International Rules for Seed Testing 2023

Validated Seed Health Testing Methods

7-007: Detection of *Alternaria linicola*, *Botrytis cinerea* and *Colletotrichum lini* in *Linum usitatissimum* (flax, linseed) seed

Including changes and editorial corrections adopted at the Ordinary General Meeting 2022, Cairo, Egypt

Effective from 1 January 2023

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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7-007: Detection of *Alternaria linicola*, *Botrytis cinerea* and *Colletotrichum lini* in *Linum usitatissimum* (flax, linseed) seed

Host: *Linum usitatissimum* L.

Pathogen(s): *Alternaria linicola* J.W.Groves & Skolko; *Botrytis cinerea* Pers. ex Pers. (Perfect state *Botryotinia fuckeliana* (de Bary) Whetzel, syn. *Sclerotinia fuckeliana* (de Bary) Fuckel.); *Colletotrichum lini* (Westerd.) Tochinai, syn. *C. linicola* Pethybr. & Laff.

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Revision history

Version 2.0, 2014-01-01: Replacement of methods 7-007, 7-017 and 7-018
Version 2.1, 2016-01-01: Corrections to text
Version 2.2, 2017-01-01: Reporting results revised
Version 2.3, 2021-01-01: Sample size and Methods revised

Background

Three ISTA methods (7-007, 7-017 and 7-018) were used to detect the three main pathogens of flax seeds, *Botrytis cinerea*, *Alternaria linicola*, *Colletotrichum lini*. The Seed Health Committee of ISTA decided to amalgamate these three methods in a simple one to detect the three pathogens. These three methods were compared and conditions which varied between these methods and also with the other ISTA existing ones were identified. A pretest was carried out in GEVES to compare the concentration of streptomycin, temperature, light and medium on four replicates of 100 seeds. All conditions tested allowed the detection of the three pathogens, and addition of streptomycin at 50 mg/l in the media allowed to avoid the development of bacteria and at the same time did not affect the detection of the three pathogens. A peer validation between the three participating laboratories was then carried out by comparing the five proposed conditions. Based on these results, a new method was proposed to detect the three pathogens of *Linum* with only one method. In this method, two media can be used: potato dextrose agar or malt agar with streptomycin, seeds are incubated at 20 °C, in darkness for 9 days and then under 12 h NUV/12 h dark to enhance sporulation if problem for pathogen identification occurs. The validation studies showed that this method allowed detection of *Alternaria linicola*, *Botrytis cinerea,* and *Colletotrichum lini* at a threshold of 1 % with 100 % specificity and a sensibility of 73, 77 and 100 % for *Botrytis cinerea*, *Colletotrichum lini* and *Alternaria linicola* respectively. The comparative test has been organised by International Seed Testing Association Seed Health Committee.

Treated seed

This method has not been validated for the determination of *Alternaria linicola, Botrytis cinerea* and *Colletotrichum lini* 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.).

Sample size

The sample (total number of seeds tested) size to be tested depends on the desired tolerance standard (maximum acceptable percentage 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
PDA or MA plates with streptomycin sulphate: 90 mm Petri dishes (one per 10 seeds)
Incubator: capable of operating at 20 ±2 °C, equipped with timer-controlled near-ultraviolet light (NUV, peak at 360 nm)

Methods

1. Pretreatment: None.
2. Plating: Aseptically place a maximum of 10 seeds per plate, evenly spaced, onto the agar surface of each PDA or MA plate.
3. Incubation: Incubate plates for 9 days at 20 °C in the dark. Alternating 12 h periods of darkness and NUV light can also be used as an option to complete darkness.
4. Reference material: Subculture a reference culture to a PDA or MA plate at the same time the seeds are plated and incubate with the test plates.
5. Examination: After 9 days of incubation, examine plates for *Alternaria linicola*, *Botrytis cinerea* and *Colletotrichum lini*. Record the number of infected seeds in each plate, for each pathogen.
6. Prolongation of incubation: Only for use without NUV light. If no sporulation is observed at 9 days, extend incubation at 20 °C with alternating 12 h periods of darkness and NUV to obtain spores until 14 days after plating. Examine plates for *Alternaria linicola, Botrytis cinerea and Colletotrichum lini*.
7. Record: Record the number of infected seeds in each plate, for each pathogen.
8. Identification criteria

8.1 *Alternaria linicola*: Examine plates for dense olive-grey colonies, 1.5–3 cm diameter. Some colonies of saprophytic *Alternaria* spp. can resemble those of *A. linicola* but the conidia of *A. linicola* are diagnostic (Fig. 1). Colonies should therefore be examined under ×50–100 magnification. Conidiophores are simple, occurring singly or in bundles, unbranched, erect, often geniculate, with 1–2 or more scars, pale olive-brown and septate. Conidia form singly, are smooth walled, olive-brown, elongated conical to ellipsoid or obclavate, gradually tapering towards the beak. The beak is long, occasionally branched, muriform 4–16 µm with transverse septa and occasionally 1–4 longitudinal septa, sometimes slightly constricted at the septa, 16–230 × 3–4.5 µm (Fig. 2) (Corlett & Corlett 1999; David 1991; Malone & Muskett 1997). The size of conidia (body and beak) is given as an indication but should not be a compulsory identification criterion. Size of conidia depends on the growth media, isolate, growth conditions: depending on references they have been described as (13–) 15–18(–22.5) × (3–)3.5–4(–4.5) µm (Damm *et al*., 2014) or 20–130 × (7–)17–24(–30) µm (anonymous, 1991). Short red streaks and water soaked areas may be visible on the hypocotyls and cotyledons of some infected seedlings (Fig. 3).

8.2 *Botrytis cinerea*: Examine for roots showing a soft rot and covered by abundant grey mycelium (Fig. 4) or just mycelium very flat, diffuse and not aerial, possibility of sclerotia producing (Fig. 5). Colonies on agar measure up to 5 cm in diameter after 5 days. Identification can be checked by high-power microscope (magnification ×200). Mycelium of tape-like hyphae producing bunches of branching conidiophores with ovoid-hyaline one-celled conidia 8–11 × 6–19 µm (Fig. 6). When analysts are familiar with the fungus, naked eye examination is sufficient for identification (Muskett & Malone 1941; Tempe 1963; Malone & Muskett 1997; Ellis & Waller 1974).

8.3 *Colletotrichum lini*: Easily recognised by visual examination. Examine the plates for shell pink to salmon coloured colonies (Fig. 7). Colonies of *C. lini* are a fine woolly-grey at the centre to salmon pink at the outer edge. Dark globose fruiting bodies (acervuli) may be scattered throughout the agar adjacent to the seed (Fig. 8). Characteristic long, black tapering hairs or setae 2–5 septate, 60–120 × 2–4 µm arise from the base of each acervulus. Bright orange conidial masses appear on the seed and agar adjacent to the seed. Conidia are hyaline; oblong to dumbell shaped, one celled, straight ends 9–15 × 3–4 µm (Malone & Muskett 1997; Kulshrestha *et al.*, 1976). Record the number of infected seeds in each plate.

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 the direct supervision of someone who has.

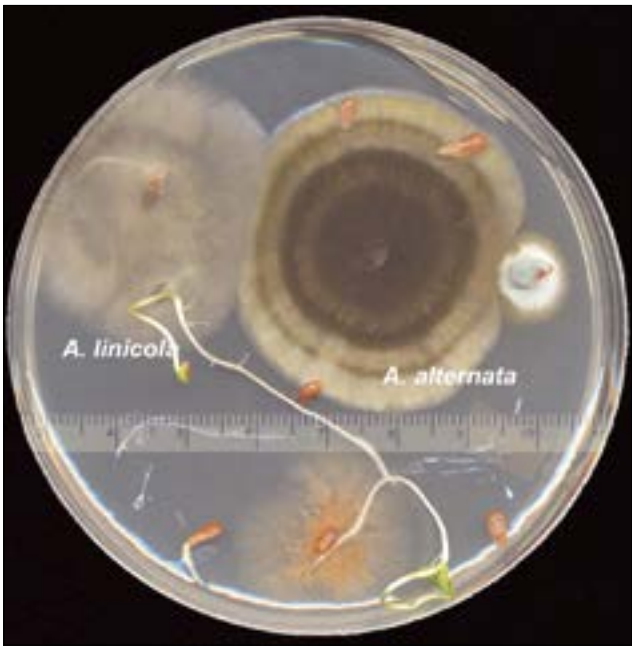

Figure 1. Olive-grey colonies of *A. linicola* and darker colonies of saprophytic *A. alternata* on malt agar.

Figure 2. Conidia of *Alternaria linicola.* ×600

Figure 3. Reddish streaks on cotyledons and hypocotyls caused by *A. linicola*.

Figure 4. Seedling showing a soft rot (arrow) and abundant sporulated grey mycelium.

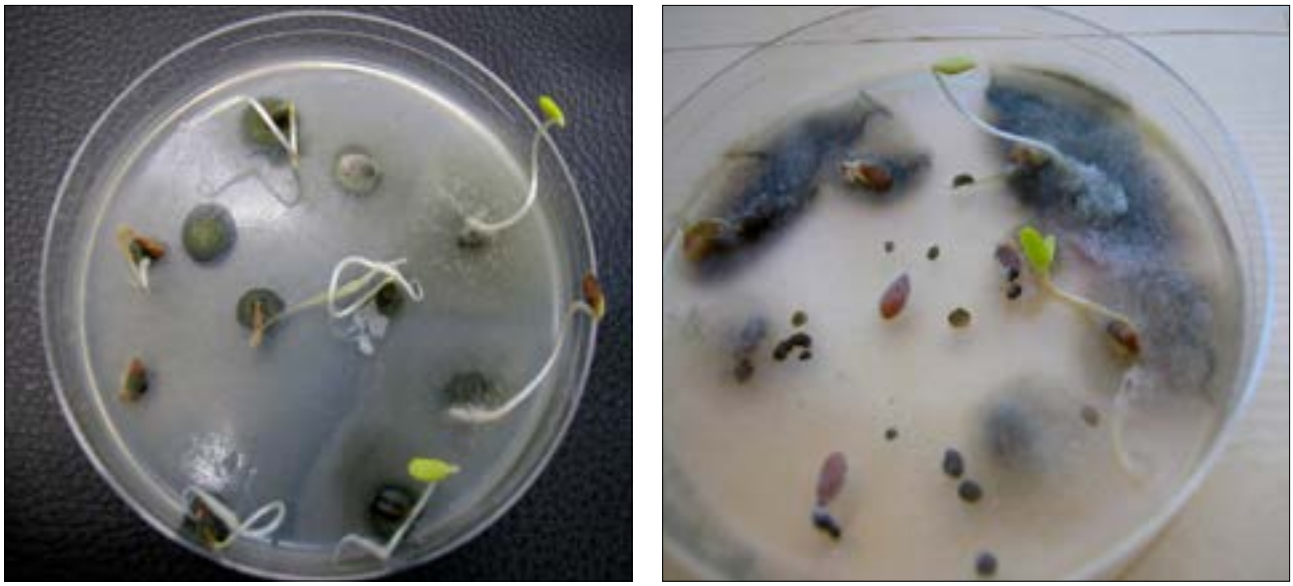

Figure 5. Colonies of *Botrytis cinerea* spreading from diseased flax seed on malt agar after 9 days of incubation. Sclerotia are visible (right).

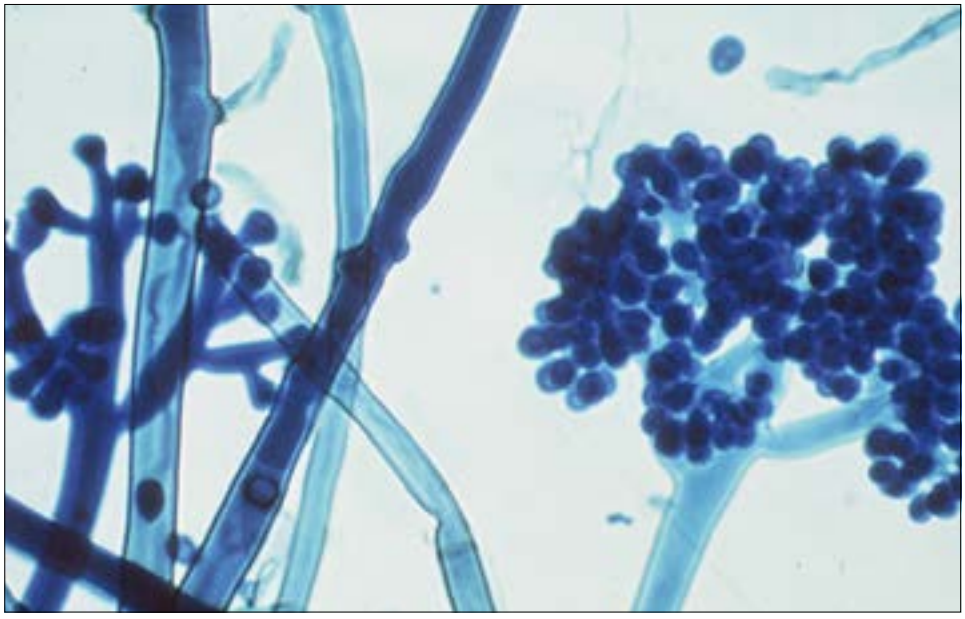

Figure 6. Conidiophores and conidia of *Botrytis cinerea* and tapelike mycelium. ×150.

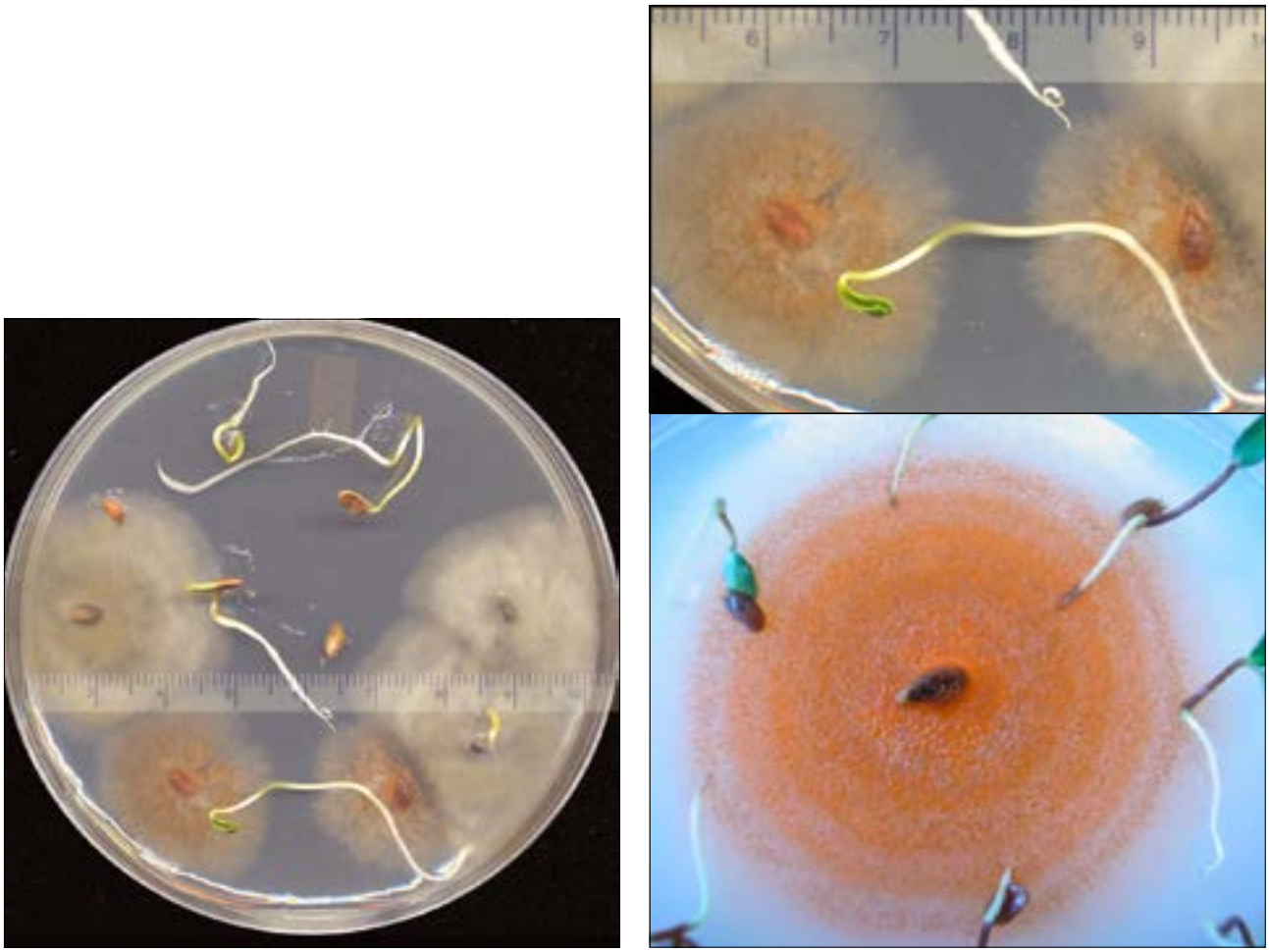

Figure 7. Salmon-coloured colonies of *Colletotrichum lini* growing from flax seed on malt agar.

Figure 8. Acervuli of *Colletotrichum lini* on flax seedling.

Critical control points (CCP)

Preparation of PDA or MA plates: the source of agar may influence the results. The level of available nutrients may vary from manufacturer to manufacturer. Both PDA and MA can be bought as a powdered medium, or MA can be made up as per recipe. Suitable products used in the comparative test include PDA, Cristomalt, agar-agar and streptomycin. Any equivalent products should be suitable. Whenever a new batch of agar is used, a check on the quality should be made, using a reference lot with a known infection level, or a reference isolate and sustainability of isolate measured. Pay particular attention to the growth characteristics of reference isolates.

Media and solutions

Potato dextrose agar + streptomycin

Potato dextrose agar (BD or equivalent) (CCP): 39 g
Distilled/deionised water: 1000 ml
Streptomycin sulphate*: 50 mg
*added after autoclaving

Streptomycin sulphate can be dissolved in sterile distilled/deionised water.

Preparation

1. Weigh out ingredients into a suitable autoclavable container.
2. Add 1000 ml of distilled/deionised water.
3. Dissolve powdered PDA in the water by stirring.
4. Autoclave at 121 °C and 15 psi for 20 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 the molten agar 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.

Malt agar + streptomycin

Agar-agar: 20 g
Malt: 10 g
Distilled/deionised water: 1000 ml
Streptomycin sulphate*: 50 mg
*added after autoclaving

Streptomycin sulphate can be dissolved in sterile distilled/deionised water. If using a commercial preparation ensure that it contains 2 % agar and 1 % malt extract.

Preparation

1. Weigh out ingredients into a suitable autoclavable container.
2. Add 1000 ml of distilled/deionised water.
3. Dissolve in the water by stirring.
4. Autoclave at 121 °C and 15 psi for 20 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 the molten agar 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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Validation references

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ISTA (2010). Validation study for the new proposed method to detect *Botrytis cinerea*, *Alternaria linicola* and *Colletotrichum lini* on *Linum*. *Method Validation Reports*. International Seed Testing Association, Bassersdorf, Switzerland.

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Figures 1–3: International Seed Testing Association. (2012). International Rules for Seed Testing, Annexe to Chapter 7: Seed Health Testing Methods. 7-017: Malt agar method for the detection of *Alternaria linicola* on *Linum usitatissimum*.

Figures 4 and 5: GEVES-SNES, rue Georges Morel, BP 90024, 49071 Beaucouzé CEDEX, France.

Figure 6: International Seed Testing Association (2012). International Rules for Seed Testing, Annexe to Chapter 7: Seed Health Testing Methods, 7-007: Detection of *Botrytis cinerea* on *Linum usitatissimum*.

Figure 7 (left): International Seed Testing Association. (2012). International Rules for Seed Testing, Annexe to Chapter 7: Seed Health Testing Methods, 7-018: Malt agar method for the detection of *Colletotrichum lini* on *Linum usitatissimum*.

Figures 7 (right) and 8: GEVES-SNES, rue Georges Morel, BP 90024, 49071 Beaucouzé CEDEX, France.