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Organización Norteamericana de Protección a las Plantas
MEXICO - USA - CANADA

NAPPO Regional Standards for Phytosanitary Measures (RSPM)

RSPM 34
Development of Phytosanitary Treatment Protocols for Regulated Arthropod Pests of Fresh Fruits or Vegetables

The Secretariat of the North American Plant Protection Organization
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Review

NAPPO Standards for Phytosanitary Measures are subject to periodic review and amendment.

The next review date for this NAPPO Standard is 2016. A review of any NAPPO standard may be initiated at any time upon the request of a NAPPO member country.

Endorsement

This Standard was approved by the North American Plant Protection Organization (NAPPO) Executive Committee on October 17, 2011 and is effective from this date.

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Implementation

See the attached implementation plans for implementation dates in each NAPPO country.

Amendment Record

Amendments to this Standard will be dated and filed with the NAPPO Secretariat.

Distribution

This standard is distributed by the NAPPO Secretariat, to the Industry Advisory Group and Sustaining Associate Members, the International Plant Protection Convention (IPCC) Secretariat, and to other Regional Plant Protection Organizations (RPPOs).

Introduction

Scope

This document describes the elements that should be considered in the development of protocols for phytosanitary treatment against regulated arthropod pests (such as mites, insects, spiders, millipedes) on or in fresh fruits or vegetables, including recommended statistical analyses.

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Definitions, Abbreviations and Acronyms

Definitions of phytosanitary terms used in this standard can be found in RSPM 5 and ISPM 5.

Outline of Requirements

The objective of phytosanitary treatment protocols is to demonstrate mortality or sterilization of the target regulated arthropod in/on a specified fresh fruit or vegetable, based on statistically valid data. Tests are conducted in the laboratory and should be representative of variability in the fruit or vegetable and target arthropod. Experiments should be replicated, statistically analyzed, and the levels of confidence reported based on sample size so that data is verifiable and reproducible.

The following items should be considered in developing treatment protocols:

- Identification of the species and variety of fruit or vegetable proposed for export.
- Specification of any defined condition(s) of the fruit or vegetable to be evaluated.
- Identification of the target arthropod species and stage of concern.
- Description of the origin and handling of the target arthropods to be used in the tests.
- Description of the fruit or vegetable and target arthropod to be used as controls in the tests.
- Implementation of separate tests for each target arthropod species and commodity combination for which determination of treatment efficacy is required.
- Description of the equipment used in the tests.
- Description, in detail, of the parameters of the test.

Background

Phytosanitary treatments sterilize or kill regulated arthropod pests on or in a fruit or vegetable to prevent their entry and establishment in areas where they are not present or are not widely distributed. Failure of treatment may result in interruption of trade and/or introduction of a new pest. Protocols to determine the efficacy of treatments, the acceptable level of effectiveness and statistical confidence for evaluations may be detailed in a bilateral agreement.

Pest Risk Analysis is used to determine phytosanitary measures required to achieve a prescribed level of quarantine security (ISPM 11: 2004). Among these measures are phytosanitary treatments of fruits and vegetables used as a stand alone measure, or in combination as part of a systems approach to eliminate or reduce the risk of introduction and spread of quarantine pests. Treatment schedules are NPPO-accepted protocols for application of chemical or physical manipulations as specified for each target regulated arthropod pest and commodity (ISPM 28: 2007). Chemical treatments include fumigation with methyl bromide, phosphine, and other gases (Neven 2010). Physical treatments include heat, cold, irradiation, controlled atmosphere, and surface barriers (Follett and Armstrong 2004; Hallman 2007; Neven 2008a, 2008b, 2010). Specifics related to irradiation are not included in this standard as they are covered in the annexes of ISPM 28.

Standardized treatment protocols should ensure uniform high quality results and should facilitate the ability to make comparisons among different commodities and pest species. Typical shortcomings of phytosanitary treatment protocols developed by researchers (Follett and Neven 2006) and submitted for consideration to NPPOs include, but are not limited to:

- Inadequate sample size of the target arthropod and/or commodity.
- Failure to treat the most tolerant target arthropod stage.
- Treatment in diet or air instead of the host commodity.
- Incomplete or inexact reporting of experimental methods.
- Inadequate or unreported treatment parameters.
- Insufficient number of treatment doses.
- Insufficient range of treatment doses.
- Absence of large-scale validation tests.
- Incorrect or inappropriate statistical analyses.

Some of these shortcomings may be related to practical difficulties in conducting this type of research. Part of the intent of this RSPM is to raise awareness of potential problems in development of quarantine treatments.

The probit 9 concept is the historical basis for designating the efficacy of treatments (Baker 1939). It is derived from probit analysis used as a statistical method to determine the dose-response relationship of a treatment. Mortality or sterilization at the probit 9 level indicates 99.9968% efficacy of the treatment. A minimum sample size of 93,613 individuals is required to demonstrate a probit 9 response at the 95% confidence level (Follett and Neven 2006). In many instances, this sample size is not feasible due to difficulty in rearing of the target arthropod or low infestation rate of the fruit or

vegetable (Follett and McQuate 2001). Couey and Chew (1986) provide quantitative methods to calculate the minimum sample size and confidence limits for other levels of treatment efficacy and confidence limits (Appendix 1).

Alternative treatment efficacy measures, such as the maximum pest limit, have been proposed (Landolt *et al.* 1984; Baker *et al*. 1990; Mangan *et al.* 1997). Risk, under this alternative measure, is defined as the probability of a mating pair, gravid female, or parthenogenic individual surviving in a shipment. The risk of survival and reproduction is a function of the treatment, the infestation rate in nature, the biology of the pest and host, and distribution systems at point of entry.

A treatment with a high level of efficacy (Probit 9) may not be the only acceptable risk management option. The primary benefit of using an alternative treatment efficacy in the development of phytosanitary treatment protocols is that a much smaller sample size may be used to demonstrate the required efficacy at a 95% confidence level (e.g., less than one mating pair survives in a shipment). A treatment with a lower efficacy level may be a component of an overall systems approach to reduce risk.

Requirements

1. Experimental Design

The purpose of these experiments is to determine the efficacy of phytosanitary treatment protocols for the target arthropod in/on the specified fruit or vegetable under defined conditions. The specific design of the experiments will be dependent upon the selected individual pest/commodity combination and statistical analyses.

1.1 General considerations

- Sampling protocols should be based on principles of independence and randomness and be appropriate for the intended statistical analysis.
- Tests should be appropriate to evaluate the target arthropod and the specified defined condition(s) of the fruit or vegetable.
- Number of replications per test should consider variability in target arthropods and fruit or vegetable over the production area. Number of replications should be representative of the range of actual production and growing conditions, for example, crop grown at high and low elevation. Adjustments may be made based on the biology of the target arthropod or characteristics of the fruit or vegetable.
- Desired level of efficacy may be the same as the maximum pest limit of less than one reproductive pair per consignment (Mangan *et al.* 1997). If the likelihood of establishment of the species in the importing country is low based on climate, host availability, or other factors; or if other phytosanitary measures are applied as part of the systems approach, a lower level of treatment efficacy may be appropriate.
- There should be a 95% confidence limit over all replicates for the required efficacy level. If a confidence limit of less than 95% is used, there should be appropriate justification.
- Number and weight of the fruit or vegetable should be sufficient to determine treatment efficacy and confidence level. The number of individual target arthropods used in each replicate and the number of individuals surviving in

controls versus treatments should be documented.

- This number should be sufficient to determine efficacy and confidence levels. The number of control fruits or vegetables to be used for dose-response, efficacy, and confirmatory tests should be documented.

1.2 Regulated arthropod pests

- When possible, target arthropod used in tests should originate from the same area as the fruit or vegetable, e.g., commercial production area.
- When possible, colonized target arthropod should be no older than three generations at the initiation of the tests, without re-stocking, and maintained on natural hosts to maintain normal physiology and behavior. Otherwise, an experiment to compare treatment efficacy between laboratory and wild populations may be necessary.
- Natural populations may be used to infest the commodity or a naturally infested commodity may be used when laboratory colonies are not available.
- Records on the origin and handling of the target arthropod should be maintained, including temperature, relative humidity, and photoperiod.
- Identified reference specimens should be kept and accessible.
- The infestation method should be documented.

1.3 Fresh fruits and vegetables

Tests should be appropriate to evaluate the treatment and any specified defined condition(s) of the fruit or vegetable.

- The fruit or vegetable used in the treatment development tests should be:
- Documented according to the species, variety (e.g., photographic documentation and identification by a botanist) and origin of the fruit or vegetable being treated.
- Classified according to a commercial grade of a defined color, size, defined stage of maturity, and physiological condition.
- Held under defined conditions that duplicate commercial handling practices prior to treatment (e.g. washed, waxed, sprayed, etc.).

1.4 Control hosts

Controls are required for all treatment development tests. The control host should be the same fruit or vegetable which is being evaluated in the dose-response tests. The control host should be infested and held under the same parameters as the treated hosts. Target arthropods used in a control and experimental replication should all come from the same group, colony, strain, or population and be of the appropriate age and condition to encourage infestation. Five percent or more of the total number of the infested fruits or vegetables should be reserved as untreated controls. The total number of target arthropods treated should be estimated from the number of target arthropods surviving in untreated controls.

Controls are used to:

- Confirm that environmental conditions were appropriate for infestation and survival.
- Indicate the normal timeframe for development of the pest
- Indicate the high level of infestation that may occur in a host.

- Verify that the target arthropod population used in the test is able to survive on the host.
- Document control mortality.

1.5 Data analysis

- The efficacy of phytosanitary treatments of fruits or vegetables and the confidence level should be calculated from the number of survivors compared to the control (Appendix 1).
- Corrections for control mortality should be calculated according to Abbott (1925).
- The sample size required for efficacy and confirmatory tests should be adjusted based on control mortality (Follett and Neven 2006, Appendix 1).

2. Tests

There are different types of testing for developing treatment protocols. The first level is determination of the most tolerant life stage; the second is efficacy testing (typically dose/mortality testing); and the third is confirmatory tests to validate the minimum treatment on a commercial scale.

2.1 Most tolerant arthropod pest life stage

Determination of the life stage that is most tolerant to the proposed treatment is mandatory. If there is sufficient data for a specific pest using a specific treatment, then this level of testing may not be necessary. This is the life stage that must be used in the efficacy and confirmatory tests.

The most tolerant life stage determination should include, but is not limited to, the following:

- Evaluation of each life stage of the target arthropod that is present or developing in/on the fruit or vegetable at and after the time of harvest.
- Generation of dose response curves for each life stage.
    - A minimum of five (5) treatment levels that gives < 100% mortality and an untreated control.
    - A minimum of 50 individual target arthropods per replicate, when possible.
    - A minimum of four (4) replicates per treatment level and untreated control.
- Documentation of environmental parameters of target arthropod rearing.
- Documentation of pre-treatment holding conditions of target arthropods and fruits or vegetables.
- Documentation of the treatment parameters.
    - Procedures for natural and/or artificial infestation of fruits or vegetables.
    - Type of treatment.
    - Specific details of the treatment:
        - Treatment level (e.g., temperature, amount of fumigant)
        - Treatment period (duration)
        - Rate of treatment application
        - Temperature range
        - Relative humidity
        - Rate of treatment removal (e.g., cooling rate)
        - Other relevant parameters required to repeat the treatment
    - Method of treatment delivery.

- Full description of the equipment used
  - Calibration of all equipment and measuring devices
    - Dose mapping of the chamber
    - Measuring devices
- Documented post-treatment parameters
  - Conditions should support survival, development, and reproduction of the target arthropod, if that status is relevant to the treatment protocol.
  - Untreated controls should survive and should reproduce normally, if that status is relevant to the treatment protocol.
  - Tests should be repeated if control mortality is ≥20%, unless the biology of the target arthropod justifies a higher mortality level (Busvine, 1971).
- In some instances two or more arthropods may be targets of the same treatment on a fruit or vegetable (Neven and Rehfield-Ray 2006). The species which is most tolerant to the treatment may be determined by a direct comparison of the species in dose response tests. The most tolerant life stage of the most tolerant species should then be used in efficacy and confirmatory tests.

2.2 Efficacy tests

Efficacy tests are conducted on the most tolerant life stage to establish the minimum treatment level for confirmatory tests. The minimum treatment level is the LT99 as calculated from the dose response curve in the most tolerant life stage tests. The efficacy tests should include, but is not limited to, the following:

- The most tolerant life stage of the target arthropod that is present or developing in/on the fruit or vegetable at and after the time of harvest will be used.
- The methodology for timing the most tolerant life stage should be documented.
- Treatment criteria:
  - A minimum treatment level equivalent to the LT99 calculated in the most tolerant life stage tests.
  - An untreated control.
  - Four (4) replicates per treatment level.
  - A minimum number of individuals of the most tolerant life stage treated and killed, with no survivors. The number chosen is dependent upon biological factors, pest load, availability of the commodity, and requirements of the importing countries.
  - The number of target arthropods in the untreated control should be equivalent to 5 to 10% of the total.
- Documentation of the treatment parameters as in Section 2.1.
- A full description of the equipment used as in Section 2.1
- Documentation of the post-treatment parameters as in Section 2.1

2.3 Confirmatory tests

Confirmatory tests are required to validate the minimum treatment level on a commercial scale. Data from efficacy tests are used to define the parameters of the confirmatory test. The confidence level, number of target arthropods, and minimum treatment level should be designated.

The confirmatory test should include, but is not limited to, the following:

- The most tolerant life stage of the target arthropod that is present in/on the fruit

or vegetable at or after harvest will be used.

- Documentation of the methodology to determine that the most tolerant life stage is present in/on the fruit or vegetable at the time of treatment.
- Treatment criteria:
  - The minimum treatment level determined in the efficacy test.
  - An untreated control.
  - A minimum number of individuals of the target arthropod killed with no survivors should be sufficient to support appropriate statistical analyses and provide acceptable confidence in the treatment to the importing country.
  - The minimum number of treated individuals can be reached by summation of the results of multiple tests.
  - The number of individuals of the target arthropod in the untreated control should be at least 5 to 10% of the total.
- Fruit or vegetable containers used during the treatment should be the same as those expected to be used in the commercial application of the treatment (as applicable).
- Documentation of the treatment parameters as in Section 2.1.
- Full description of the equipment used as in Section 2.1.
- Documentation of the post-treatment parameters as in Section 2.1.

3. Post-treatment Fruit and Vegetable Handling

Treated and control fruits or vegetables may be held until the target arthropod matures, if it is relevant to the treatment protocol. Fruit or vegetable holding conditions should optimize target arthropod survival and be specified in the experimental design.

Fruit or vegetable handling criteria should be documented, including, but not limited to:

- Temperature
- Relative humidity
- Photoperiod
- Holding medium
  - pesticide-free
  - sterile
  - well-drained to prevent arthropod mortality from excess moisture
- Restricting access by other organisms which can interfere with any of the target arthropod life stages.
- Recording the number of target arthropod individuals and life stages from each piece of fruit or vegetable for each replicate.
- Holding controls separately from treated fruits and vegetables.

Data to be recorded include, but are not limited to:

- Daily environmental conditions during the fruit or vegetable holding period.
- Number, life stage, and emergence dates of target arthropods developing in or on the fruit or vegetable and control host, as relevant to the treatment (Appendix 1).
- Ability of surviving target arthropods to develop and/or reproduce, if relevant to the treatment.
- Deviation from normal morphology and behavior.

- Numbers emerging from, or developing in, the fruit or vegetable compared to those from controls

4. Interpretation of Results

The following items, among others, should be considered in interpretation of data from treatment protocols:

- The minimum treatment level can be confirmed in statistically validated efficacy and commercial scale tests with the most tolerant life stage and commercial grade commodity.
- The defined conditions of the treatment evaluated and confirmed in the tests can be designated as a requirement to meet the appropriate level of protection.
- High mortality (≥20%) in untreated controls may indicate a problem with the experimental conditions, the infestation procedures, or other issues that negatively impact arthropod survival. The test should be repeated and test conditions may require modification.
- Other risk management options (e.g., systems approach).are normally required to achieve an appropriate level of protection, as determined by the importing NPPO, if treatment efficacy is below the LT99 level.

5. Recordkeeping

The NPPO should keep appropriate records of phytosanitary treatment development tests. Information kept should be appropriate for the intended purpose of demonstrating the efficacy of treatments. Information in the records should include, but is not limited to:

- scientific name of target arthropod
- scientific name and variety of fruit or vegetable
- location of reference specimens
- tests conducted, defined conditions, experimental design, dates, locations, data, statistical calculations, and results
- references.

For each target arthropod species and fruit or vegetable combination, the NPPO of the exporting country should provide the NPPO of the importing country with reports on results of treatment development tests in accordance with this standard. The publication of treatment tests in peer-reviewed scientific journals is encouraged.

Appendix 1: Statistical analyses for developing phytosanitary treatment protocols for arthropod pests in fresh fruits and vegetables

1.1 Control mortality

An estimate of the treatment response should be made after correcting dose response data for control mortality (Abbott 1925). Corrected dose response data should be used in comparisons between treatments.

1.2 Dose response

Statistical analyses to detect significant differences between treatment levels may include, but are not limited to:

- Probit analysis
- Factorial analysis of variance
- Linear regression and analysis of covariance by standard least squares model
- Means should be separated by an appropriate post-hoc test (e.g., Tukey's honest significant difference, Duncan's multiple range).

Corrected dose response data (percent mortality data) may be transformed to normalize the distribution prior to statistical analysis where a normal distribution is an assumption (Follett and Armstrong 2004; Neven 2008a,b).

1.3 Sample size estimation

The effectiveness of phytosanitary treatments of fruits or vegetables and its confidence level should be calculated from the level of infestation, which is the number of target arthropods pests surviving the treatment and the control (Couey and Chew 1986). In developing fruit or vegetable quarantine treatments, such as hot water treatments, the level of confidence associated with treating a number of arthropod pests with zero survivors is given by the equation,

$$C = 1 - (1 - pu)n \qquad (1)$$

where *pu* is the maximum allowable infestation proportion (e.g. 0.0001 for 99.99% mortality) and *n* is the number of trial insects (Couey and Chew 1986). Equation 1 can be rearranged to determine the number of insects that are required for trials for a given level of confidence.

$$n = [\log(1-0.95)/\log(1- pu)] \quad (2)$$

Equation 2 calculates how many insects (*n*) there must be in trials with no survivors to achieve a 95% confidence (C, as a proportion) that the survival proportion is below a predetermined level (*pu*) (Couey and Chew 1986).

The sample size required for efficacy and confirmatory tests should be adjusted to account for control mortality (Follett and Neven 2006). The adjusted sample size a,

$$a = n/(sc) \qquad (3)$$

where n is derived from equation 2 and sc is the percentage of survivors in the control divided by 100.

1.4 Confidence level estimation

Couey and Chew (1986) provide an equation to estimate the confidence levels for effectiveness when only a few insects survive on a host,

$$\sum_{X=0}^{X=S} e\text{-}mmx/x! = 1 - C \qquad (4)$$

where m is $n \times pu$, n is the number of insects or fruit or vegetable sampled, s is the number of survivors, and C is the confidence level. This equation uses the Poisson distribution law and assumes large n and small pu (Couey and Chew 1986). It is expected that most fruits and vegetables in treatment protocol development trials will have 0 or 1 survivors and a Poisson distribution, which these models assume, may be the most appropriate (Baker *et al.* 1990).