monitoring, number of sensors for fruit core temperature of non-infested fruits in the chambers in each replication, intervals of temperature recording and determination of start point of treatment), information of stacking of infested/non-infested fruits in the chamber, storage conditions of fruits after treatment (temperature, humidity and period), criteria for the determination of live/dead insects for each stage, data analysis, number of the replicates (date of replication))*

*- The methods employed should be explained in detail and include figures and pictures (preparation of infested fruit, location and the arrangement of infested fruits and sensors for the core temperature of fruits in the chambers).*

*(3) Results and discussion*

*- Results should be described based on the obtained data.*

*- Data on the number of live/dead insects and calculated mortality rates in each replicate should be tabulated. Data of non-treated control should be also included in the table.*

*- Temperature records will be required to demonstrate that all developmental stages in each experimental plot received equivalent heat treatment.*

## 8. Exploratory testing

### 8.1. Purpose

[45] This test is carried out to determine the treatment conditions required to achieve complete mortality of the most tolerant stage of the target fruit fly species.

### 8.2. Methods

#### *8.2.1 Developmental stage of fruit fly for testing*

[46] The most tolerant stage of fruit fly found in the aforementioned tests should be used.

#### *8.2.2 Preparation of fruit infested with fruit fly*

[47] Preparation of fruit infested with the most tolerant stage should follow the method described in section 6.

#### *8.2.3 Treatment condition*

[48] The choice of the treatment parameters (fruit core temp and dwell time) to be used in the exploratory trials should be high enough to result in complete mortality but low enough that it does not result in reduced fruit quality. Dwell times should be increased in several sequential steps.

### *8.2.4 Number of insects and fruits tested*

[49] The number of test fruits should be adjusted to obtain approximately 3 000 individuals of the test insects per treatment condition.

[50] The number of test insects should be calculated on the basis of the infestation rate in non-treated control, as follows.

[51] Infestation rate = No. of survivors in untreated fruits / No. of untreated fruits

[52] Estimated number of test insects = Infestation rate X No. of treated fruits.

### *8.2.5 Replication of test*

[53] Two or more replications are required and should be conducted separately rather than concurrently.

### *8.2.6 Treatment method and measurement of temperature*

[54] Treatment and measurement of temperature should be conducted as per sections 7 and 5. The treatment starts from the time when half of the fruit core sensors have reached the target temperature.

### *8.2.7 Determination of mortality*

[55] Mortality is determined as described in section 7.2 7.

## 8.3. Test results

[56] Test results are recorded in a table such as the following. (Example**: Table 5**)

[57] Based on the results recorded below a treatment of 46.5°C for 20 minutes would be selected as the treatment regime to be used in the confirmatory trials.

**Table 5** Exploratory testing: Mortality of oriental fruit fly (most tolerant stage: 27hr-old egg) in fruit treated at 46.0 and 46.5°C for 0-30 minutes.

| Replicate | Fruit temperature and dwell time | No. of Fruit | Estimated No. of treated insects* | Total No. of survivors | Observed Mortality (%) |
|---|---|---|---|---|---|
| 1 (February 15, 2016) | 46.0°C | 40 | 3,520 | 148 | 95.8 |
| | 46.5°C | 40 | 3,520 | 82 | 97.6 |
| | 46.5°C + 10 min. | 40 | 3,520 | 0 | 100 |
| | 46.5°C + 20 min. | 40 | 3,520 | 0 | 100 |
| | 46.5°C + 30 min. | 40 | 3,520 | 0 | 100 |
| | Control | 40 | - | 3,520 | |
| 2 (February 25, 2016) | 46.0°C | 40 | 3,640 | 193 | 94.7 |
| | 46.5°C | 40 | 3,640 | 59 | 98.4 |
| | 46.5°C + 10 min. | 40 | 3,640 | 5 | 99.9 |
| | 46.5°C + 20 min. | 40 | 3,640 | 0 | 100 |

| | | | | | |
|---|---|---|---|---|---|
| | 46.5°C + 30 min. | 40 | 3,640 | 0 | 100 |
| | Control | 40 | - | 3,640 | |

*Estimated total number of treated larvae = Infestation rate X No. of treated fruits.

Infestation rate = No. of survivors in untreated fruits / No. of untreated fruits.

*< Documentation >*

*(1) Purpose*

*(2) Materials and methods*

*- Details of methods (preparation of fruits infested with most tolerant stage, fruit cultivar, treatment conditions, thermometry (number of sensors for fruit core temperature of non-infested fruits in the chambers in each replication, intervals of temperature recording and determination of start point of treatment etc.), information of stacking of infested/non-infested fruits in the chamber, storage conditions of fruits after treatment (temperature, humidity and duration), criteria for the determination of mortality etc. (refer to "<Documentation> of 7. Most heat tolerant life stage (comparison test for heat tolerance of insects)").*

*(3) Results and discussion*

*- Results should be described based on obtained data.*

*- Data of exploratory tests; date of each replication, number of fruits per experimental plot, effective number of test insects, number of insects per fruit, number of survived and/or dead insects, and survival rate (mortality), etc. in each replicate should be tabulated. Data of non-treated control should be also included in the table.*

*- temperature records for each replicate (refer to "5. Measurement of temperature").*

## 9. Large scale testing

### 9.1. Purpose

[58] The level of efficacy and associated confidence level derived from the test results is a product of the test population used (cumulative number of test insects used). It is preferable that replicated trials be undertaken to accumulate large numbers of test insects as opposed undertaking a single test. An example of a procedure that has been widely used is mortality trials testing 30,000 individuals. These trials are normally designed using 3 replicates with each replicate treating 10,000 insects. As such the cumulative total of treated insects is >30,000.

### 9.2. Methods

#### *9.2.1 Developmental stage of fruit fly for testing*

[59] The most tolerant life stage of the fruit fly found in the aforementioned susceptibility test will be used.

#### *9.2.2 Preparation of fruit infested with fruit fly*

[60] The commodity infested with the most tolerant life stage should be produced as described in section 7.

### *9.2.3 Treating condition*

[61] The treatment conditions should be based on the results of the small-scale disinfestation test that caused 100% mortality or on other data indicating the treatment condition that provides quarantine security.

### *9.2.4 Number of insects and fruits tested*

[62] Treatment plots: Number of test fruits should be adjusted to obtain e.g. 10 000 individuals per replication.

[63] The number of test insects is to be estimated on the basis of survival observed in untreated control as follows.

[64] Infestation rate = No. of survivors in untreated fruits / No. of untreated fruits.

[65] Estimation of number of test insects = Infestation rate X No. of treated fruits.

[66] Untreated control: More than 1/5 of treated fruits will be provided for untreated control.

### *9.2.5 Replication of tests*

[67] Treatment from heating through to the final exposure period will be separately repeated 3 times or more.

### *9.2.6 Stacking of fruits in treatment chamber*

[68] Infested test fruits are placed with sensor-inserted fruit and filler fruit in the chamber. The use of filler fruit can assist in obtaining a more uniform temperature distribution around treated fruit but can also be used to manipulate the loading rate in the treatment chamber.

### *9.2.7 Treatment method and measurement of temperature*

[69] Un-infested fruit are used for measurement of fruit core temperature during vapour heat treatment, and these sensor-fruits should have a similar average weight and size to the infested fruit.

### *9.2.8 Determination of mortality*

[70] Mortality should be determined as described in sections 7 and 8.

## 9.3. Test results

[71] Test results should be recorded in a table such as the following. (Example**: Table 6**)

**Table 6** Confirmatory testing; Mortality of oriental fruit fly (most tolerant stage: e.g. 27hr-old egg) in the fruit vapour heat treated at 46.5 °C for 30 min.

| Replicate | Date | Control | | Treated | | | | | Remark |
|---|---|---|---|---|---|---|---|---|---|
| | | No. of Fruit | No. of live insects | No. of Fruit | Estimated No. of treated insects* | Total No. of survivors | Observed Mortality | True Mortality (95% CI) | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 1 | March 29, 2016 | 50 | 4,619 | 121 | 11,178 | 0 | 100 | 99.9732 | full-load 150kg/$m^3$ |
| 2 | April 20, 2016 | 50 | 4,788 | 129 | 12,353 | 0 | 100 | 99.9757 | full-load 150kg/$m^3$ |
| 3 | May 15, 2016 | 35 | 3,107 | 86 | 7,634 | 0 | 100 | 99.9608 | half-load 75kg/$m^3$ |
| 4 | May 20, 2016 | 35 | 3,199 | 86 | 7,860 | 0 | 100 | 99.9619 | half-load 75kg/$m^3$ |
| Total | | 170 | 15,713 | 422 | 39,025 | 0 | 100 | 99.9923 | |

*Estimated total number of treated insects = Infestation rate X No. of treated fruits.

Infestation rate = No. of survivors in untreated fruits / No. of untreated fruits.

### 9.4. Evaluation of mortality data and temperature data for conversion to phytosanitary treatment schedule

[72] When setting treatment parameters for trials it is important to remember that the highest fruit core temperature recorded may become the minimum temperature recommend in any proposed treatment schedule. As such precautions should be taken so that the fruit core temperature does not rise far from the target temperature. An example is starting the dwell time (= holding time) when half of the sensors inserted into fruits have reached the target temperature rather than waiting until all probes have reached the target temperature. While temperature sensors usually show a uniform heating rate there are occasions when then can be a significant time from when the first sensor and the last sensor reaches the target temperate. In this situation the first probe will inevitably have reached a temperature above the target temperature. As mentioned above the highest fruit core temperature recorded may become the minimum temperature recommend in any proposed treatment schedule (e.g. the target temperature of 46.5°C may become 46.7°C if there is a time lag between the first and last probes reaching the target temperature).

*< Documentation >*

*(1) Purpose*

*(2) Materials and methods*

*- Details of methods (refer to"<Documentation> of 8. Exploratory Testing").*

*(3) Results and discussion*

*- Results should be described based on data recorded during the confirmatory testing (refer to "<Documentation> of 8. Exploratory test").*

*- Temperature data for each replicate (refer to "5 .Measurement of temperature")*

## 10. Fruit quality testing

### 10.1. Purpose

[73] Once an effective treatment has been determined fruit quality trials should be undertaken. Evaluation of effects of the vapor heat treatment on the fruit quality does not constitute part of the treatment

development procedure, but due to its importance for successful commercial adoption, some procedural recommendations are provided. The following is an example of how such testing may be undertaken.

### 10.2. Methods

[74] Fruit quality trials should be conducted on export quality fruits using the same treatment schedules as the confirmatory testing or more severe conditions (such as higher temperatures, longer exposure times or starting the dwell time when all of the sensors reached the target temperature rather than half the sensors reaching the target temperature) than can be expected from commercial application of the treatment.

[75] Factors such as harvest season, maturity, cultivar, treatment schedules and other factors are usually considered when assessing the effects of the treatment on commodity, such as external damage, shelf-life, flavor and aroma. Test fruits should be stored under simulated trade (transport) conditions such as storage temperature, humidity and freight transport times from exporting country to importing country and distribution conditions in the importing country.

### 10.3. Test results

[76] All fruit quality results should be summarized and tabulated. If the treatment regime used does result in reduced fruit quality it is recommend that photographs clearly identifying the type and severity of injury be provided.

*< Documentation >*

*1. Purpose*

*2. Materials and methods*

*- Details of methods (treatment condition (temperature, period), number of the test fruits (control group and treated group) in each replicate, thermometry, storage condition (temperature, humidity and period etc.) after the treatment.*

*- Data analysis (criteria for determination of the cold injuries related to taste, smell, external and internal appearance, etc.).*

*- Number of replicates*

*3. Results and discussion*

*- Results should be described based on obtained data.*

*- Data from fruit quality test; date each replicate undertaken, number of the fruits per experimental plot, etc. in each replicate should be tabulated. Data of non-treated control should be also included in the table.*

*- Data of temperature records for each replicate (refer to 5. Measurement of temperature).*