International Rules for Seed Testing 2025

Validated Seed Health Testing Methods

7-033: Detection of *Ascochyta rabiei* in *Cicer arietinum* (chickpea) seed

Including changes and editorial corrections adopted at the Ordinary General Meeting 2024 in Cambridge, United Kingdom

Effective from 1 January 2025

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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7-033: Detection of *Ascochyta rabiei* in *Cicer arietinum* (chickpea) seed

Host: *Cicer arietinum*
Pathogen(s): *Ascochyta rabiei*

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Revision history

Version 1.0, 2025-01-01: New method

Background

Ascochyta rabiei (syn. *Phoma rabiei*), also known by its teleomorph name *Didymella rabiei* (syn. *Mycosphaerella rabiei*), is responsible for *Ascochyta* blight on chickpea seeds. In 2019, the French project 'AsCoLuP' was initiated with the ambition to develop better management and understanding of two diseases on chickpea and lupin. One of the aims of this project was to provide a validated method to detect, identify and test the pathogenicity of *A. rabiei* on chickpea.

Chickpea blight, caused by the fungus *A. rabiei* is one of the most serious diseases of the crop; severe epidemics have been reported worldwide (Nene, 1982; Nene and Reddy, 1987; Collard *et al*., 2001). This pathogen is very aggressive on chickpea crops and can spread quickly in the field once established and when weather conditions are suitable (Pearse, 2005). The crop reaction is dependent on the weather conditions, specific cropping practices and cultivar (Pearse, 2005). The fungus selectively attacks chickpea, then persists in the crop's residue, seeds and weeds.

There is a high rate of seed-to-seedling transmission of *Ascochyta* in chickpea; even a small number of infected seeds can result in significant seedling infection in the field. Reports have indicated that a 0.1 % *Ascochyta*-infected seed lot (one infected seed in 1000), could potentially result in 175 infected seedlings per acre (Pearse, 2005).

The use of *Ascochyta* blight-free seed and seed treatment with effective fungicides reduce the probability of transmitting seed-borne disease to the seedlings (Gan *et al*., 2006). The infected seeds are often symptomless; therefore, a reliable seed health detection method is crucial to avoid plant infection.

Initial crop infection is due to the introduction of either infected seed or from movement of infected plant debris, through wind-borne spores (ascospores), machinery or animals. Spores of the fungus can survive for a short time on skin, clothing and machinery. Subsequent in-crop infection occurs when inoculum is moved higher in the canopy or to surrounding plants by wind or rain splash during wet weather (GRDC, 2009).

This method includes a detection procedure to detect and identify *A. rabiei* and an optional pathogenicity test to confirm the pathogenicity of the isolates (Fig. 1).

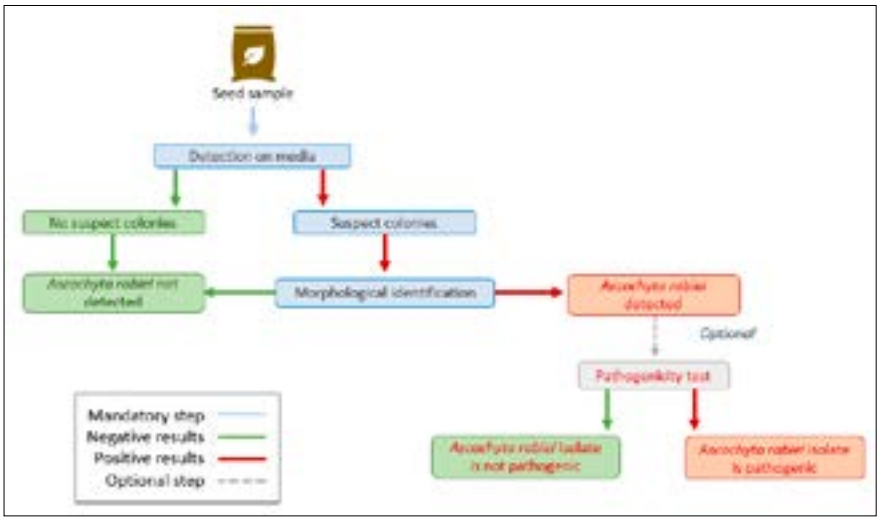

Figure 1. Process flow diagram explaining method assays and decisions taken, depending on intermediate results until final result, for detection and pathogenicity of *Ascochyta rabiei* in *Cicer arietinum* (chickpea) seed.

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. autoclaving, disinfection) and in accordance with local health, environmental and safety regulations.

Treated seed

This method has not been validated for the detection of *Ascochyta rabiei* on treated seed. (Definition of 'treatment': any process, physical, biological or chemical, to which a seed lot is subjected, including seed coatings. See 7.2.3.)

Sample size

The sample size (total number of seeds tested) and subsample size depend on the desired tolerance standard (maximum acceptable number of seeds infested) and detection limit (theoretical minimum number of pathogen propagules per seed which can be detected). The minimum sample size should be 400 seeds.

Materials

Reference material: reference cultures or other appropriate material to use as positive process control
Media: malt agar (MA) or potato dextrose agar (PDA) (for detection); chickpea seed meal dextrose agar (CSMDA) (for pathogenicity test)
Autoclave
Sodium hypochlorite solution: (1 % available chlorine) for seed disinfection
Sterile distilled/deionised water
Sterile blotter paper: for blotting seed dry and for germination of seedlings in pathogenicity test
Plates: 90 mm sterile Petri dishes (one per ten seeds)
Steriliser
Forceps
Incubator: operating at 20 ±2 °C in darkness (for incubation of samples)
Incubator: operating at 20 ±2 °C under near-ultraviolet (NUV) light (for growth of reference material)
Stereomicroscope: ×6.5–50 magnification
Compound microscope: ×100–400 magnification
Chickpea seedlings: susceptible to all races of *Ascochyta* for pathogenicity test (e.g. 'Benito')
Filter
Beakers
Microscope slides
Sterile blades
Malassez cell (haemocytometer)
Sterile pipettes
Plastic bags
Steamed potting soil
Trays, cups and cover
Growth chamber: operating at 20 ±2 °C and 80 % relative humidity (RH)

Methods

Note: All the steps described here are important and should be followed as written. Critical control points are indicated by 'CCP'.

1. Detection method
1.1 Pretreatment: Immerse seeds in a solution of sodium hypochlorite (NaOCl) (1 % available chlorine) for 10 min, then drain, rinse well in sterile water and drain again. Dry the seeds on sterile blotter paper.
1.2 Plating
1.2.1 Aseptically place a maximum of ten seeds onto the agar surface of each MA or PDA plate, evenly spaced around the perimeter.
1.2.2 Positive control (reference material): Aseptically plate a reference culture on media.
1.3 Incubate for 7 d at 20 ±2 °C, in darkness.
1.4 Examination (CCP): After 7 d, examine each seed by naked eye (Fig. 2a, b). *Ascochyta rabiei* is a slow growing fungus and might be located on the seed coat or under the seeds. To allow an accurate record, it is important to move the seeds away to reveal the presence of mycelium and pycnidia. On both media, white- to green-coloured mycelium develops slowly; development of pale brown to dark brown pycnidia could occur on or below the surface. At 7 d, the colony diameter varies between 10–35 mm on both media. Pycnidia usually release an orange mucilaginous mass of pycnidiospores (cirrus). Doubtful colonies may be confirmed by the presence of pycnidia on the seed coat or on the media surface when examined at ×25 magnification using a stereomicroscope; pycnidia are 65–245 µm diameter, ostiole 30–50 µm wide. Further confirmation can be made by examining pycnidiospores present in pycnidia using a compound microscope (×100–1000 magnification); pycnidiospores are hyaline, straight or slightly curved (0–)1 septate, some unicellular, slightly (or not)

constricted at the septum, rounded at each end, mostly 7–16 × 3–5 µm (Figs 3 and 4).

2. Pathogenicity test (optional)

2.1 Inoculum multiplication: Plate the isolates on CSMDA media (Fig. 5); incubate at 20 °C under NUV for at least 7 d.

2.2 Pretreatment: Immerse seeds of a chickpea cultivar know to be highly susceptible to *A. rabiei* (e.g. 'Benito') in a solution of NaOCl (1 % available chlorine) for 10 min, then drain, rinse well in sterile water and drain again. Dry the seeds on sterile blotter paper.

2.3 Germination: Place the disinfected seeds on wet blotter paper. Roll the paper with the seeds and place it in a plastic bag. Incubate the closed bags at 20 °C in darkness for 2–3 d to allow seed germination. Ensure to germinate enough seeds for all the suspect colonies to be tested.

2.4 Inoculum preparation: Pour 2 ml of sterile water onto the CSMDA plates containing the grown isolate and scrape the surface with a microscope slide. Filter the inoculum and estimate the concentration using a Malassez cell. Dilute, if necessary, in sterile water to obtain a concentration from 1.10^4 to 1.10^5 conidia/ml. Make sure to have a sufficient volume of inoculum to soak the seeds.

2.5 Inoculation: Cut the root tips (approximately 1 cm) of 2–3-day-old germinated chickpea seeds (Fig. 6) and soak three seeds in each inoculum for 10 min (Fig. 7). Sow the seeds in potting soil.

2.6 Negative control: Repeat with the soaking of three seeds in sterile water.

2.7 Incubation: 10 d at 20 ±2 °C; RH = 100% (using a cover), 12 h light, 12 h dark.

2.8 Examination: After 10 d, take the plantlets out, check the presence of symptoms and compare to the positive (Fig. 8) and negative controls (Fig. 9) (CCP). Symptoms caused by *A. rabiei* are necrosis on the stem (always) (Fig. 10), seed blackened (most of the time), leaf wilting (often) and plantlet rotting (sometimes).

2.9 Record the suspect colonies as pathogenic if at least one of the seedlings presents black necroses on the stem.

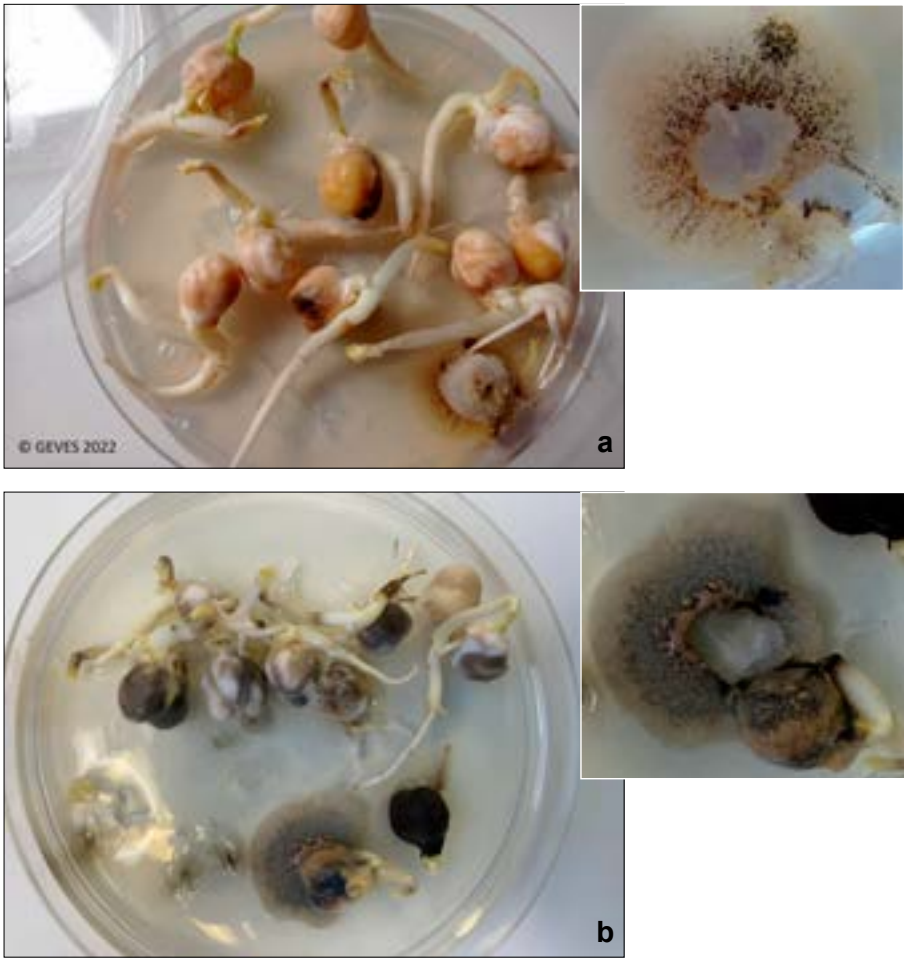

Figure 2. Colonies of *Ascochyta rabiei* after 7 days' incubation in darkness: **a** malt agar; **b** potato dextrose agar.

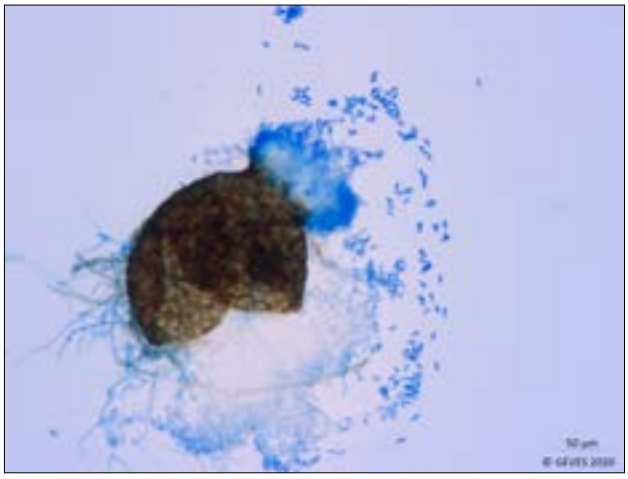

Figure 3. Pycnidia and pycnidiospores of *Ascochyta rabiei* (×200).

Figure 4. Pycnidiospores of *Ascochyta rabiei* (×400).

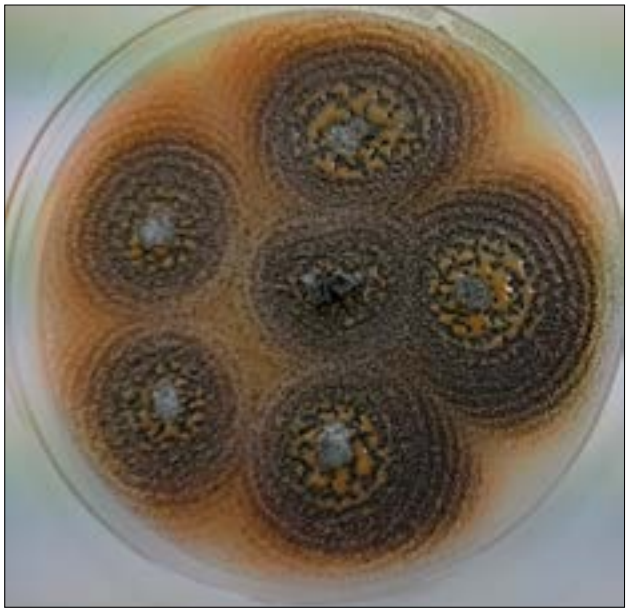

Figure 5. Colonies of *Ascochyta rabiei* on chickpea seed meal dextrose agar.

Figure 6. Cutting of root tips of 2-day-old germinated seeds.

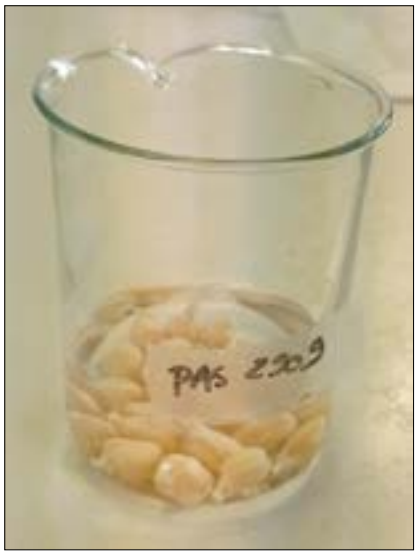

Figure 7. Inoculation by soaking cut germinated seeds in a 1.105 conidia/ml *Ascochyta rabiei* suspension.

Figure 8. *Cicer arietinum* seedlings 10 days after inoculation with pathogenic isolate of *Ascochyta rabiei*.

Figure 9. *Cicer arietinum* seedlings 10 days after inoculation with negative control.

Figure 10. Example of stem necrosis due to *Ascochyta rabiei* on a *Cicer arietinum* seedling.

General methods

Checking tolerances: Tolerances provide a means of assessing whether 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 Table 5B Part 1 of Chapter 5 of the ISTA Rules or in 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.

For the detection method:

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 'detected' and give the percentage of infected seeds.

For the pathogenicity test:

In the case of a negative result (absence of symptoms on all seedlings), the results must be reported as 'not pathogenic'.

In the case of a positive result (presence of symptoms on at least one of the seedlings), the results must be reported as 'pathogenic'.

Quality assurance

Specific training

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

Critical control points (CCP)

When the wording of the method suggests that an action is critical this has been marked with 'CCP'.

- The examinations should be carried out by trained technical staff, as morphological identification of Ascochyta rabiei and its symptoms require experience (Steps 1.4 and 2.8)
- The plantlets of the positive control seed sample should give typical symptoms (at least black necrosis on the stem) and the plantlets of the negative control seed sample should give no symptoms (at least absence of black necrosis on the stem) (Step 2.8).

Media and solutions

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.

Malt agar + streptomycin

Agar: 20 g
Malt: 10 g
Distilled/deionised water: 1000 ml
Streptomycin sulphate*: 50–130 mg

*added after autoclaving

Use 50–130 mg of streptomycin sulphate, depending on the level of saprophytic bacterial contamination commonly encountered. Streptomycin sulphate can be dissolved in distilled/deionised water. Filter sterilisation is required.

Preparation

1. Weigh out ingredients into a suitable autoclavable container.
2. Add 100 ml distilled/deionised water.
3. Dissolve the ingredients completely in water by stirring.
4. Autoclave at 121 °C and 15 psi for 15 min.
5. Allow the agar to cool to approximately 50 °C and add streptomycin sulphate dissolved in sterile distilled/deionised water.
6. Pour 18–20 ml of MA into 90 mm Petri dishes and allow to solidify before use.

Storage

Prepared plates may be stored at 4 °C for up to 6 weeks.

Potato dextrose agar + streptomycin

Potato dextrose agar: according to manufacturer's instructions
Distilled/deionised water: 1000 ml
Streptomycin sulphate*: 50–130 mg

*added after autoclaving

Use 50–130 mg of streptomycin sulphate, depending on the level of saprophytic bacterial contamination commonly encountered.

Preparation

1. Weigh out ingredients into a suitable autoclavable container.
2. Add 1000 ml of distilled/deionised water.
3. Dissolve the ingredients completely in water by stirring.
4. Autoclave at 121 °C and 15 psi for 15 min.
6. Allow the agar to cool to approximately 50 °C and add streptomycin sulphate dissolved in sterile distilled/deionised water.
7. Pour 18–20 ml of PDA into 90 mm Petri dishes and allow to solidify before use.

Storage

Prepared plates may be stored at 4 °C for up to 6 weeks.

Chickpea seed meal dextrose agar

Chickpea seed meal: 40 g
D-glucose: 20 g
Agar: 14 g
Distilled/deionised water: 1000 ml

Preparation

1. Weigh out ingredients into a suitable autoclavable container.
2. Add 1000 ml distilled/deionised water.
3. Dissolve the ingredients completely in water by stirring.
4. Autoclave at 121 °C and 15 psi for 15 min.
5. Allow the agar to cool to approximately 50 °C.
6. Pour 18–20 ml of CSMDA into 90 mm Petri dishes and allow to solidify before use.

Storage

Prepared plates may be stored at 4 °C for up to 6 weeks.

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