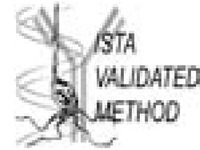
International Rules for Seed Testing
Annexe to Chapter 7: Seed Health Testing Methods



7-001a: Blotter method for the detection of *Alternaria* dauci on *Daucus carota*

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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.

Crop: *Daucus carota* (carrot)

Pathogen: *Alternaria dauci*

Prepared by: Sheppard, J.W., Cockerell, V. and Roberts, S.J.
ISTA-PDC Method Validation Sub-committee

Sponsored by: ISTA-PDC Method Validation Sub-committee

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Background

This method was originally published in the *ISTA Handbook of Seed Health Testing* in November 1964 as S.3. No. 4 and revised in 1987 (Gambogi, 1987). It was incorporated into the *Annexe to Chapter 7: Seed Health Testing Methods* as method 7-001 (Sheppard and Cockerell, 2002). It has been renumbered (7-001a) and slightly modified following studies conducted by the International Seed Health Initiative - Vegetables in 1999 and 2001 (Van Bilsen, 2003). The studies compared blotter and malt agar methods and concluded that the two were equivalent. Note that seeds can be simultaneously tested for the presence of *Alternaria radicina* using the same method (see method 7-002a).

Validation studies

Van Bilsen (2003)
Copies are available: by e-mail from ista.office@ista.ch; or by mail from the ISTA Secretariat.

Please send comments, suggestions or reports of problems relating to this method to the leader of the ISTA-PDC Mycology Working Group, c/o ISTA Secretariat

Safety Precautions

Ensure you are familiar with hazard data and take appropriate safety precautions. 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. autoclave, disinfect) and in accordance with local health, environmental and safety regulations.

Treated seed

Seed treatments may affect the performance of this test. It should only be performed on untreated seed.

Materials

Reference material	- The use of reference cultures or other appropriate material is recommended.
Substrate	- Blotters or filter papers, 9.0 cm, circular (e.g. Whatman No 1 or equivalent), free from micro-organisms and inhibitors (3 per plate).
Plates	- 9.0 cm sterile Petri dishes (one per ten seeds).
Incubator	- Operating at 20 ± 2°C, equipped with timer-controlled near-ultraviolet lights (NUV, peak at 360 nm, e.g. colour number 08, Philips; BLB, Sylvania).
Freezer	- Operating at –20 ± 2°C.

Sample Preparation

1. It is vital to exclude any possibility of cross-contamination between seed samples. This can be achieved by swabbing/spraying equipment and gloved hands with 70% ethanol.
2. The test is carried out on a working sample as described in Section 7.4.1 of the International Rules for Seed Testing.

Method

[Critical control points are indicated by **CCP**]

1. Place three 9.0 cm filter papers in each plate and soak with sterile distilled/de-ionised water. Drain away excess water.
2. Aseptically place 10 seeds, evenly spaced (**CCP**), on the surface of the filter paper in each plate.
3. Incubate for 3 d at 20 ± 2°C in the dark.
4. Transfer plates to freezer and maintain at –20 ± 2°C for 24 h.
5. After freezing, incubate for 6 d at 20 ± 2°C with alternating 12 h periods of darkness and NUV light (ISTA,1984; Tempe, 1968). Plates should be approx. 25 cm below the lights and should not be stacked.
6. Examine seeds under a stereoscopic microscope at x30 for fungal growth and up to x80 magnification for identification of conidia. Conidiophores are simple or slightly branched (Fig. 1), arising singly or in small groups from the surface of the seed or on aerial mycelium. Conidia are usually solitary, obclavate, up to 450 µm long (including beak), pale olivaceous brown at first, becoming brown with age, with a long pale

beak up to 3 times the length of the body (Ellis, 1971). Groups of sunken conidia are sometimes visible by the emerging clusters of their bright long beaks (Fig. 1, bottom left). Record the number of infected seeds in each plate (**CCP**).

General Methods

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. A tolerance table, which can be applied to most direct seed health tests, can be found in Table 5.1 of Annexe 16 of the International Rules for Seed Testing or in Table G1 of the *Handbook of Tolerances and Measures of Precision for Seed Testing* (Miles, 1963)

2. *Reporting Results*

   The result of a seed health test should indicate the scientific name of the pathogen, and the test method used. When reported on an ISTA 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 (e.g. 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 by **CCP** in the methods]

Spreading hyphae may lead to contamination of other seeds. Seeds must therefore be spaced at least 20 mm from each other with not more than 10 seeds per 9.0 cm Petri dish (Step 2).

Samples may be difficult to examine due to the growth of contaminants, especially *Alternaria tenuis*, and/or *A. radicina*. Experience and great care is required for the detection of all occurrences (ISTA 1984) (Step 6).

Supplementary examination may be required 13-14 d after plating of seeds to allow hampered or deeper inoculum to exceed contaminants by its fructification (Hewett, 1964) (Step 6).

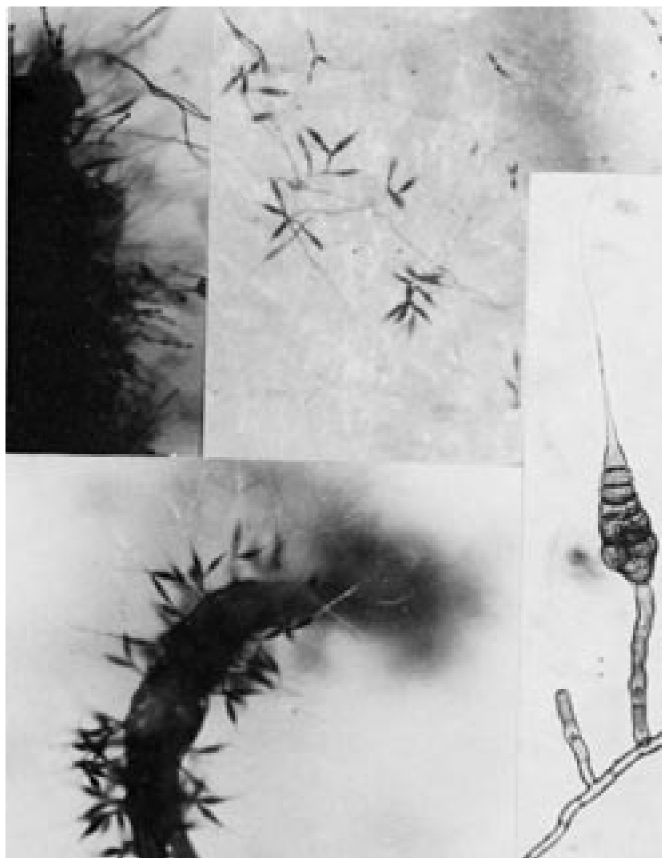

Fig. 1. Top: single conidia of *Alternaria dauci* and chains of conidia of the saprophyte *A. tenuis* on seed surface (left) and simple or slightly branched conidiophores with conidia of *A. dauci* developing from creeping hyphae on the blotter (right), x80 magnification. Bottom: conidia of *A. dauci* on simple or slightly branched conidiophores borne on a single rootlet initial at x80 magnification (left); conidium and simple conidiophores at x350 magnification (right).

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