International Seed Federation
Seed is Life

INTERNATIONAL SEED HEALTH INITIATIVE (ISHI)

Best Practices for Blotter and Agar Plating Assays in Fungal Seed Health Tests

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This document describes best practices for the use of blotter and agar plating (hereinafter 'plating') assays in seed health testing to ensure accurate and reliable results. Best practices include process controls and assay conditions that should be applied to all trials.

Controls and conditions are designated as essential (must/shall be included) or recommended (can be included).

I. BLOTTER AND PLATING: DIRECT TESTS FOR DETECTING PATHOGENIC FUNGI

Blotter and plating assays are used to identify pathogenic fungi. Seeds are incubated, with or without first being treated to prevent germination (with cold temperature or a chemical application), and then evaluated for visual indicators of fungal growth.

II. CONTROLS AND THEIR PURPOSE

The types of controls for blotter and plating assays are defined in Table 1. Their purpose is to verify both the quality of the material used in the assays and proper test execution. Proper negative and positive controls should be included in every assay to ensure reliable test results. Unless exceptions are stated, these process controls and assay conditions should be applied to all blotter and plating assays.

Table 1: Controls to be included in blotter and plating assays.

Control type	**Positive Process Control (PPC) - Essential**
Definition	A well characterized seed lot, naturally or artificially infected with the target pathogen tested using the same assay at the same time as the test samples. Alternatively, for the agar plating method, a culture plate with a reference isolate of the target can be used. In the case of obligate parasites, and when naturally infected seed is not available, an alternative host/pathogen control can be used as PPC.
Expected Result	Detection of the target pathogen.
Description	The PPC serves to check the whole process and ensure that the target pathogen properly grows in the test conditions.

Note: When handling the PPC, it is important to ensure no cross-contamination occurs. This can be done by e.g., handling the positive control the last after working with negative controls and sample, or use of filter tips and a different set of pipettes when using a dissolved reference isolate.

Control type	Negative Process Control (NPC) - Essential
Definition	A known negative seed sample (with respect to the target pathogen) tested using the same assay at the same time as the test samples.
Expected Result	No detection of the target pathogen.
Description	The NPC checks for cross-contamination of blotter and/or plating materials as well as lab equipment during the process.

III. ASSAY SET-UP

The essential and recommended conditions for the set-up of a blotter and plating assays are described in Table 2.

Table 2: Set-up and determination of results for blotter and plating assays.

Description	Essential	Recommended
Substrate: The blotter/filter paper or agar media used must be sterile.	x	
Incubation container: Prior to re-use, blotter boxes/Petri dishes (incubation containers) must be sanitized using a validated sanitization method (e.g. bleach or soap or washing liquid) to prevent cross-contamination from the previous test.	x	
Sanitization practices: To avoid cross-contamination between seed samples during distribution of seed and seed/seedling evaluation, appropriate sanitization practices must be used (e.g., changing gloves, sanitizing the seed spreader between seed samples with e.g., ethanol, sodium hypochlorite or other).	x	
Saturation of substrate: The quantity of liquid (water or fungicide) used to saturate the substrate in each incubation container must be optimized for the blotter assay as performed in each laboratory. Uniform conditions between incubation containers are necessary to maintain uniformity in growth of the target pathogen, disease development, or seedling germination.	x	
Application of antimicrobial agents to substrate: Validated antimicrobials, as defined by the protocol, may be used to saturate the filter paper (blotter assay) or added to the media (plating assay) to minimize the growth and spread of saprophytic microorganisms. Validation of the antimicrobial substances must show that recovery of the target pathogen is not affected when applied to substrate. It is the responsibility of the user to evaluate any possible effect of these compounds on target growth due to variations in formulation, provider, etc.		x
Seed sanitization: A validated seed sanitization step, as defined by the protocol (e.g., ethanol, dilute sodium hypochlorite treatment or other), may be used to minimize the growth of saprophytic microorganisms. Validation of the seed sanitization technique must show that recovery of the target pathogen is not affected. It is the responsibility of the user to		x

Description	Essential	Recommended
evaluate any possible effect of the seed sanitization step on target growth.		
Distribution of seed on substrate: Seed must be distributed evenly over the substrate surface within each incubation container with seed spacing as defined by the protocol.	x	
Target confirmation: Examine the seeds visually and, if necessary, under stereoscopic microscope to identify the target fungi by comparing its morphology with the positive control. **Morphological examination** should only be performed by experienced persons in the recognition of typical structures used for fungal identification. When required by the detection method, a **pathogenicity assay** is used for final confirmation. Use the appropriate controls, hosts and environmental conditions referred to in ISHI-Veg's Best Practices for Biological Assays in Seed Health Tests. Finally, if **PCR assays** are used during identification/confirmation process, then include all the appropriate controls defined in ISHI-Veg's Best Practices for PCR Assays in Seed Health Tests. See https://www.worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg-method-development/ for best practice documents.	x	

IV. ENVIRONMENTAL CONDITIONS

Environmental conditions can greatly influence the outcome of the test and must be controlled and monitored for the duration of each test as described in Table 3.

Table 3: Environmental conditions for a blotter and plating assay.

Description	Essential	Recommended
Temperature: For the duration of the test, the temperature of the test location must be set according to the requirements specified in the protocol. It must also be monitored using temperature probes placed in relevant positions, and recorded for the entire period. It must not deviate from the acceptable range by more than ± 2°C	x	
Photoperiod: In the test location, photoperiod must be maintained as per the requirements of the protocol for the duration of the test	x	
Light conditions: Light of appropriate intensity and spectrum must be supplied to the seeds/seedlings for adequate growth of the target pathogen, disease development, or seedling germination. The quality of bulbs or LEDs should be monitored for functionality and overheating. Care must be taken when stacking incubation containers or plates to ensure that light intensity and spectrum are not decreased below the acceptable limit for the assay.	x	
Relative humidity: The relative humidity must be maintained as per the requirements of the protocol.	x	