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

7-005: Detection of *Ascochyta pisi* on *Pisum sativum* (Pea)

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Crop: *Pisum sativum* (Pea)

Pathogen: *Ascochyta pisi* Lib.

Prepared by: ISTA-PDC Method Validation Sub-committee

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Revised 19.11.2001 J. Sheppard, V. Cockerell
Reprinted 2003
Version 1.1 2008-01-01
"Treated seed" revised; "Reporting results" revised

Submitted by: ISTA-PDC Method Validation Sub-committee

Background

This method was originally published in the ISTA Handbook of Seed Health Testing in November 1964 as S.3. No. 16 revised 1987 by P.D. Hewett, Official Seed Testing Station for England and Wales, Cambridge, United Kingdom. The method was incorporated into the newly revised Annexe to Chapter 7 in 2002 from the 1999 edition of the ISTA Rules. The method was reviewed by the ISTA-Seed Health Committee in 2006 (Cockerell & Koenraadt, 2007) with the recommendation to accept for a further five years.

Studied in International Comparative Testing: 1960, 1966, 1967, 1968-71, 1973-1975.

Summary of Validation Studies

Agar tests detect approximately 50% more infection by *Ascochyta* spp. than blotter tests (Anselme and Champion, 1962; Tempe, 1968).

International comparative tests (Hewett, 1987) showed that of over 350 results obtained by experienced stations, 95% fell within tolerance limits used for germination tests

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 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 safety regulations.

Treated Seed

This method has not been validated for the determination of *Ascochyta pisi* on treated seed. Seed treatments may affect the performance of the method.
(Definition of treatment: any process, physical, biological or chemical, to which a seed lot is subjected, including seed coatings. See 7.2.3)

Materials

Reference Material	-	The use of reference cultures or other appropriate material is recommended when ever possible.
Media	-	Malt Agar or Potato Dextrose Agar.
Sodium hypochlorite solution	-	(1% available chorine) for seed disinfection.
Petri dishes	-	When sowing density is given by a number of seeds per Petri dish, a diameter of 90 mm is assumed.
Incubator	-	Capable of operating in the range 20 ± 2 ºC.

Sample Preparation

The test is carried out on a working sample of 400 seeds as described in Section 7.4.1 of the ISTA Rules as appropriate.

Method

1. *Pretreatment*

   10 minutes in a 1% (available chlorine) sodium hypochlorite solution followed by draining.
2. *Agar Method*

   Malt or potato dextrose agar. Place 10 seeds on the agar surface in each Petri dish.
3. *Incubation*

   7 days at 20 ºC in darkness.
4. *Examination*

   After 7 days examine each seed by naked eye for abundant white mycelium which often covers infected seeds (Fig. 1). Doubtful colonies may be confirmed by the presence of wavy hyphae at the edge of the colony when examined at ×25 magnification. Colony diameter typically 20-30 mm, occasionally smaller or incompletely surrounding the seed. Reverse of colonies medium to dark orange-brown centrally, opaque and even, becoming lighter in colour towards the edge of the colony. Gelatinous-looking orange-brown pycnidia often present (although only sometimes clearly visible), particularly

where seed touches agar. Under STM at ×20–25 magnification, using both transmitted and incident light, hyphae are curled, often several running together, typically with moisture drops (although these evaporate easily) (Fig. 2). Very limited growths from some seeds may only be seen if dishes tilted to get lighting at best angle, or under STM examination or after extended incubation. Pycnidia are up to 250 µm in diameter. Spores, hyaline, cylindric, of slightly curved with rounded ends, 1-septate, slightly constricted at septum, mostly 12 × 4.5 µm (Punithalingam, and Holliday, 1972).

Notes:

Samples frequently bear *A. pinodes* (*Mycosphaerella pinodes* (Berk. & Blox.) Westerg.) and, occasionally, *A. pinodella* (*Phoma medicaginis* Malbr. & Roum. var. *pinodella* (Jones) Boerema). These pathogens differ markedly from *A. pisi* in their colony and mycelial characters and in spore morphology (see CMI descriptions Nos. 340 and 518, respectively).

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. Suitable tolerances, which can be applied to most direct seed health tests, can be found in Tables 5B of Chapter 5 of the ISTA Rules, or in the *Handbook of Tolerances and Measures of Precision for Seed Testing* by S.R. Miles (*Proceedings of the International Seed Testing Association* 28 (1963) No 3, p 644).

2. 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 International Seed Analysis Certificate*, results are entered under Other Determinations.

Preparation of Media and Solutions

1. 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. 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:

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

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

2. Malt Agar

Compound	g/L
Malt Agar[1]	According to manufacturer's instructions
Distilled/de-ionized water	1000 mL

[1] **CCP** *Malt agar constituents should be equivalent to those of the following manufacturers Difco, USA or Oxoid, UK*

Preparation
1. Weigh out ingredients into a suitable autoclavable container.
2. Add 1000 mL of distilled/de-ionized water.
3. Dissolve powdered Malt Agar in distilled/de-ionized water by stirring.
4. Autoclave at 15 p.s.i. and 121 °C for 15 min.
5. Allow agar to cool to approx. 50 °C.
6. Pour 15–22 mL of molten agar into 90 mm Petri plates and allow to solidify before use.

Storage
Prepared plates may be stored at 4 ºC for up to 6 weeks.

3. Potato Dextrose Agar

Compound	g/L
Potato Dextrose Agar[1]	According to manufacturer's instructions
Distilled/de-ionized water	1000 mL

[1] **CCP** *Potato Dextrose Agar constituents should be equivalent to those of the following manufacturers Difco, USA or Oxoid, UK*

Preparation
1. Weigh out ingredients into a suitable autoclavable container.
2. Add 1000 (or 500) mL of distilled/de-ionized water.
3. Dissolve powdered PDA in distilled/de-ionized water by stirring.
4. Autoclave at 15 p.s.i. and 121 °C for 15 min.
5. Allow agar to cool to approx. 50 °C.
6. Pour 15–22 mL of molten agar into 90 mm Petri plates and allow to solidify before use.

Storage
Prepared plates may be stored at 4 ºC for up to 6 weeks.

Quality Assurance

Critical Control Points

Where the wording of the original Working Sheet suggests that an action is critical this has been marked with ***CCP***.

References

The following references are extracted from the ISTA Handbook on Seed Health Testing, Working Sheet No. 16, P. D. Hewett, 1987.

Anselme, C. and Champion, R. (1962). L'analyse sanitaire des semences de pois. Proceedings of the International Seed Testing Association, 27, 829-842.

Hewett, P.D. (1979). Pretreatment in seed health testing. 2. Duration of hypochlorite pretreatment in the agar plate test for *Ascochyta* spp. Seed Science and Technology, 7, 83-85

Hewett, P.D. (1987). Detection of seed-borne *Ascochyta pisi* Lib. and test agreement within and between laboratories. Seed Science and Technology, 15, 271-283.

Leach, C.M. (1962). The quantitative and qualitative relationship of ultra violet and visible radiation to the induction of reproduction in *Ascochyta pisi*. Canadian Journal of Botany, 40, 1577-1602.

Punithalingam, E. and Holliday, P. (1972). C.M.I. Descriptions of pathogenic fungi and bacteria No. 334. Commonwealth Mycological Institute, Kew.

Tempe, J. de (1968). The quantitative evaluation of seed-borne pathogenic infection. Proceedings of the International Seed Testing Association 33, 573-581.

Fig. 1. Colonies of *A. pisi*, face (left) and reverse (right), from test on PDA, following hypochlorite pretreatment. Incubation for 7 days at 21ºC in darkness

Fig. 2. Typical appearance of hyphae of *A. pisi*, test conditions as for Fig. 1.