International Rules for Seed Testing 2027

Validated Seed Health Testing Methods

7-004: Detection of *Plenodomus lingam* and *P. biglobosus* in *Brassica* spp. seed

Including changes and editorial corrections adopted at the Ordinary General Meeting 2026 in Calgary, Canada

Effective from 1 January 2027

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.

Published by
The International Seed Testing Association (ISTA)
Richtiarkade 18, CH-8304 Wallisellen, Switzerland

© 2027 International Seed Testing Association (ISTA)

Online ISSN 2310-3655

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.

7-004: Detection of *Plenodomus lingam* and *P. biglobosus* in *Brassica* spp. seed

Host: *Brassica* spp.

Pathogen(s): *Plenodomus lingam* (Tode) Höhnel (previously *Leptosphaeria maculans* [Tode ex Fr.] Ces. & de Not; *Phoma lingam*) or *Plenodomus biglobosus* (Shoemaker & H. Brun) (previously *Leptosphaeria biglobosa*)

Authors: Orgeur, G.[1], McEwan, M.[2], Chamaille, A.[1], Andro, C.[1], Rolland, M.[1], Politikou, L.[3] & Grimault, V.[1]

[1]GEVES, rue Georges Morel, CS90021, 49071 Beaucouzé, France

[2]SASA, Roddinglaw Road, Edinburgh, EH12 9FJ, UK

[3]ISHI-Veg, Hosted by: GEVES SNES, 25 rue Georges Morel, C.S. 90024, 49071 Beaucouze Cedex, France

Revision history

Version 1.0, 2001-11-19
Revised 2001-11-19: J. Sheppard, V. Cockerell
Reprinted 2003
Version 1.1, 2008-01-01: Treated seed revised; Reporting results revised
Version 2.0, 2018-01-01: Method revised
Version 2.1, 2021-01-01: Sample size revised
Version 2.2, 2024-01-01: Sample size revised
Version 2.3, 2025-01-01: Pathogens, Background, Treated seed, Materials, Methods and figure captions revised
Version 2.4, 2027-01-01: Flowchart added in Methods

Background

Phoma lingam is no longer the officially accepted name for the causal agent of black leg, stem canker and dry rot in *Brassica* species and other crucifers. The currently correct pathogen names are *Plenodomus lingam* (Tode) Höhnel (previously *Leptosphaeria maculans*) and *Plenodomus biglobosus* (Shoemaker & H. Brun) (previously *Leptosphaeria biglobosa*).

Plenodomus lingam has historically colonised countries where *Plenodomus biglobosus* is prevalent, such as Poland and central Canada (Fitt *et al*., 2008). *Plenodomus lingam* isolates are responsible for basal stem damage (crown canker), whereas *Plenodomus biglobosus* causes pale brown lesions with a dark margin on the upper stem (Dilmaghani *et al*., 2009). Differentiation between the two fungal species was based on phytopathology and molecular criteria.

The reference method for the detection of *Phoma lingam* is the ISTA Rule 7-004 version 1.1 (ISTA, 2014). Seeds were placed on blotter paper (Whatman No. 1 or equivalent) containing 2.4 Dichlorophenoxyacetic acid (2.4-D) to prevent seed germination. As the 2.4-D is toxic its use is not recommended in routine tests. With the objective of proposing an alternative, two methods were tested during the TESTA project in 2015: the deep freezing method (ISHI-Veg, ISF 2011) and the malt agar method (GEVES-SNES laboratory) were tested.

The results from both methods were compared in an international comparative test, between ten laboratories, organised by GEVES. A 0.1 % limit of detection (analytical sensitivity) was shown based on a test performed in one laboratory. The other criteria (accordance/repeatability, concordance/reproducibility, diagnostic sensitivity, diagnostic specificity and accuracy) were calculated based on results of all ten laboratories in the comparative test. Similar results were obtained using all three methods. Using deep freezer method showed a better inhibition of seed germination compared to 2.4-D. Therefore in version 2.0 of the method, the deep freezing method replaces the 2.4-D method.

PCR and pathogenicity testing were performed during the comparative test (Orgeur *et al*., 2015) to distinguish *Plenodomus lingam* (previously *Leptosphaeria maculans*) from *Plenodomus biglobosus*. The primer set (Liu *et al*. and Plant Research International [PRI], Wageningen) to be used needs to be chosen using an internal validation test by laboratories. In cases where morphological identification of a suspect colony is difficult, a PCR confirmation and a pathogenicity test confirmation can be useful. The PCR is carried out, and if PCR is negative, a pathogenicity test is carried out on cotyledons. The pathogenicity test can be performed directly after identification of a suspect colony.

Treated seed

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

Sample size

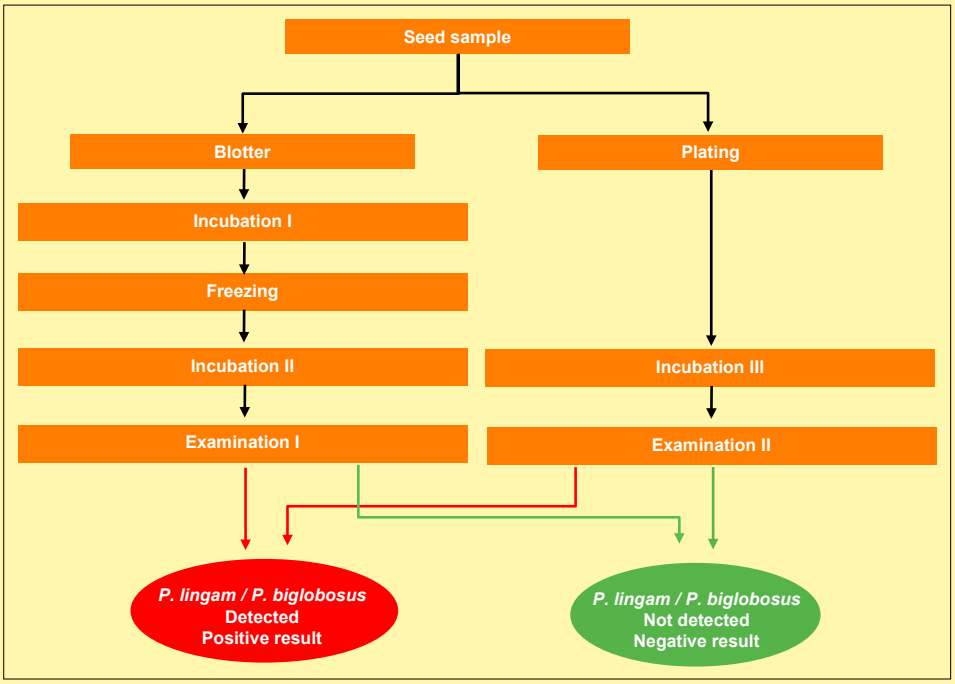
The sample size (total number of seeds to be tested) and subsample size depend on intended use, the maximum acceptable infection level and the analytical sensitivity of the method. The minimum sample size should be 400 seeds.

Materials

Reference material: known strain of *Plenodomus lingam* or *P. biglobosus,* or standardised reference material

Blotter: filter paper, e.g. Whatman No. 1 or equivalent; malt agar (CCP); Petri dish (diameter of 90 mm) or plastic box with lid (e.g. 20 cm × 14 cm); freezer –20 ±2 °C; incubator 20 ±2 °C with near-ultraviolet light (NUV); stereomicroscope

Malt agar (CCP): Petri dish diameter of 90 mm; incubator 20 ±2 °C; stereomicroscope



Methods

1. Blotter:

1.1 Plating of seeds: Place three pieces of sterilised filter paper (Whatman No. 1 or equivalent) in each Petri dish and add 5 ml of sterilised water. Pour off the excess and place 25 seeds on each plate or alternatively 100 seeds in each plastic box.

1.2 Pretreatment: None.

1.3 Incubation:

1.3.1 Incubate for 24 h at 20 ±2 °C in darkness.

1.3.2 Transfer dishes to freezer and maintain at –20 ±2 °C for 24 h.

1.3.3 After freezing, incubate for 14 d at 20 ±2 °C with alternating 12 h periods of darkness and NUV light.

1.4 Examination: After 11 d, examine at ×25 magnification for loose growing silver white mycelium and pycnidial primordia of *Plenodomus* on the seed and substrate. After 14 d, make a second examination for pycnidia on seeds and on the filter paper close to suspected seeds. Pycnidia are relatively large, about 250 µm, with papilla, sometimes developed as a neck (Figs 1a, b) and often have purple (amethyst) exudate. If the seed is covered with mycelium it is recommended to use a sterile botanical needle or any other sterile device to expose the seed and look for typical secreting pycnidia.

The ubiquitous saprophyte *Phoma herbarum* occurs also on *Brassica* seed, but has smaller pycnidia formed superficially on the seed coat (Fig 1c), not papillate, with white yellow or pink but not purple (amethyst) exudates. Another fungus, *Stemphylium* sp. produces perithecia on blotter and can be confused with pycnidia of *Plenodomus*. Seeds from which pycnidia of *Plenodomus* spp. have developed are recorded as infected. Results can be recorded as percentage of seeds infected by *Plenodomus* spp.

2. Malt agar:

2.1 Plating of seeds: Aseptically place 10 seeds per malt agar plate.

2.2 Pretreatment: None.

2.3 Incubation: Incubate for 7 d at 20 ±2 °C with 24 h darkness.

2.4 Examination: After 7 d examine at ×25 magnification for loose growing silver brownish white mycelium of *Plenodomus* on substrate (Fig 2). Examination for pycnidia on infected seeds and on media: pycnidia are relatively large, about 250 µm, with papilla, sometimes developed as a neck. The ubiquitous saprophyte *Phoma herbarum* occurs also on *Brassica* seed, but has smaller pycnidia formed superficially on the seed coat, not papillate, with white yellow or pink but not purple (amethyst) exudate. Seeds on which typical mycelium with or without pycnidia of *Plenodomus* have developed are recorded as infected. Examination can be postponed up to 11 d if detection cannot be concluded only by the presence of typical mycelium. If the seed is covered with mycelium or if the pycnidia is inside the media it is recommended to use a sterile botanical needle or any other sterile device to expose the seed and look for typical secreting pycnidia. It is also optional to examine the plates on a light table, making it easier to locate suspected pycnidia.

Unlike option 1 (the blotter test) *Stemphylium* sp. produces perithecia and conidia on malt agar and cannot be confused with pycnidia of *Plenodomus* spp. Seeds from which mycelium and/or pycnidia of *Plenodomus* spp. have developed can be recorded as percentage of seeds infected.

Overview of methods

	Blotter paper	Malt agar
Media	Blotters (filter paper), e.g. Whatman No. 1 or equivalent.	Malt agar (CCP)
Plates	Sowing density of 25 seeds per plate, 90 mm Petri dishes are recommended (40 plates per sample). Alternatively, sub sample of 100 seeds can be placed in one 20 cm × 14 cm sized blotter with distilled water in a clean similar sized plastic box with lid.	Sowing density of 10 seeds per plate, 90 mm Petri dishes are recommended (100 plates per sample).
Incubator	Operating range 20 ±2 ºC. Alternating 12 h periods of darkness and near-ultraviolet light (NUV) during incubation is recommended to stimulate sporulation. The recommended source is the black light fluorescent lamp (peak at 360 nm).	Operating range 20 ±2 ºC. To stimulate sporulation, 24 h of darkness is recommended.
Freezer	Operating range –20 ±2 ºC.	Operating range –20 ±2 ºC.

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, the test method used and the number of seeds tested. When reported on an ISTA Certificate, results are entered under 'Other Determinations'.

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.

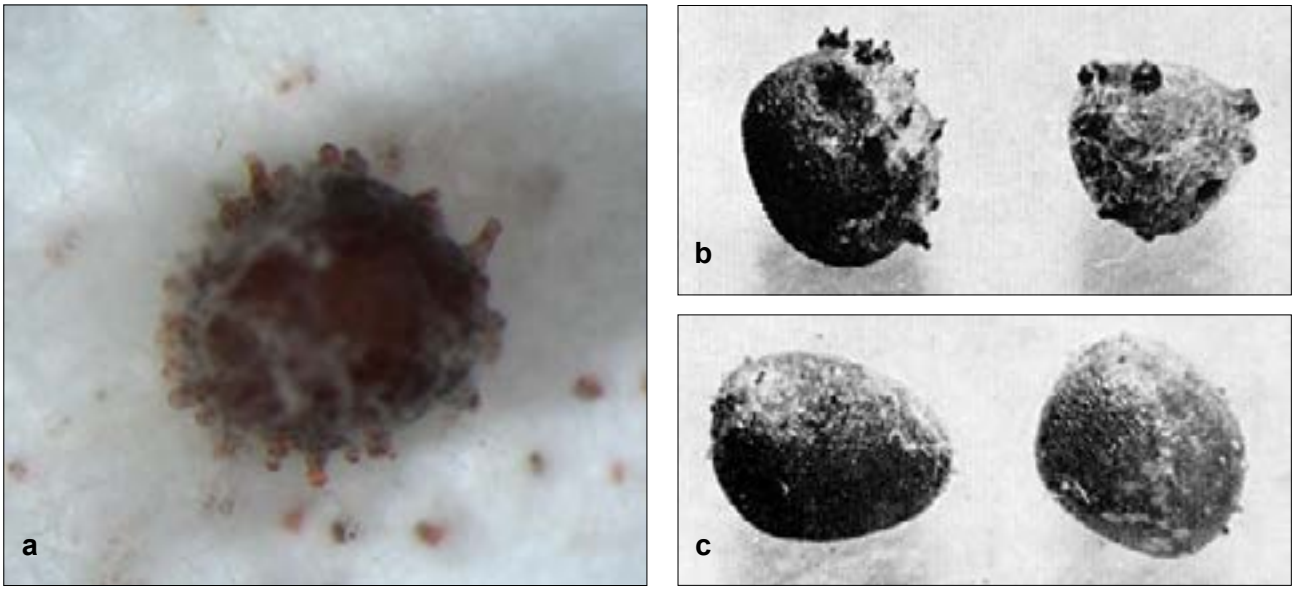


Figure 1. a (photograph courtesy of SASA), **b** (photograph courtesy of ISTA) *Brassica* seeds with pycnidia of *Plenodomus* spp. amethyst exudate from pycnidia. **c** With Phoma herbarum (ISTA, 2014).

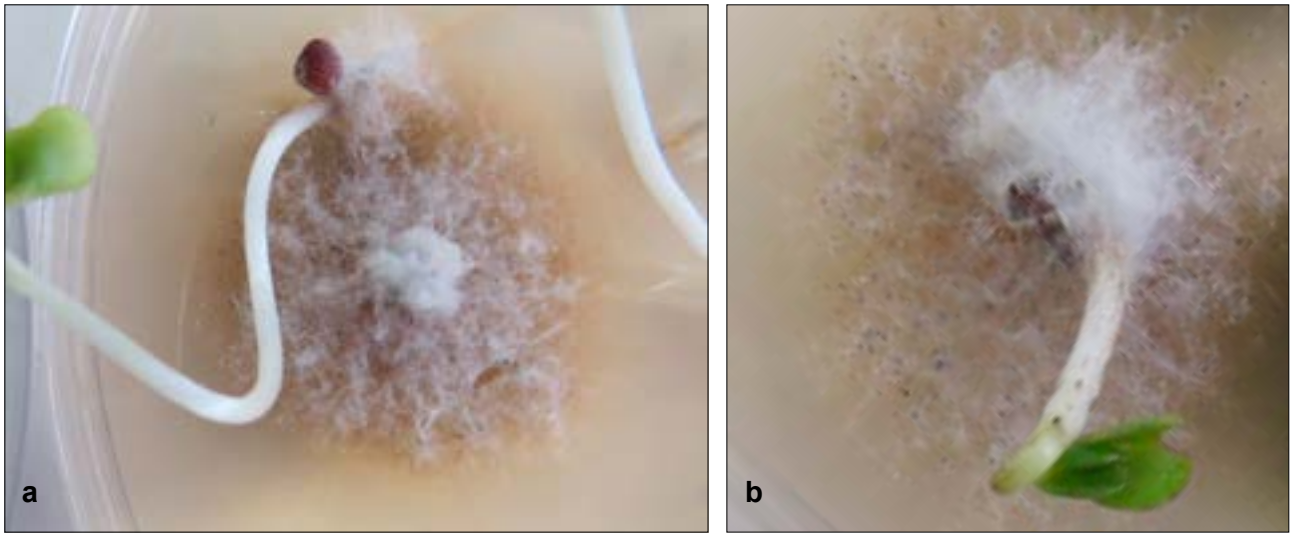


Figure 2. a Aspect of *Plenodomus* spp. colony. **b** Pycnidia at 7 d on media: silver brownish white mycelium with first pycnidia formation (photographs courtesy of GEVES).

Quality assurance

Critical control points (CCP)

The malt source can influence the results. The level of available nutrients may vary from manufacturer to manufacturer. Whenever a new batch of malt is used a check on the quality must be made using a reference lot with a known infection level, or a reference isolate and the sustainability of the isolate measured. Pay particular attention to the growth characteristics of reference isolates.

Media and solutions

Malt agar

Compound	Amount/1000 ml of deionised/ distilled water	Amount/500 ml of deionised/ distilled water
Malt (CCP)	10 g	5 g
Agar	17 g	8.5 g
Streptomycin sulphate (add after sterilisation)	0.05 g	0.025 g

Preparation

1. Weigh out ingredients into a suitable autoclavable container.
2. Add 1000 (or 500) ml of deionised/distilled water.
3. Dissolve the ingredients in deionised/distilled water by stirring.
4. Autoclave at 121 °C and 15 psi for 15 min.
5. Allow agar to cool to approximately 50 °C and add Streptomycin sulphate dissolved in sterile water.
6. Pour 20 ml of malt agar into 9 cm Petri dish and allow to solidify before use.

Storage

Plates may be stored for 2 months at 5 °C.

References

Dilmaghani, A., Balesdent, M. H., Didier, J. P., Wu, C., Davey, J., Barbetti, M. J., Hua, L., Moreno-Rico, O., Phillips, D., Despeghel, J. P., Vincenot, L., Gouta, L. and Rouxel, T. (2009). The *Leptosphaeria maculans–Leptosphaeria biglobosa* species complex in the American continent. *Plant Pathology*, **58**, 1044–1058.

Fitt, B. D. L., Hu, B. C., Li, Z. Q., Liu, S. Y., Lange, R. M., Kharbanda, P. D., Butterworth, M. H. and White, R. P. (2008). Strategies to prevent spread of *Leptosphaeria maculans* (*Phoma* stem canker) onto oilseed rape crops in China. *Plant Pathology*, **57**, 652–664.

International Seed Federation (2011). Method for the detection of *Phoma lingam* on *Brassica* spp. seed, version 2. International Seed Health Initiative for Vegetable Crops, Nyon, Switzerland.

International Seed Testing Association (2014). Annexe to Chapter 7: Seed Health Testing Methods. 7-004: Detection of *Phoma lingam* on *Brassica* spp. Basserdorf, Suisse. *International Rules for Seed Testing*. International Seed Testing Association, Bassersdorf, Switzerland.

International Seed Testing Association (2016). *International Rules for Seed Testing*. International Seed Testing Association, Bassersdorf, Switzerland.

Liu, S. Y., Lui, Z., Fitt, B. D. L., Evans, N., Foster, S. J., Huang, Y. J., Latunde-Dada, A. O., Lucas, J. A. (2006). Resistance to *Leptosphaeria maculans* (*Phoma* stem canker) in *Brassica napus* (oilseed rape) induced by *L. biglobosa* and chemical defence activators in field and controlled environments. *Plant Pathology*, **55**, 401–412. Doi: 10.1111/j.1365-3059.2006.01354.x

Miles, S. R. (1963). Handbook of tolerances and of measures of precision for seed testing. *Proceedings of the International Seed Testing Association*, **28** (3), 525–686.

Orgeur, G., McEwan, M., Chamaille, A., Andro, C., Rolland, M., Politikou, L. and Grimault, V. (2015). Task 5.7: Validation of detection methods of *Leptosphaeria maculans* (*Phoma lingam*) on *Brassica* seeds on blotter and agar in comparison to the actual ISTA 7-004 method, in order to avoid use of 2,4-D. GEVES, International Seed Health Initiative for Vegetable Crops [ISHI-Veg] (International Seed Federation)/ TESTA WP5 (International Seed Testing Association).

Validation references

ISTA (2017). *Method validation reports on Rules proposals for the International Rules for Seed Testing 2018 Edition*. International Seed Testing Association, Bassersdorf, Switzerland. Republished from GEVES, International Seed Health Initiative for Vegetable Crops [ISHI-Veg] (International Seed Federation)/ TESTA WP5 (International Seed Testing Association) validation report.