International Rules for Seed Testing
Annexe to Chapter 7: Seed Health Testing Methods

7-016: Acidified PDA method for the detection of *Phomopsis* complex on *Glycine max*

Published by: International Seed Testing Association (ISTA), Bassersdorf, Switzerland
2003

Crop: *Glycine max* (Soya bean)

Pathogens: *Phomopsis longicolla*, *Diaporthe phaseolorum* var. *sojae* (conidial state: *Phomopsis phaseoli,* syn. *P. sojae*), *D. phaseolorum* f. sp. *caulivorum, D. phaseolorum* var. *caulivora.*

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Sponsored by: International Seed Health Initiative - Field Crops

Revision History: Version 1.0, 01 January 2003.

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.

Validation Studies

Walcott *et al.* (2003).
Copies are available: by e-mail from ista.office@ista.ch; by mail from the ISTA Secretariat

Please send comments, suggestions or reports of problems relating to this method to the leader of the ISTA-PDC Mycology Working Group, c/o ISTA Secretariat

Safety Precautions

Ensure you are familiar with hazard data and take appropriate safety precautions, especially during preparation of media, autoclaving, and weighing out of ingredients. It is assumed that this procedure is being carried out in a microbiological laboratory by persons familiar with the principles of Good Laboratory Practice, Good Microbiological Practice, and aseptic technique. Dispose of all waste materials in an appropriate way (e.g. autoclave, disinfect) and in accordance with local health, environmental and safety regulations.

Treated Seed

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

Materials

Reference material	- The use of reference cultures or other appropriate material is recommended.
Acidified PDA plates	- 9.0 cm Petri dishes (one per ten seeds).
Incubator	- operating at 25 ± 2°C.
Sterile paper towels	- For blotting seed dry.

Sterile distilled/de-ionised water

1% NaOCl

Forceps (tweezers)

Timer

pH meter

Sample Preparation

1. 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.

2. The test is carried out on a working sample as described in Section 7.4.1 of the International Rules for Seed Testing.

Method

[Critical control points are indicated by **CCP**]

1. *Pre-treatment*

   Gently rinse seeds in NaOCl solution (1% available chlorine) for 30 s, then rinse for 30 s in sterile water. Blot the seed dry on sterile paper towels.

2. *Plating*

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

3. *Incubation*

   Incubate plates for 7 d at 25 ± 2°C in the dark.

4. *Control*

   Sub-culture 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 x5 to x10 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 x 1.5-3.5 µm. Beta conidia are long and needle-shaped, sometimes curved or hooked, hyaline, 7.5-35.0 x 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 x 1.6-2.1 µm to 8.6-11.8 x 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 x 2.0-2.7 µm.

General Methods (common to many test procedures)

1. *Checking Tolerances*

   Tolerances provide a means of assessing whether or not the variation in result within or between tests is sufficiently wide as to raise doubts about the accuracy of the results. A tolerance table, which can be applied to most direct seed health tests, can be found in Table 5.1 of Annexe 16 of the *ISTA Rules* or in Table G1 of the *Handbook of Tolerances and Measures of Precision for Seed Testing* (Miles, 1963).

2. *Reporting Results*

   The result of a seed health test should indicate the scientific name of the pathogen, and the test method used. When reported on an ISTA Certificate, results are entered under *Other Determinations.*

   In the case of a negative result (pathogen not detected), the results should be reported in terms of the tolerance standard (e.g. infection level less than 1% with 95% probability). The tolerance standard depends on the total number of seeds tested, n, and is approximately 3/n (P=0.95) (see Roberts *et al.,* 1993).

   In the case of a positive result the report should indicate 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

[Identified by **CCP** in the method]

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).

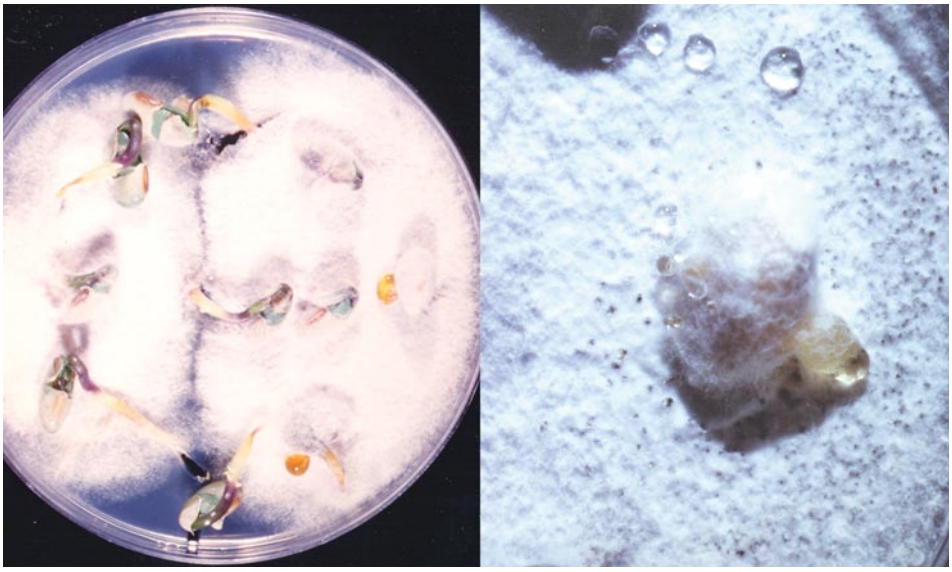

Fig. 1. Infected seeds overgrown by a dense, white, floccose mycelium (left) which often contains black, globose fruiting bodies (pycnidia) and/or black stromatic bodies (right).

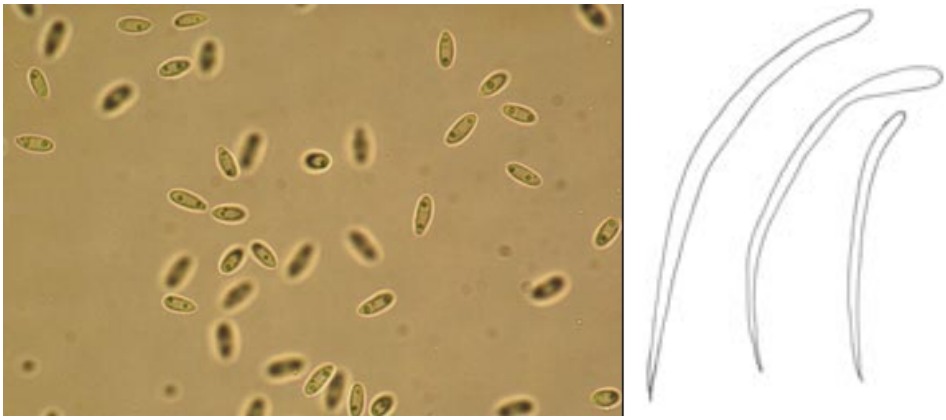

Fig. 2. Micrograph of readily produced alpha conidia *of Phomopsis longicolla,* x400 (left) and drawing of long needle-like beta conidia of *Phomopsis longicolla,* x1000 (right).

Preparation of Acidified Potato Dextrose Agar (APDA)

(McGee, 1986, 1991)

Compound	g/l	g/500 ml
Potato Dextrose Agar (Difco)	39	19.5
Distilled/de-ionised water	1000 ml	500 ml
Lactic acid	variable	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, 115 psi for 15 min.
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 9.0 cm 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.

Preparation of Sodium Hypochlorite Solution

Sodium hypochlorite for pre-treatment of seed can be prepared from fresh 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 pre-treatment.

Example

To prepare 1000 ml of a solution of sodium hypochlorite containing 1% chlorine from a stock of commercial bleach containing 12% available chlorine:

$$V_{stock} = 1000 \times 1/12 = 83$$

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

Alternatively, sodium hypochlorite solutions can be prepared by using sodium dichroroisocyanurate tablets (e.g. Presept, Johnson and Johnson Medical Products) according to the manufacturer's instructions.

References

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