International Rules for Seed Testing 2024

Validated Seed Health Testing Methods

7-016: Detection of *Phomopsis* complex in *Glycine max* (soybean, soya bean) seed

Including changes and editorial corrections adopted at the Ordinary General Meeting 2023 in Verona, Italy

Effective from 1 January 2024

Validation reports

See References. Copies are available by e-mail from the ISTA Secretariat at ista.office@ista.ch.

Please send comments, suggestions or reports of problems relating to this method to the ISTA Seed Health Committee, c/o ISTA Secretariat.

Disclaimer

Whilst ISTA has taken care to ensure the accuracy of the methods and information described in this method description, ISTA shall not be liable for any loss or damage, etc. resulting from the use of this method.

Safety precautions

Ensure you are familiar with hazard data and take appropriate safety precautions, especially during weighing out of ingredients. It is assumed that persons carrying out this test are in a laboratory suitable for carrying out microbiological procedures and familiar with the principles of Good Laboratory Practice, Good Microbiological Practice, and aseptic techniques. Dispose of all waste materials in an appropriate way (e.g. autoclaving, disinfection) and in accordance with local health, environmental and safety regulations.

Note on the use of the translations

The electronic version of the International Rules for Seed Testing includes the English, French, German and Spanish versions. If there are any questions on interpretation of the ISTA Rules, the English version is the definitive version.

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7-016: Detection of *Phomopsis* complex in *Glycine max* (soybean, soya bean) seed

Host: *Glycine max* (L.) Merr.
Pathogen(s): *Phomopsis longicolla* Hobbs, *Diaporthe phaseolorum* var. *sojae* (Lehm.) Wehm. (Imperfect state *P. phaseoli* (Desm.) Sacc., syn. *P. sojae* Lehmann); *Diaporthe phaseolorum* (Cke. & Ell.) Sacc. f. sp. *caulivora* (DPC), syn. *D. phaseolorum* var. *caulivora* Athow & Caldwell

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Revision history

Version 1.0, 2003-01-01
Version 1.1, 2010-01-01: Editorial change to APDA preparation: correction of autoclaving pressure
Version 1.2, 2013-01-01: Definition of sample size
Version 1.3, 2014-01-01: Addition of positive control; common name of host added
Version 1.4, 2017-01-01: Reporting results revised
Version 1.5, 2021-01-01: Sample size added; Methods revised; Media and solutions revised
Version 1.6, 2022-01-01: Methods (pretreatment) revised
Version 1.7, 2024-01-01: Sample size and Figures revised

Background

This method for the detection of *Phomopsis* seed decay in soya beans uses direct plating on acidified potato dextrose agar (APDA) with visual identification of subsequent fungal growth. It replaces the original working sheet published in the *ISTA Handbook of Seed Health Testing* in November 1964 as S.3. No. 27 (Wallen, 1964) and the second and third editions prepared by Kulik (1981, 1989). This version of the assay contains several changes to the previous method descriptions following the results of a study comparing two methods using four replicates of four seedlots in six independent laboratories in Argentina, Australia, Canada, France and the USA (Walcott, *et al.*, 2003). The changes include: the use of APDA instead of malt agar or amended water agar; surface disinfestation with 1 % NaOCl for 30 s instead of 2 % for 10 min, followed by a 30 s rinse in sterile water; plates are incubated in the dark at 25 °C for 7 d before evaluation.

Treated seed

Seed treatments may affect the performance of this test. It should only be performed on untreated seed.

Sample size

The sample size (total number of seeds to be tested) depends on intended use, the maximum acceptable infection level and the analytical sensitivity of the method. The minimum sample size should be 400 seeds.

Materials

Reference material: reference cultures or other appropriate material
Acidified PDA plates: 90 mm Petri dishes (one per ten seeds)
Incubator: operating at 25 ±2 °C
Sterile paper towels: for blotting seed dry
Sterile distilled/deionised water
1 % NaOCl
Forceps (tweezers)
Timer
pH meter

Sample preparation

It is vital to exclude any possibility of cross-contamination between seed samples. This can be achieved by swabbing/spraying equipment and gloved hands with 70 % ethanol.

Methods

Critical control points are indicated by CCP.

1. Pretreatment: Gently rinse seeds in a solution of sodium hypochlorite (NaOCl) (1 % available chlorine) for 30 to 60 s, then rinse for 30 s in sterile water. Blot the seeds dry on sterile paper towel.

2.1 Plating: Using aseptic technique, evenly space seeds on the surface of the acidified PDA plates (10 seeds per plate).

2.2 Positive control (reference material): Aseptically place seeds in an appropriate number of plates to obtain the reference culture, or plate a reference culture on media. The number of plates required will depend on the level of contamination of the positive-control seed lot.
3. Incubation: Incubate plates for 7 d at 25 ±2 °C in the dark.
4. Control: Subculture a reference culture onto a plate of acidified PDA and incubate with the test plates. Alternatively, a sample of seed known to be infested may be surface sterilised, plated on acidified PDA and incubated under the same conditions as the test samples.
5. Examination: Examine the plates after 3 and 7 d incubation using a dissecting microscope or hand lens at ×5 to ×10 magnification. Compare with control and record the number of infected seeds on each plate. Infected seeds are usually overgrown by a dense, white, floccose mycelium which often contains black, globose fruiting bodies (pycnidia) and/or black stromatic bodies (Fig. 1). Pycnidia are usually short-beaked, but the length of the beak may vary considerably. Spore exudate usually accumulates as a straw-yellow droplet at the tip of the beak, but occasionally in the form of a cirrhus. At high magnification two kinds of conidia may be seen. Alpha conidia are hyaline, non-septate, rounded at both ends (although some may have one end more drawn out than the other), straight to ellipsoidal (Fig. 2, left), 4.5–11.0 × 1.5–3.5 µm. Beta conidia are long and needle-shaped, sometimes curved or hooked, hyaline, 7.5–35.0 × 0.8–1.8 µm (Fig. 2, right). The majority of pycnidia produce only alpha conidia, a few produce alpha conidia with some beta conidia, rarely beta conidia are produced alone (see also: McGee, 1991)(CCP).
The long-beaked, black, globose perithecia of *Diaporthe phaseolorum*, the perfect state of *Phomopsis phaseoli*, are not usually encountered on plated seeds from lots produced in areas free of stem canker. Ascospores are hyaline, 1-septate but may appear to be 3-septate, with 1–2 drops in each half of the spore, which is either ellipsoidal with the ends slightly pointed and slightly to noticeably constricted at the septum, 4.4–6.3 × 1.6–2.1 µm to 8.6–11.8 × 3.0–3.9 µm, or sometimes broadly fusiform and then often curved with no constriction and obtuse at each end, 7.5–10.5 × 2.0–2.7 µm.

General methods

Checking tolerances: Tolerances provide a means of assessing whether or not the variation in results within or between tests are sufficiently wide as to raise doubts about the accuracy of the results. Suitable tolerances, which can be applied to most direct seed health tests, can be found in Table 5B Part 1 of Chapter 5 of the ISTA Rules, or Table G1 in Miles (1963).

Reporting results: The result of a seed health test should indicate the scientific name of the pathogen detected and the test method used. When reported on an ISTA Certificate, results are entered under 'Other Determinations'.
The report must indicate the number of seeds tested.
In the case of a negative result (pathogen not detected), the results must be reported as 'not detected'.
In the case of a positive result, the report must indicate the percentage of infected seeds.

Quality assurance

Specific training

This test should only be performed by persons who have been trained in fungal identification or under their direct supervision.

Critical control points (CCP)

Care should be taken not to mistake the acervuli of *Colletotrichum dematium* (Pers. ex Fr.) Grove var. *truncata* (Schw.) Arx, or the perithecia of *Glomerella glycines* (Hori) Lehman & Wolf, for the perithecia of *D. phaseolorum* or the pycnidia of *P. phaseoli*. These fruiting bodies are also dark but they are usually not in a dense, white, floccose mycelium as is the case with those of *D. phaseolorum* and *P. phaseoli*. In addition, there are no dark setae (sterile hairs) associated with the fruiting bodies of *Phomopsis* spp. (Step 5).

Media and solutions

Acidified potato dextrose agar (APDA)

(McGee, 1986, 1991)

Potato dextrose agar (BD Difco™): 39 g
Distilled/deionised water: 1000 ml
Lactic acid: variable

Preparation

1. Weigh out all ingredients into a suitable container.
2. Add 1000 ml (or 500 ml) of distilled/deionised water.
3. Steam to dissolve.
4. Autoclave at 121 °C, 15 psi for 15 min.

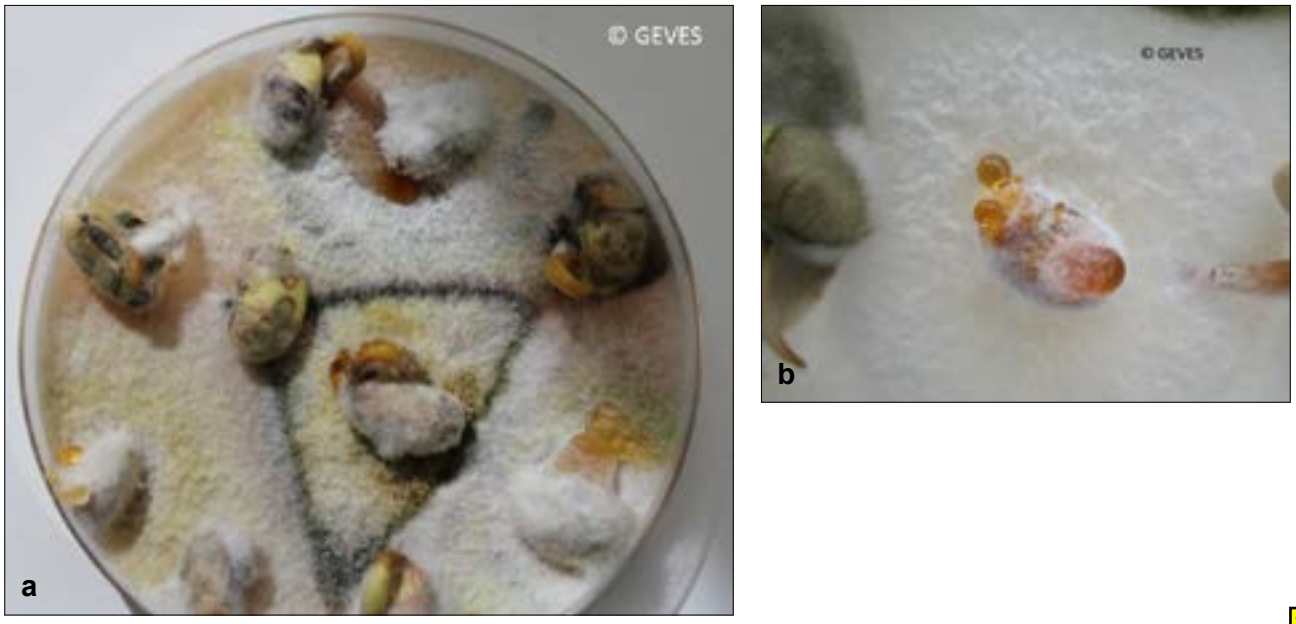

Figure 1. a Colonies of *Phomopsis* complex on acidified potato dextrose agar. **b** Exudate on seed.

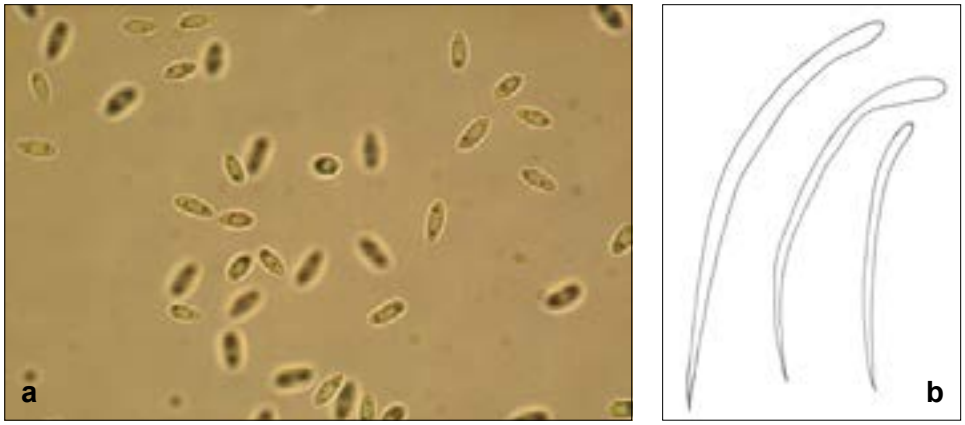

Figure 2. a Micrograph of readily produced alpha conidia of *Phomopsis longicolla,* ×400.
b Drawing of long needle-like beta conidia of *Phomopsis longicolla,* ×1000.

5. Cool to approx. 50 °C.
6. Adjust pH to 4.5 with 85 % lactic acid. It is usually assumed that the lactic acid is sterile, filter sterilise if contamination is suspected.
7. Pour plates (22 ml per 90 mm Petri dish) and allow to solidify at room temperature (ca. 22 °C) for 24 h before use.

Storage

Prepared plates may be stored at room temperature or at 4 °C for up to three weeks.

Sodium hypochlorite solution

Sodium hypochlorite for pretreatment of seed can be prepared from commercial bleach diluted to 1 % available chlorine. The concentration of chlorine in commercial bleach varies considerably and declines with storage. Use the formula:

$$V_{stock} = V_{final} \times C_{final} / C_{stock}$$

(where V = volume and C = % available chlorine) to calculate the volume of commercial bleach stock solution required to prepare sodium hypochlorite solutions for use in seed pretreatment.

To prepare a 1 l solution of sodium hypochlorite containing 1 % chlorine from a stock of commercial bleach containing 12 % available chlorine:

$$V_{stock} = 1 \times 1/12 = 0.083$$

Thus add 83 ml of the 12 % stock to 917 ml water.

The percentage of active chlorine decreases rapidly in solution so, NaClO 1 % solution must be stored in the dark and used within 3 days of preparation. It is possible to check chlorine concentration with chlorine strip tests.

References

Kulik, M. M. (1981) *ISTA Handbook of Seed Health Testing*, *Working sheet No. 27 (2nd ed.)*: *Soybean pod and stem blight*, *stem canker*, Phomopsis *seed decay*. International Seed Testing Association, Zurich, Switzerland

Kulik, M. M. (1989) *ISTA Handbook of Seed Health Testing*, *Working sheet No*. 27 (3*rd ed*): *Soybean Stem Canker*. International Seed Testing Association, Zurich, Switzerland.

McGee, D. C. (1986) Prediction of *Phomopsis* seed decay by measuring soybean pod infection. *Plant Disease*, **70**, 329–333

McGee, D. C. (1991) *Phomopsis* Seed Decay. In: McGee, D. C., *Soybean diseases*: *A Reference Source for Seed Technologists*, pp 13 - 20. APS Press, St. Paul, Minnesota, USA.

Miles, S. R. (1963). Handbook of tolerances and of measures of precision for seed testing. *Proceedings of the International Seed Testing Association*, **28** (3), 525–686.

Roberts, S. J., Phelps, K., Taylor, J. D. & Ridout, M. S. (1993) Design and interpretation of seed health assays. In: Sheppard, J. W., (Ed.) *Proceedings of the First ISTA Plant Disease Committee Symposium on Seed Health Testing*, *Ottawa*, *Canada*. pp. 115–125. Agriculture Canada, Ottawa, Canada.

Wallen, V. R. (1964) *ISTA Handbook of Seed Health Testing*, *Working sheet No*. 27: *Soybean Stem Canker*. International Seed Testing Association, Zurich, Switzerland.

Validation references

ISTA (2003). Comparative evaluation of the Botran-amended blotter and acidified potato dextrose agar assays for the detection of *Phomopsis* seed decay in soybean seed. *Method Validation Reports*. International Seed Testing Association, Bassersdorf, Switzerland.