International Seed Federation
Seed is Life

Detection of *Botrytis allii* in Onion Seed by Plating

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Developed by ISHI

Detection of *Botrytis allii* in Onion Seed by Plating

Crop: Onion (*Allium cepa*)

Pathogen(s): *Botrytis allii*

Version: 1 (November 2023)

PRINCIPLE

Detection of viable *Botrytis allii* in onion seed is done by plating NaOCl surface sterilized seeds on a semi-selective media (half strength lactic acid PDA containing PCNB). Suspect fungal colonies are confirmed by morphological examination.

The method process workflow is presented in Figure 1.

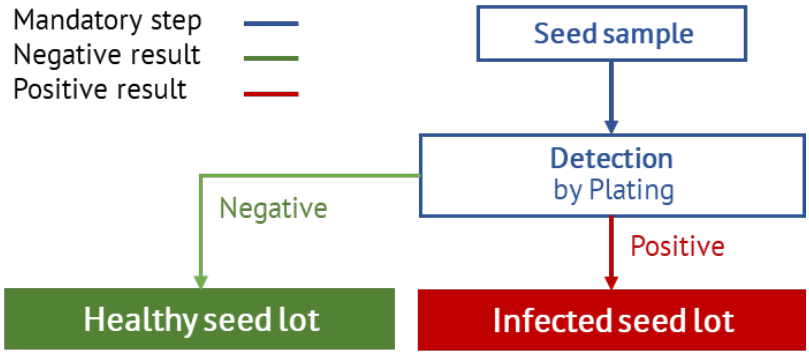

Figure 1. Method process workflow

METHOD VALIDATION

The validation report for the detection of *Botrytis allii* in onion seed (Hiddink *et al*., 2023) can be found at https://www.worldseed.org/our-work/seed-health/ishi-method-development-and-validation/. The report concludes that the method is fit for purpose at the qualitative level and can be used for testing of onion seeds for the presence of *Botrytis allii*. It should be noted that both *Botrytis allii* and *B. aclada* are morphologically undistinguishable (Garfinkel, 2021) and cannot be separated with this method.

RESTRICTIONS ON USE

Before using this protocol routinely, it is necessary to verify its performance, especially when material and consumables from different suppliers are used. Technical details on the reagents/material used in the validation study (e.g., supplier's information) are provided in the protocol and the validation report.

This test method is suitable for untreated seed or physically disinfected seed lots (such as hot water, heat). This test method has not been validated for seed treated with protective chemicals or biological substances or chemical processes with the aim of disinfestation/disinfection. If

treated seed is tested using this method, it is the responsibility of the user to determine empirically (through analysis, sample spiking, or experimental comparisons) whether the protective chemicals or biological substances have an effect on method results.

METHOD EXECUTION

To ensure process standardization and valid results, it is strongly recommended to follow the Best practices for Blotter and Agar Plating Assays in Fungal Seed Health Tests developed by ISHI.

SAMPLE AND SUBSAMPLE SIZE

The recommended minimum sample size is 400 seeds.

REVISION HISTORY

Version	Date	Changes (minor editorial changes not indicated)
1	November 2023	First version of the protocol.

Protocol for detecting *Botrytis allii* in Onion Seed by Plating

I. DETECTION BY PLATING

Materials

- Container for sterilizing seed
- NaOCl (1% (v/v) active ingredient)
- Autoclaved blotter paper
- Laminar flow hood
- Sterilized forceps
- Distilled or de-ionized water
- Plates of half strength lactic acid PDA semi-selective medium (see Table I.1)
- Incubator: operating at 20-23 °C, with 12 hours near ultraviolet (NUV) light/12 hours dark
- Stereo microscope: capable of magnifying at ×25
- Sterile spreader
- Controls (Table I.2)
- Lab disposables

Table I.1. Half strength lactic acid PDA semi-selective medium.

Compounds	Amount/L
Potato dextrose agar (e.g., Sigma or equivalent)	19.5 g
Agar	5.0 g
Distilled/deionized water to a final volume of	1,000 mL
Filter sterilized (0.2 µm bacterial filter) lactic acid (85%)[a]	1.4 mL
Pentachloronitrobenzene (PCNB)[a] (10 mg/mL methanol)	20.0 mg (2.0 mL)

[a] Added after autoclaving. Storage conditions and duration may affect PCNB activity, which can influence the performance of the test.

Table I.2. Types of controls used.

Control type	Description
Positive control (PC)	A known strain of *B. allii*
Positive process control (PPC)	A known *B. allii* infected seed sample
Negative process control (NPC)	A known *B. allii* free seed sample

1. Seed sterilization and plating

1.1. Place each onion seed subsample, the positive process control (PPC), and negative process control (NPC), in a labelled perforated sterilization container (e.g., an enclosed tea strainer made of woven screen).

1.2. Place the container for 2 min in NaOCl solution (1% (v/v) active ingredient, ensure concentration with test strips: e.g., activate or equivalent (10.000 ppm)). Shake a few times and rinse in sterile water for 30 sec (e.g., swirling the strainer in a 1 L beaker of sterile water under a laminar flow hood, works well).

1.3. While still in the laminar flow hood, dry the seeds on sterile labelled blotter papers. Use sterile forceps to place aseptically, for example 10 seeds per medium plate and divide them evenly over the plate.

1.4. Transfer a pure culture of *B. allii* (positive control, PC) onto two plates of the semi-selective medium.

Note: It is recommended to also transfer pure cultures for both *Botrytis cinerea* and *B. squamosa* for discrimination.

1.5. Incubate all plates at 20-23 °C with 12 hours NUV light/12 hours dark.

2. Evaluation of plating

2.1. Count the number of suspect *B. allii* colonies on each plate of tested subsamples and control samples at 7 and 12 days.

Note: In case of seed samples with highly developed saprophytic growth overgrowing the plates and covering all the seeds at 12 days, use the number of suspect *B. allii* colonies at 7 days as the final reading. Only when *B. allii* colonies are detected at 7 days, the test is valid. When no *B. allii* colonies are detected at 7 days the test is invalid due to saprophytic overgrowth at 12 days.

2.2. Use a stereo microscope with × 25 magnification to identify suspect colonies. *Botrytis allii* colonies on half strength lactic acid PDA appear as grey fast growing and sporulate profusely (Figure I.1). *Botrytis allii* develops tall slender branched conidiophores bearing clusters of single celled ovoid conidia 5-6 × 7-11 µm (Figure I.2).

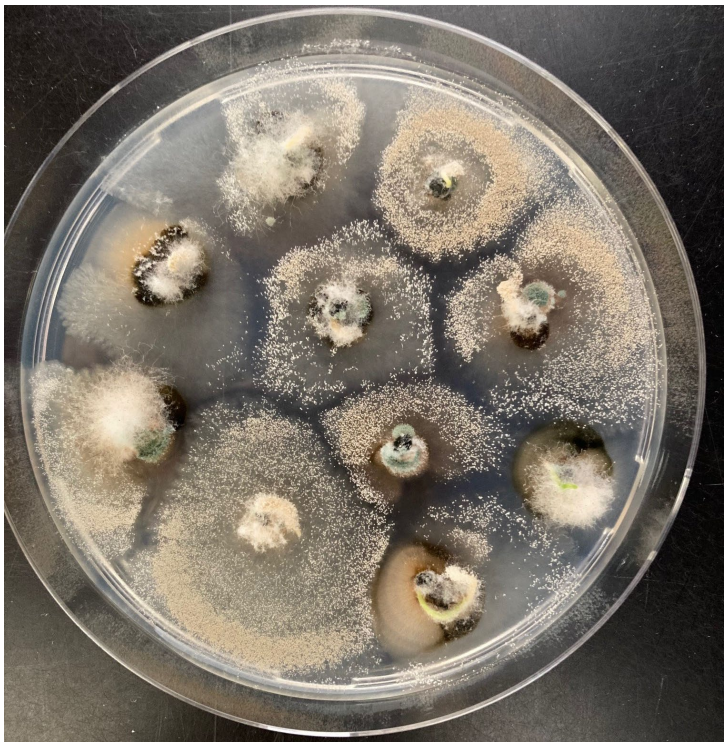

Figure I.1. Onion seeds infected with *B. allii* grown on half strength lactic acid PDA medium, 12 days after plating.

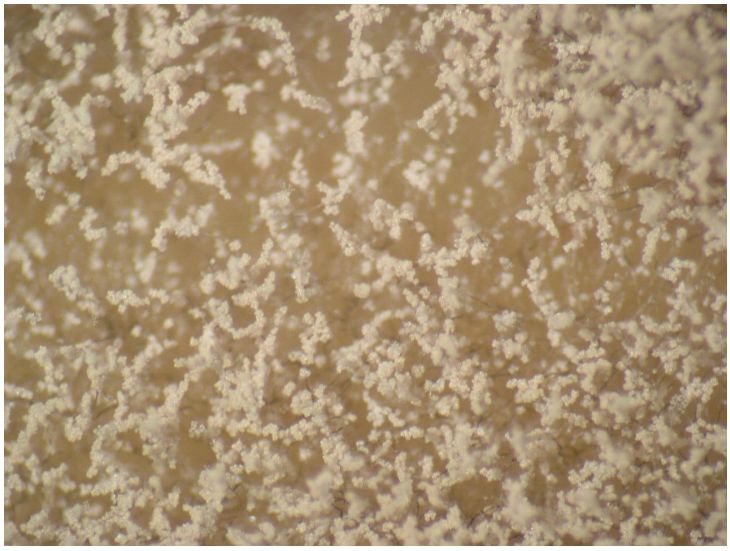

Figure I.2. Typical *B. allii* conidiophores and conidia, 12 days after plating.

2.3. Compare suspect colonies detected in plates of tested subsamples to colonies identified in plates of the PPC sample and PC.

Note: Test results are only valid when all included controls presented in Table I.2 give the expected results. Ensure the reference culture has grown properly onto the semi-selective medium.

Botrytis squamosa can be easily distinguished as it does not produce conidia easily (Steentjes *et al*., 2021) and produces much larger conidia when sporulating (Chilvers and du Toit, 2006).

Compared to *B. cinerea*, *B. allii* produces more abundant and shorter conidiophores (~1 mm long), while *B. cinerea* has dematiaceous and branched conidiophores, frequently 2 mm or more in length (Figure I.3). Furthermore, *Botrytis cinerea* shows a faster growth compared to *B. allii* (Chilvers and du Toit, 2006).

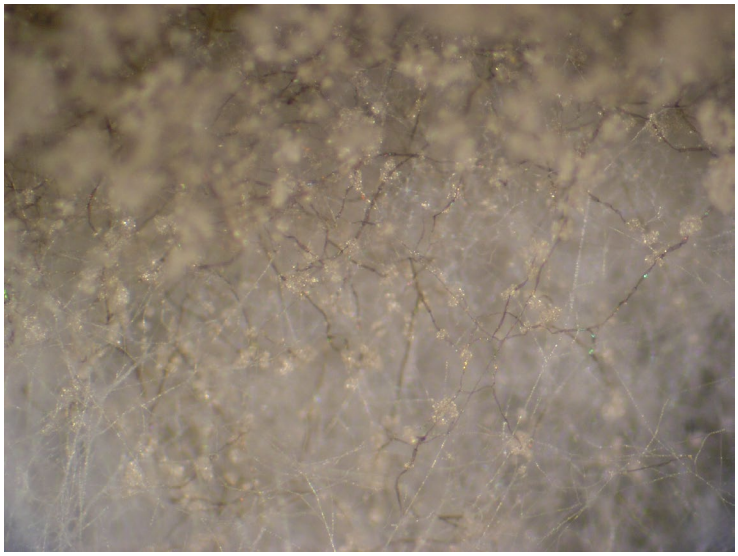

Figure I.3. Typical *B. cinerea* conidiophores and conidia, 12 days after plating.

REFERENCES

Chilvers, M. I. and du Toit, L. J. (2006). Detection and identification of *Botrytis* species associated with neck rot, scape blight, and umbel blight of onion. *Plant Health Progress,* **7(1)**. https://doi.org/10.1094/PHP-2006-1127-01-DG.

Garfinkel, A. R. (2021). The history of *Botrytis* taxonomy, the rise of phylogenetics, and implications for species recognition. *Phytopathology,* **111(3)**, 437-454. https://doi.org/10.1094/PHYTO-06-20-0211-IA.

Hiddink, G., Asma, M., Khosravifar, A., Saito, S., Ueyama, H. and Woudenberg, J. H. C. (2023). Detection of *Botrytis allii* in Onion Seed by Plating Assay. Validation report, International Seed Federation (ISF), Nyon, Switzerland. https://worldseed.org/our-work/seed-health/ishi-method-development-and-validation/

Steentjes, M. B. F., Scholten, O. E. and Kan van, J. A. L. (2021). Peeling the onion: Towards a better understanding of *Botrytis* diseases of onion. *Phytopathology*, **111(3)**, 464-473. https://doi.org/10.1094/PHYTO-06-20-0258-IA