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

**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 damage etc., resulting from the use of this method.

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

Published by The International Seed Testing Association (ISTA)
Zürichstr. 50, CH-8303 Bassersdorf, Switzerland

Copyright© 2014 by the International Seed Testing Association

All rights reserved. No part of this publication may be reproduced, stored in any retrieval system or transmitted in any form or by any means, electronic, mechanical, photocopying, recording or otherwise, without prior permission in writing from ISTA.
**Crop:** *Linum usitatissimum* L. (flax, linseed)

**Pathogens:** *Alternaria linicola* J.W. Groves & Skolko, *Botrytis cinerea* Pers. (Teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel), and *Colletotrichum lini* (Westerd.) Tochinai, (= *Colletotrichum linicola* Pethybr. & Laff.)

**Authors:** V. Grimault¹, I. Serandat¹, C. Brochard¹, R. Kohen², S. Brière³

¹GEVES-SNES, rue Georges Morel, BP 90024, 49071 Beaucouzé CEDEX, France
E-mail: valerie.grimault@geves.fr

²Official Seed Testing Laboratory, The Volcani Center A.R.O., Bet-Dagan 50520, Israel
E-mail: rkohen@volcani.agri.gov.il

³Canadian Food Inspection Agency, 3851 Fallowfield Road, Ottawa, Ontario, Canada
E-mail: stephan.briere@inspection.gc.ca

**Revision history**
Version 2.0, 2014-01-01: Replacement of methods 7-007, 7-017 and 7-018

**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 organized by International Seed Testing Association Seed Health Committee.

**Validation studies**


Grimault V., Serandat I., Brochard C. (2010). Validation study for the new proposed method to detect *Botrytis cinerea*, *Alternaria linicola* and *Colletotrichum lini* on *Linum*.

Copies are available by e-mail from ista.office@ista.ch, or by mail from the ISTA Secretariat.

Annexe to Chapter 7: Seed Health Methods: 7-007-2
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 health, safety and environmental regulations.

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) or subsample 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). In any case, the minimum sample size should be of 400 seeds.

Materials

Reference material: reference cultures or other appropriate material  
PDA or MA plates with streptomycin sulphate: 9.0 cm 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)

Sample preparation

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

Method

1. Plating  
   Aseptically place a maximum of 10 seeds per plate, evenly spaced, onto the agar surface of each PDA or MA plate.

2. Incubation  
   Incubate plates for 9 days at 20°C in the dark.

3. 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.
4. 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.

5. Prolongation of incubation

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*.

Record the number of infected seeds in each plate, for each pathogen.

Identification criteria

*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 x50 – x100 magnification. Conidiophores are simple, occurring singly or in bundles, pale olivebrown, septate, and variable in length 5-8 μm. Conidia form singly, are smooth walled, olive-brown, obclavate with long, tapering occasionally branched beaks muriform 4-16 μm with transverse septa and occasionally 1-4 longitudinal septa, sometimes slightly constricted at the septa (Fig. 2)(Corlett and Corlett 1999; David 1991; Malone and Muskett 1997). Short red streaks and water soaked areas may be visible on the hypocotyls and cotyledons of some infected seedlings (Fig. 3).

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

**Figure 2.** Conidia of *Alternaria linicola*. x600
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 and Malone 1941; Tempe 1963; Malone and Muskett 1997; Ellis and Waller 1974).

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).
Alternaria, Botrytis and Colletotrichum on Linum usitatissimum

Colletotrichum lini

C. lini is easily recognised by visual examination. Examine the plates for shell pink to salmon coloured colonies (Fig. 7). Colonies of C. lini are a fine wooly-grey at the centre to salmon pink at the outer edge. Dark globose fuiting 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 x 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 x 3-4 μm (Malone and Muskett 1997; Kulshrestha et al., 1976).

Record the number of infected seeds in each plate.
Figure 7. Salmon-coloured colonies of *Colletotrichum lini* growing from flax seed on malt agar.

Figure 8. Acervuli of *Colletotrichum lini* on flax seedling.
General methods
(common to many test procedures)

1. 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 Tables 5B of Chapter 5 of the ISTA Rules, or in Miles (1963).

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.

In the case of a negative result (pathogen not detected), the results should be reported in terms of the tolerance standard (for example 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 the direct supervision of someone who has.

Critical control points

(identified in the methods by 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.

Preparation of PDA + streptomycin

**PDA (CCP i.e. Difco or equivalent):** 39 g  
**Distilled/deionized water:** 1000 mL  
**Streptomycin sulphate**: 50 mg  
*added after autoclaving*

**Preparation**

1. Weigh out ingredients into a suitable autoclavable container.
2. Add 1000 mL of distilled/deionized 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 water.
6. Pour 18–20 mL of the molten agar into 9.0 cm Petri dishes and allow to solidify before use.

**Streptomycin sulphate**

Streptomycin sulphate can be dissolved in sterile distilled water.

**Storage**

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

**Preparation of MA + streptomycin**

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

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/deionized 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 water.
6. Pour 18–20 mL of the molten agar into 9.0 cm Petri dishes and allow to solidify before use.

**Streptomycin sulphate**

Streptomycin sulphate can be dissolved in sterile distilled water.

**Storage**

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


Photograph credits


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.