NAPPO
North American Plant Protection Organization
Organización Norteamericana de Protección a las Plantas
MEXICO - USA - CANADA

DD 09: Criteria for Evaluating Phytosanitary Seed Treatments

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The purpose of this project was the **development of a list of criteria for evaluating phytosanitary seed treatments** as well as the identification of data gaps and research needs where they may exist. The document covers the following topics:

- What constitutes an effective treatment;
- When should treatments be applied;
- How would countries agree that a treatment is (or is not) sufficient for managing risk;
- When (or if) verification through testing is justified;
- When (or if) additional testing after a treatment is appropriate;
- Whether seed is an epidemiologically significant pathway (what is the likelihood of pathogen introduction) after treatment; and,
- What impacts do the treatments have on the commodity?

1. Background

International Standard for Phytosanitary Measures 28 (ISPM 28 - *Phytosanitary treatments for regulated pests*) provides general guidance on the development of phytosanitary treatments. However, the standard does not provide any specific guidance on phytosanitary treatments for seeds. Evaluating phytosanitary treatments for seeds may differ from other commodities; therefore, this paper presents additional considerations for evaluating phytosanitary treatments for seeds specifically.

The goal of phytosanitary seed treatment research is to subject infected seed to one or more treatments that reduce and/or devitalize the pathogen from the seed pathway. Treatments for seed represent a special case because of the difficulty involved in verifying that treatment efficacy has met the appropriate level of protection identified by the importing country. In some cases, treatments for seeds may affect the vitality of the seed being treated.

Depending on the type of diagnostics used to verify treatment efficacy, seed may still test positive for the presence of a pest even if it has undergone an efficacious treatment to inactivate a pest (e.g. serological tests may still detect pathogen proteins or nucleic acid-based tests may detect residual DNA or RNA after effective treatment). Furthermore, seed treatments – as with most other phytosanitary treatments for other types of commodities – may not achieve 100% devitalization of the pest. The risk associated with the pest, and the appropriate level of protection of the importing country will inform the necessary level of efficacy of the treatment. A combination of factors may help to evaluate the reduced risk of a particular seed lot; biology of the pest, minimum founder rate, efficacy of the seed treatment and acceptable level of risk.

Note: The scope of this project is focused on both seed borne and seed transmitted pests. It is understood that invasive weeds (seeds) can pose a potential phytosanitary risk in the seed pathway, however the criteria and considerations to mitigate quarantine weeds (seeds) are considerably different than those for pathogens. Therefore, we have not tried to incorporate the criteria for mitigating quarantine weeds (seeds) throughout this document. Seed certification, inspection and cleaning to remove quarantine weeds and testing to certify seed lots free from these weed (seeds)

are commonly utilized practices to mitigate the presence of regulated weed (seeds) in seed lots. Seed testing certificates produced by laboratories with internationally recognized accreditation systems and/or laboratories testing according to internationally recognized seed testing methods are credible sources to provide evidence of freedom from regulated weed species. If National Plant Protection Organizations (NPPO) are willing to mutually accept these sources, it could eliminate the need for testing seed lots for the presence of regulated weed seeds in both the country of origin and the destination country.

2. Types of Treatments

According to the adopted International Plant Protection Convention (IPPC) standard on *International Movement of Seeds*, "seed treatments include, but are not limited to:

- pesticides (fungicides, insecticides, nematicides and bactericides)
- disinfectants, generally used against bacteria and viruses and some fungi; disinfection may take place during various steps in seed processing (e.g. seed extraction, seed priming) or during a dedicated disinfection process
- physical treatments (e.g. dry heat, steam, hot water, irradiation by ultraviolet light, high pressure, deep-freezing)
- biological or biochemical treatments based on different modes of action, such as antagonism, competition and induced resistance."

Appendix 1 includes examples of pesticide, disinfectant and physical treatments. Treatments may be systemic or contact treatments. A particular treatment may be effective against one or more pathogens, or two or more treatments may be combined to target one or more pathogens.

3. Purpose of Treatments

Treatments for seeds are often applied as protective treatments intended to prevent a soil borne pest from infecting the growing seedling (e.g. seeds are treated with a fungicide to prevent fungal infection when the seed sprouts) or remove microbial pests that may be on the surfaces of seeds. For phytosanitary purposes, a treatment is applied to kill or devitalize a pest that is associated with the seed. Additionally, seed biological control treatments may directly (e.g., by antibiotic production or niche competition) or indirectly (by inducing plant resistance) reduce the risk of pathogen introduction and epidemic development.

4. Developing a Research Protocol for Phytosanitary Seed Treatments

Experimental materials and methods should be described in detail. Research protocols should be submitted to the NPPO that will be considering the treatment before the research begins. This is to ensure that the protocol is agreed upon prior to the initiation of research and that the protocol meets requirements of the importing country. Research completed without a protocol having been reviewed may result in rejection of the research results.

5. Determining Efficacy of Treatments

As with any other phytosanitary treatment, efficacy is demonstrated through small scale testing followed by large scale confirmatory tests.

5.1 Small Scale Tests

Small scale testing or preliminary tests are usually done to determine the best treatment. All treatments are subject to pesticide regulations and should conform to any specific requirements related to such regulations. Several doses should be used to determine the optimum treatment. Each treatment rate must be tested in a replicated fashion (minimum 3 replicates per treatment) with an appropriate negative control to allow for statistical analysis of the effects of the treatment. If possible, different seed infestation rates should be tested (e.g. low, medium and high percentages on infested seeds/lot).

The researcher should choose the most appropriate detection assay method for the pest and commodity. This will be dependent on the biology of the pathogen and the type of treatment being tested. Multiple assays can be used if required. The assay should be described in detail. Researchers are also required to submit all results from the small-scale dose-response testing to the NPPO prior to the initiation of confirmatory trials. This step will ensure that there are no discrepancies between NPPO and the researchers as to the best treatment.

5.2 Large Scale Confirmatory Tests

Large scale confirmatory tests are done to establish statistical confidence in the efficacy of a proposed treatment. The sample size for the confirmatory testing should be mutually agreed upon by NPPO and researchers. An example of a specific test size is provided in Appendix 2. Seed pests are diverse and guidelines for confirmatory test size will be dependent on the pathogen biology, risk of establishment in environment, and host.

Obtaining naturally infected seed in sufficient numbers to conduct statistically significant tests can be challenging. Where appropriate, spiked seed samples can be used. If spiked seed samples are used, the method of spiking should mimic natural infection of seed by that particular pest. Additional information on testing requirements can be found in Appendix 3.

5.3 Measuring Efficacy

Researchers should subject data to appropriate statistical analyses, such as probit analysis at the LD50, LD95, LD99, and probit-9 levels. The type of statistical analysis used to determine the efficacy of the treatment should be described. Researchers are also encouraged to provide "raw" data in table format. Acceptable measures used for pathogen detections include colony forming units (cfus), often used for treatments that are considered to be disinfectants. The experimental design for a study should take into account the need to quantify or measure efficacy, and account for the mode of action of the treatment.

5.4 Determining Effects of the Treatment

Plant pathogenic microorganisms (e.g. fungi, bacteria, viruses, etc.) are the most common type of pest associated with seed. Because of the biology of these organisms, it may be difficult to assess the effect (inactivation, devitalization, etc.) and efficacy of a treatment. This may also require extended growth and observation of the planted seed in a growth chamber, greenhouse or small field plots. This is due to the complexity of the life-cycle of the pathogens which may only produce symptoms on mature plants.

In conducting confirmatory tests, researchers should demonstrate that the pest is non-viable (inactivated, devitalized, etc.) on/in the seed post-treatment. This may be demonstrated through direct detection of the pathogen, seedling/plant grow-out assays or a combination of pathogen detection and plant grow-out. See the section below on testing treated seed.

Some biological seed treatments do not actually kill or devitalize pests; they are applied as seed coatings and, once the seed is planted and germinates, these biological agents rapidly colonize the rhizosphere and developing root systems to limit seed-to-seedling transmission of disease. In these situations, efficacy could vary depending on environmental conditions and soil microflora. Hence, seed treatment efficacy may have to be determined by evaluating effects on the resulting plants through plant grow-out studies in different locations/conditions. In such cases, these issues should be discussed with the NPPO prior to testing.

6. Feasibility of Treatments

International Standard for Phytosanitary Measures 28 (*Phytosanitary treatments for regulated pests*) provides guidance on issues related to feasibility of treatments. For seeds, in particular, one special consideration is the effect of treatments on viability or germination rate of the seed. Treatments that negatively impact the commodity should be carefully considered before they are used. Information about the impacts of treatments on the following attributes of seed should be included in the outcomes of the efficacy research as they are an important consideration for the users of seed treatments:

- Vigor
- Viability
- Physical damage
- Physiological alterations of the seed
- Promoting seed latency or premature germination
- Effects on seed storage
- Shelf-life of the treatment

Other aspects of feasibility to consider include cost, practicality of application and accessibility of inputs (equipment and materials). It should be noted that pesticide treatments used on seeds may need to be registered in both the exporting and importing countries by the responsible authorities.

7. Special Considerations for Research on Phytosanitary Treatments of Seeds

7.1 Testing Treated Seed

During research, it may be necessary to confirm that the treatment has devitalized, killed or otherwise inactivated the pest, or as noted in Section 5.4 where biological treatments prevent transmission of disease. This may be done through an appropriate diagnostic test if the test can indicate that the pest is devitalized or killed.

However, the following information from ISPM 38 *International Movement of Seeds* should be noted: “The test results of treated seeds should be interpreted carefully, as the following situations may be encountered:

- The treatment inactivates the pest but the detection method detects both viable and non-viable pests. This may be the case with some serological or molecular tests or when detection is based on morphological identification of pests or pest structures that may remain even after treatment (e.g. nematodes, spores). In such cases, determination of the efficacy of the treatment is conclusive only if a test validated for treated seeds is used.
- The treatment physically or chemically inhibits the detection method; for example, some detection methods for bacteria are affected by fungicide treatments.
- The treatment adversely affects the detection method; for example, a method detects only pests present externally and any pests remaining internally after the treatment cannot be detected. In these situations, other detection methods that are able to detect internal infection should be used".

In some cases, it may be appropriate to grow out seed under conditions conducive for disease to determine if viable pathogens are still present. Before growing out seeds, there are different types of *in vitro* methods that can be used to screen for the presence of pathogens (e.g. germinating seeds in culture medium or on blotters moistened with sterile water).

To preclude any additional testing of treated seed lots the NPPO(s) could enter into a trade agreement where both sides have reviewed the treatment research data and agree that it is sufficient to mitigate the phytosanitary risk. If further testing of treated seed is deemed to be required it would be at the discretion of the NPPO and should only be done if it is technically justified according to relevant standards (ISPM No. 1, 11, 28, 38, etc.). After a treatment has been agreed upon to be used in trade, the NPPO(s) may need to verify periodically the efficacy of the treatment through testing.

8. Sharing Research Protocols and Data among NAPPO NPPOs

All results, including research protocol used, raw data, and statistical analyses, should be shared with the NPPO. The quality and consistency in data will determine if the research result(s) is (are) accepted. The NPPO that has approved the treatment should consider providing the details of the treatment to importing NPPOs for their consideration. If one NAPPO member country approves a treatment that was developed according to an appropriate research protocol, other NAPPO member countries may want to consider approving that treatment for their own purposes.

9. Knowledge Gaps

Although seed trade has increased dramatically in the last 40 years, the tools available for regulators to mitigate seed transmitted pests remain limited and is an area that needs further research investment. Many treatments currently available for phytosanitary purposes are not appropriate for dealing with pathogens in or on the seed coat of seed being used for propagative purposes as it dramatically decreases seed germination. Development of additional treatment options for seed borne and seed transmitted pathogens localized in or on the seed coat or in the endosperm is needed by regulators.

Several private industries are investigating various new technologies to treat seed borne pathogens, but the treatments need to be validated by NPPOs in order to use these treatments for phytosanitary

purposes. Additional options for both internal and external pathogens need to be developed by both industry and government scientists and validated for use by NPPOs.

Seed tests can be used to check the phytosanitary status of shipments at the port of entry, but there are many gaps that need to be filled relating to testing. First, seed tests can give erroneous results if conducted on seed that has been treated. Development and validation of seed tests for high priority pathogens that can be used after seed has been treated is a need of NPPOs. Having an effective seed test available will allow regulators to make informed regulatory decisions at the port of entry regarding infection status of the imported consignment.

Additionally, sampling methodologies for seeds is an area for further research. Sampling can influence the detectability of pathogens in seed lots. Required sample sizes could vary depending on the pathogen and seed type.

Many lots of seeds that move around the world contain less than 20,000 seeds. These small seed lots pose unique challenges to NPPOs in terms of sampling and testing. Often, seed lots are too small for a test outcome that provides a statistically significant result. Developing effective treatments for quarantine pathogens may facilitate movement of small lots of seeds.

10. References

ISPM 11. 2016. *Pest risk analysis for Quarantine Pests*. Rome, IPPC, FAO.
ISPM 28. 2016. *Phytosanitary Treatments for Regulated Pests*. Rome, IPPC, FAO.
ISPM 38. 2017. *International Movement of Seed*. Rome, IPPC, FAO.
RSPM 36. 2013. *Phytosanitary Guidelines for the Movement of Seed*. Ottawa, Canada, NAPPO.
USDA. 2016. *Treatment Manual*.
https://www.aphis.usda.gov/import_export/plants/manuals/ports/downloads/treatment.pdf

APPENDIX 1 - Examples of different types of seed treatments

Pesticides

An example of chemical treatments is using Thiram to treat alfalfa seed:
Seeds of alfalfa (*Medicago falcata, M. gaetula, M. glutinosa, M. media, and M. sativa*)
Pest: *Verticillium albo-atrum*
Alternative treatments:
Treatment: Dust with 75 percent Thiram at the rate of 166 grams per 50 kilograms of seed (3.3g/kg).
Treatment: Treat with a slurry of Thiram 75 WP at a rate of 166 grams per 360 milliliters of water per 50 kilograms of seed (3.3g pesticide/7.2ml water/kg seed).
(note: chemical treatment is treating with Thiram 75 WP).

Disinfectants

An example of disinfectant is using hot water plus chemical dip for treating citrus seeds for citrus canker (*Xanthomonas axonopodis*).
Seeds of Citrus (Rutaceae family)
Pest: Citrus Canker (*Xanthomonas axonopodis*)
Treatment: Hot water plus chemical dip

1. Wash the seed if any mucilaginous material, such as pulp, is adhering to the seed.
2. Immerse the seed in water heated to 125 °F (51.6 °C) or higher for 10 minutes.
3. Then, immerse the seed in a solution containing 200 parts per million sodium hypochlorite at a pH of 6.0 to 7.5 for at least 2 minutes. (Note: chemical treatment is treating with sodium hypochlorite).

Physical treatments include heat treatment and forced hot air

An example of a physical treatment is using steam to treat corn seed:
Corn (seed) (Small lots for propagation but not for food, feed, or oil purposes)
Pest: Various corn-related diseases
Treatment: Treat seeds with a dry application of Mancozeb in combination with Captan. Disinfect bags by: 1) Dry heat at 212 °F for 1 hour. Treat small bales only; or 2) Steam at 10 pounds pressure at 40 °F for 20 minutes.

APPENDIX 2 - Example of specific test sizes for seed treatments

Example 1: An example of a specific test size that is approved by USDA-APHIS is 30,000 infected seed or more. No pathogen propagates should survive out of 30,000 infected seeds tested. Statistically, test results defined here provide probit 8.72 control with 99.990015 percent mortality at a 95% confidence level at 0.0001000 infection rate. Smaller numbers may be used if approved by USDA-APHIS depending on the biology of the host and pathogen. The untreated control lot size must be at least 25% as large as the lot of the seed lot used to test treatments. Use at least 4 repetitions. Each repetition should have the equivalent of 100% infected seed.

APPENDIX 3 - Additional considerations for developing seed treatments

Test Plant Pathogen

Use isolates/strains of the plant pathogen of interest that represents the most virulent populations in the country for which the treatment is being developed. Virulence should be demonstrated and maintained throughout the experimental period. The research should supply complete information on 1) identity of the taxon of the pathogen, 2) date of isolation, 3) pathogenicity, and 4) methodology for maintenance of the pathogen. Use natural or artificial inoculation and include a full description of inoculation method(s). When artificial methods are used, the method should mimic natural infection as closely as possible. Naturally infested or infected seed is preferred but the inoculum level should be considered carefully.

Host Seed

Conduct all preliminary tests, dose-response research, and confirmatory trials on the commodity for which the treatment is being developed.

Use seed of market quality that has not been treated with fungicides or other chemicals.

Use a cultivar that is highly susceptible to infection. Each preliminary treatment including the non-treated control repetition should include at least 100 infected seed.

Seed Treatments

Pesticide treatment - When possible, use fungicides registered in both the exporting and importing countries for the crop to be tested to avoid pesticide use issues, or trade barriers. In cases where the fungicide is not registered, appropriate regulatory authorizations may need to be obtained. Choose fungicides that have a high level of efficacy.

Physical treatment - hot water or oil treatments can be used on several seed commodities for various diseases. Provide a detailed description of thermal bath and procedures for conducting thermal treatment. This treatment has been known to give quarantine level of control for some pathogens. It may be used in conjunction with chemical treatments. Temperature readings cannot be averaged.

Disinfectants - There are several chemical seed treatments in addition to fungicides, including chlorine, quaternary ammonium chloride, hydrogen peroxide, peroxyacetic acid that are used as disinfectants. Researchers should give a detailed description of the equipment and procedures when testing disinfectants.

Equipment

Equipment - The researchers should provide a thorough description of the treatment monitoring equipment used during the study, e.g., sensor type, number, and placement; accuracy of monitoring equipment; specifications of treatment equipment. Diagrams and pictures should also be supplied for all these factors for both small-scale and confirmatory trials, as appropriate.