PMRG RESEARCH GUIDELINE:

GUIDELINES FOR THE DEVELOPMENT OF COLD DISINFESTATION TREATMENTS FOR FRUIT FLY HOST COMMODITIES

CONTENTS

INTRODUCTION

Scope

[1] This research guideline outlines technical procedures that might be used to support cold treatments against quarantine fruit fly species in host commodities.

References

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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 phytosanitary cold treatments involves a number of distinct steps. For the TPPT to evaluate a submission and the CPM to adopt annexes to ISPM 28 detailed information on experimental facilities and equipment, target pest, regulated articles, and the methodology employed should be available. The following steps are provided as a general guideline for the development of a cold phytosanitary treatment.

  i. Determination of the most cold tolerant life stage of the target fruit fly species.
  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 cold treatment using the most cold-tolerant stage (confirmatory testing).

[7] General information (research provider, test insect, test fruit and treatment facility etc.) should be described as in Part I. In Part II, the details of disinfestation test methods (experimental design) mentioned above (i -iii) are to be described (an example is given using Mediterranean fruit fly *Ceratitis capitata* and a host plant). Evaluation of effects of the cold 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.

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

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 insect species 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 1000 individuals), preferably collected from a large quantity of field infested host fruit in 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 variety or 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 cold treatment chambers or facilities used for most cold tolerant life stage, exploratory testing and large scale testing should be reported.

< Documentation >

(1) Location and organization responsible for operation of the cold chambers

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

- Procedure and time intervals of the defrosting and its influence on the temperature in the chambers, i.e. a range of temperature fluctuations.

(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 sensor & temperature recorder

[15] Temperature sensors and recording devices to be used for experiments must be calibrated prior to the start of each trial. Temperature sensors are calibrated by placing them in an ice slurry (e.g. 80% ice, 20% water). Sensors reading more than ±0.3 should not be used (for more details refer e.g. ASTM E563-11).

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

Table 1 Calibration of sensors (Ice water immersion) March 29, 2017

Time	Sensor Number							
	1	2	3	---	12	13	14	15
9:05	0.0	0.0	0.0	---	0.0	0.0	0.1	0.0
9:10	0.0	0.1	0.0	---	0.0	-0.1	0.1	0.0
9:15	0.0	0.1	0.0	--	0.0	-0.1	0.1	0.0
9:20	0.0	0.1	0.0	---	0.0	-0.1	0.0	0.0
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: 0°C) - (Reading)

5.2. Measurement of temperature during treatment

[16] Fruit core temperature and air temperature in the cold chamber during treatment should be measured continuously or at regular intervals (e.g. every 60 minutes) by using temperature sensors and recording devices. Fruit used to measure core temperature should be not be infested.

5.2.1. Air temperature

[17] Air temperature in the chamber should be measured at least at two points, one at the cold air delivery position and the other at the air return position. Air temperatures are not an essential component in developing treatment schedules but may provide useful information for interpreting the research results.

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.

[19] 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 10 sensors should be used for the confirmatory testing and 5 sensors for exploratory and most tolerant stage testing.

5.2.3. Recording of temperature data

[20] Recorded temperatures may be summarized but all raw data should be provided (**Example: Table 2** for a treatment at 2.0°C for exposure of 18 days).

[21] Temperature recordings for fruit core temperatures must be recorded from the time fruit is loaded into the chamber treatment chamber until the treatment is completed. It is important to clearly identify

when the cool down period finishes and when the treatment commences and concludes. 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 9.4.

Table 2 Chamber air and fruit core temperatures during the treatment at 2.0°C. Replicate 1 29 March, 2016

Date	Time	Air Temperature (°C)		Fruit Temperature (°C)						Remarks
		Sensor Number		Sensor Number						
		1	2	3	--	5	--	9	10	
		Supply	Return	Top		Middle		Bottom		
29-3-2016	9:00	1.0	1.4	25.4	--	25.3	--	25.5	25.1	Loading fruit into chamber (cooldown starts)
29-3-2016	10:00	1.1	1.5	20.0	--	20.8	--	20.3	20.4	
29-3-2016	11:00	1.0	1.3	19.5	--	19.9	--	19.0	19.2	
--	--	--	--	--	--	--	--	--	--	
30-3-2016	7:00	0.9	1.4	2.2	--	2.1		2.2	2.2	
30-3-2016	8:00	1.0	1.4	2.1	--	2.0		2.2	2.1	
30-3-2016	9:00	1.0	1.3	2.0	--	2.0		2.1*	2.0	← Start of treatment when half the sensors reach 2.0°C
30-3-2016	10:00	1.1	1.5	2.0	--	2.0		2.0	2.0	
--	--	--	--	--	--	--	--	--	--	
17-4-2016	7:00	1.1	1.6	2.0	--	1.9		2.0	1.9	
17-4-2016	8:00	1.1	1.5	1.9	--	2.0		2.0	2.0	
17-4-2016	9:00	1.1	1.4	1.9	--	2.0		2.0	1.9	← End of treatment and fruit removed from cold room

* Each sensor must be adjusted to account for the calibration factor i.e. probe 9 has a calibration factor of -0.1°C so a reading of 2.1°C is actually 2.0°C

< Documentation >

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

- Procedures and pictures, for example ice slurry calibration 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

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 (e.g. 27±1°C) used to store infested fruit for the development trials needs to the same temperature regime used to hold infested fruit before and after treatment in subsequent efficacy trials.

6.2. Methods

[22] 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.

[23] For **artificial inoculation,** eggs are collected by placing an oviposition receptacle (e.g. a hollowed-out fruit dome) in cages with gravid females (oviposition time: e.g. 1-2 hours). Eggs are then counted and placed into the fruit e.g. 50 eggs per fruit are artificially inoculated into 70 fruits (= 5 fruit/day x 10 days for larval development observation and 5 fruit/day x 4 days for pupal observations) and incubated at a constant temperature (e.g. 27±1°C). The number of inoculated eggs per fruit should be adjusted according to the size of fruit to ensure that fruit are not over infested.

[24] 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).

[25] Observations:

- **Larvae**: After egg-inoculation, at least 5 fruit are dissected every day for 10 days to check the life stage present and the number of insects of each developmental stage.

- **Puparia and adult**: Infested fruits are placed in rearing cages on a bed of sand, sawdust or paper as a pupation medium and held for 10 days. After storage, 5 fruit a day are inspected and the pupation medium is sieved to determine if pupae are present. Adult emergence from recovered puparia should also be recorded.

- **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).

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

6.3. Test results

[27] Test results from artificial inoculation (e.g. 50 eggs/fruit x 5 fruit/day x 10 days of observation) can be recorded as follows. (Example: Table 3).

Table 3. Development of Mediterranean fruit fly in the infested fruit (at 25 °C)

Replicate (date)	Stage	Number of larvae at each day after egg-inoculation/oviposition										
		0*	1*	2	3	4	5	6	7	8	9	10
1 (Feb. 1, 2016)	Egg	250	250	250	25(3)**	10(0)**	17(0)**					
	1st instar	0	0	0	225	91	34	5	3	4	0	0
	2nd instar	0	0	0	0	115	116	80	40	26	14	5
	3rd instar	0	0	0	0	0	50	98	172	175	192	207
	Total	250	250	250	250	216	217	211	215	205	206	212

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

**The number in parenthesis indicates the number of hatched eggs after removal and further incubation.

< 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 cold tolerant life stage

7.1. Purpose

[28] 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.

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

7.2. Methods

7.2.1 Developmental stage of fruit fly for testing

[30] When undertaking trials with larvae it is not always possible to choose a time where 100% of the larvae present are the same stage (e.g. 100% second instar). More often there will be an overlap or stages present (e.g. results from development rates of *C. capitata* on day four show there was 10 eggs, 91 first instar and 115 second instars present). As such infestation times should be chosen so that the majority of the larvae present are the target life stage.

[31] 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

[32] 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.

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

[34] 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 condition

[35] Treatment temperature (fruit core temperature) should be selected from a range of temperatures which would feasibly be used in quarantine treatments without inducing negative impact on fruit quality. Time of exposure should be specified so that susceptibility of different life stages can be compared using different exposure periods e.g., 1, 3, 5, 7, 9, 11, 13 days.

7.2.4 Number of test insects and fruits

[36] 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 example in large fruit (e.g. citrus) 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 per fruit per dose.

7.2.5 Replication

[37] 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

[38] When placing infested fruit in the treatment chamber, fruit are normally grouped together based on the treatment time. For example, all fruit to be removed on day 1 (eggs and all larval stages) will be placed together in the chamber. This makes it easier to remove fruit from the chamber and more importantly tries to ensure that each life stage at a particular exposure time has received similar treatment conditions. While there is always some temperature variations within a treatment chamber the grouping of samples should minimize these variations within each treatment group.

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

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

7.2.7 Mortality

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

[42] 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

[43] Test results are recorded in a table such as follows. (Example**: Table 4**).

Table 4 Most tolerant stage testing; Mortality of each life stage of Mediterranean fruit fly in the fruit treated at 2°C for various exposure periods. Replicate 1; March 15, 2016 – Example for artificial inoculation (50 eggs per fruit)

Stage	Exposure period (days)	No. of fruit	Total no. of inoculated eggs	Total no. of insects alive	Mortality (%)	Corrected mortality*
24 hour old eggs	Control	6	300	234	22.0	-
	1	6	300	70	76.7	70.1
	3	6	300	14	95.3	94.0
	--	--	--	--	--	--
	15	6	300	0	100	100
Immature larvae (1st-2nd instar)	Control	6	300	220	26.7	-
	1	6	300	92	69.3	58.2
	3	6	300	23	92.3	89.5
	--	--	--	--	--	--
	15	6	300	0	100	100
Mature larvae (3rd instar)	Control	6	300	217	27.7	-
	1	6	300	91	69.7	58.1
	3	6	300	33	89.0	54.8
	--	--	--	--	--	--
	15	6	300	0	100	100

* Abbot's correction (Abbott (1925))

[44] 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.

< 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 treatment period), 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 duration), 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 cold 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 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 temperature and treatment durations) to be used in the exploratory trials should be low enough to result in complete mortality but should not result in reduced fruit quality. The exposure period should be provided in several sequential steps e.g. 10, 12, 14 16 and 18 days.

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 fruit 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 7.2.7.

8.3. Test results

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

[57] Based on the results recorded below a treatment of 16 days would be selected as the treatment regime to be used in the confirmatory trials.

Table 5 Exploratory testing: Mortality of Medfly (most tolerant stage) in fruit treated at temperature of 2.0 °C for a range of exposure periods

Replicate	Fruit temperature and dwell time	No. of Fruit	Estimated No. of treated insects*	Total No. of survivors	Observed Mortality (%)
1 (April 15, 2016)	10	80	3,230	148	95.4
	12	80	3,230	82	97.5
	14	80	3,230	3	99.9
	16	80	3,230	0	100
	18	80	3,230	0	100
	Control	80	-	3,230	-
2 (May 5, 2016)	10	80	3060	108	96.5
	12	80	3060	34	98.9
	14	80	3060	2	99.9
	16	80	3060	0	100
	18	80	3060	0	100
	Control	80	-	3,060	-

*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 period), criteria for the determination of mortality etc. (refer to "<Documentation> of 7. Most cold tolerant life stage").

(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 live/dead insects per fruit, and survival rate (mortality), etc. in each replicate should be tabulated. Data of non-treated control should be also included in the table.

- Temperature data 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] Fruits infested with the most tolerant stage should be produced as described in section 7.

9.2.3 Treatment condition

[61] The treatment conditions should be based on the results of the exploratory test that resulted in   100% mortality or on other data indicating the treatment condition   provides quarantine security.

9.2.4 Number of insects and fruits tested

[62] Treatment plots: Number of test fruits should be adjusted to obtain 10 000 test 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 precooling 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 the treatment, and these sensor-fruits should have a similar average weight and size to the infested fruit.

[70] Temperatures should be monitored regularly to observe if the average fruit core temperature is falling below the target temperature. This is very important as the minimum average temperature will become the minimum temperature that can be recommend in any proposed treatment schedule. So even if 9 out of 10 sensors are above the target temperature the minimum proposed temperature will be based on the lowest average temperature from sensor with the lowest readings.

9.2.8 Determination of mortality

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

9.3. Test results

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

Table 6. Confirmatory testing; Mortality of Medfly (most tolerant stage: e.g. 3rd instar larvae) in the fruit treated at 2 °C for 18 days.

Replicate	Date	Control		Treated				
		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, 2017	80	3,220	350	14,087	0	100	99.9787
2	April 20, 2017	70	2,980	300	12,771	0	100	99.9765
3	May 15, 2017	70	3,055	300	13,092	0	100	99.9771
Total		220	9,255	950	39,950	0	100	99.9925

*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.

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

[73] When interpreting trial results it is important to remember that proposed treatment schedules will use the lower temperature threshold recorded and the maximum exposure period recorded. Two examples of how the experimental results have been converted into treatment schedules are as follows: (1) If the lowest average fruit core temperature for a specified duration (e.g. 18 days) over three replicates is 2.0°C, then it would be appropriate to propose a treatment schedule of 2.0 °C or below for 18 days (see Table 7). However, if one of replicates has a treatment duration longer than 18 days (e.g. 18 days and 1 hour), the schedule should be 2.0°C for longer than 18 days (e.g. 19 days). Similarly, if one of replicates includes an average fruit core temperature for 18 days that is lower than 2.0°C (e.g. 1.9 °C), the proposed treatment schedule would reflect this e.g. 1.9 °C or below for 18 days.

Table 7 Summary of the temperature data of confirmatory testing at 2 °C for 18 days).

Replicate	Fruit loaded into chamber (Date and Time)	Start of treatment (Date and time)	End of treatment (Date and time)	Total Duration	Average of fruit temperatures (°C)
1	March 29, 2017 (10:00)	March 30 (17:00)	April 17 (17:00)	18 days	2.07°C
2	April 20, 2017 (10:00)	April 21 (18:00)	May 9 (17:00)	17 days 23 hrs	2.04°C
3	May 15, 2017 (17:00)	May 17 (00:00)	June 3 (21:00)	17 days 21 hrs	2.01°C
Average fruit temperature for the 3 replicates was 2.04°C Proposed phytosanitary treatment schedule: Fruit temperature at 2.0°C or below for 18 days					

*Removal date, time of infested fruit from the cold chamber.

**Average of fruit temperatures recorded (by sensors) from the start to end of the treatment.

< 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 Large scale 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

[74] Once an effective treatment has been determined fruit quality trials should be undertaken. Evaluation of effects of the cold treatment on the 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

[75] Fruit quality trials should be conducted on export quality fruit using the same treatment schedules as the confirmatory trials or more severe conditions (such as lower temperature and longer exposure periods) that can be expected from commercial application of the treatment.

[76] 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 the exporting country to importing country and distribution conditions in the importing country.

10.3. Test results

[77] 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).