International Rules for Seed Testing 2024

Validated Seed Health Testing Methods

7-032: Detection of *Verticillium dahliae* in *Spinacia oleracea* (spinach) seed

Including changes and editorial corrections adopted at the Ordinary General Meeting 2023 in Verona, Italy

Effective from 1 January 2024

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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7-032: Detection of *Verticillium dahliae* in *Spinacia oleracea* (spinach) seed

Host: *Spinacia oleracea* L.
Pathogen(s): *Verticillium dahliae* Kleb.

Prepared by: du Toit, L. J.[1], Gilijamse, E.[2], Oosterhof, J.[2], Hiddink, G.[3] and Politikou, L.[4]
[1] Department of Plant Pathology, Washington State University Mount Vernon NWREC, 16650 State Route 536, Mount Vernon, WA 98273-4768, USA. Email: dutoit@wsu.edu
[2] Rijk Zwaan Breeding B.V.,Burgemeester Crezéelaan 40, P.O. Box 40, 2678 ZG De Lier, The Netherlands. Email: j.oosterhof@rijkzwaan.nl, e.gilijamse@rijkzwaan.nl
[3] Enza Zaden Seed Operations B.V., P.O. Box 7, 1600 AA Enkhuizen, The Netherlands. Email: g.hiddink@enzazaden.nl
[4] ISF, 7 Chemin du Reposoir, 1260 Nyon, Switzerland. Email: liana.politikou@ufs-asso.com

Revision history

Version 1.0, 2018-01-01: New method
Version 1.1, 2021-01-01: Sample size revised; Media and solutions revised
Version 1.2, 2024-01-01: Sample size revised

Background

Two incubation methods are described for the detection of *Verticillium dahliae* on spinach seed; agar test on the NP-10 agar medium (du Toit *et al*., 2005; Iglesias-Garcia *et al*., 2013) and the deep-freeze blotter test (Derie *et al*., 1988; du Toit and Hernandez-Perez, 2005; du Toit *et al*., 2005; du Toit and Derie, 2008; Block and Shepherd, 2008; Cummings *et al*., 2009; du Toit, 2011; Standaard protocol NAKtuinbouw, 2011; Villarroel-Zeballos *et al*., 2012). Both methods were validated in each of two comparative tests organised by the International Seed Health Initiative for Vegetables, ISF (ISHI-Veg).

The NP-10 agar method was shown to be suitable for testing non-treated seed and is not recommended for fungicide-treated seed as fungicides may diffuse into the medium, potentially resulting in an overestimation of the relevant *V. dahliae* levels on the seeds when the objective is to determine if the seed treatment is effective against seedborne *V. dahliae* (du Toit, 2011). Likewise, the antibiotics in NP-10 agar medium may affect some biological control seed treatments adversely, potentially resulting in overestimation of the levels of infected seed. The blotter assay is suitable for both treated and non-treated spinach 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 techniques. Dispose of all waste materials in an appropriate way (e.g. by autoclaving or disinfecting) and in accordance with local health, safety and environmental regulations.

Treated seed

The NP-10 method is suitable for non-treated seed and seed that has been treated using physical (e.g. hot water) or chemical (e.g. chlorine) processes with the aim of disinfestation/disinfection, provided that any residue, if present, does not influence the assay. It is the responsibility of the user to check for such antagonism and/or inhibition by analysis, sample spiking, or experimental comparisons.

The NP-10 method is not recommended for fungicide-treated seeds (du Toit, 2011), or seeds treated with biological control agents if part of the objective of the assay is to assess how well a treatment on the seed works against seedborne *V. dahliae*. If seeds are treated with biological control agents, the biological treatments may be affected adversely by antibiotics in the NP-10 agar medium, potentially diminishing the efficacy of the treatment against any seedborne *V. dahliae* and resulting in elevated counts. Seeds treated with fungicide(s) may interfere with the reading of the test because fungicides readily diffuse into the agar medium, potentially diminishing the efficacy of the treatment against any seedborne *V. dahliae* and resulting in increased levels of detection (du Toit, 2011).

The blotter method is suitable for non-treated seeds, seeds treated with physical (e.g. hot water) or chemical (e.g. chlorine) processes (du Toit and Hernandez-Perez, 2005) with the aim of disinfestation/disinfection, provided that any residue, if present, does not influence

the assay. It is the responsibility of the user to check for such antagonism and/or inhibition by analysis, sample spiking, or experimental comparisons. Blotter method is also suitable for seed treated with fungicides (du Toit *et al*., 2005; du Toit *et al*., 2007; du Toit *et al*., 2009; du Toit *et al*., 2010) and seeds treated with biological control agents (Cummings *et al*., 2009).

The method has been validated for thiram and metalaxyl-M (also named mefenoxam) treated seeds (du Toit, 2011; Gillijamse and Politikou, 2015). In published literature this method also has been used to test seeds treated with other fungicides and biological control agents (du Toit *et al*., 2007; Cummings *et al*., 2009; du Toit *et al*., 2009; du Toit *et al*., 2010).

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

Note: All of the following materials are critical control points (CCP).

NP-10 method

NP-10 agar in acrylic boxes: 10 cm × 10 cm, up to 34 seeds per box (Hoffman Manufacturing, Inc.) *or*
Petri dishes: 9.0 cm diameter, 25 seeds per plate

Blotter method

Acrylic boxes:
– 20 cm × 14 cm (DBP plastics), 100 seeds per box *or*
– 10 cm × 10 cm (Hoffman Manufacturing, Inc.), up to 34 seeds per box *or*
– Petri dishes, 9.0 cm diameter, 25 seeds per plate

Blotter-paper for acrylic boxes: Steel blue germination blotter paper, Anchor Paper Co., e.g. D1.360.560, All Paper BV, or equivalent
Blotter paper for plates: Steel blue germination blotter paper, Anchor Paper Co., e.g. Cns 2.84.r, All Paper BV or equivalent
Freezer: capable of operating at –20 ±2 °C

Common material for both methods

Reference material: use of reference cultures of *V. dahliae* or seed lots known to be infected with *V. dahliae*
Sterilised forceps
Incubator: capable of operating at 20–24 °C, equipped with near ultraviolet light (NUV, peak at 360 nm) and cool fluorescent white light
Dissecting microscope: 8–100× magnification

Methods

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

1. Preparation of spinach seeds
   Spinach seeds, except those treated with fungicides, biological control agents and disinfectants like bleach, or hot water, need to be surface sterilised as follows:
1.1 Place the seeds in a tea strainer.
1.2 Immerse the tea strainer with the seeds in 1.2 % NaOCl solution for 60 s with constant manual agitation of the strainer to keep the seeds swirling throughout the 60 s. Make sure that the volume of 1.2 % NaOCl solution covers the tea strainer fully (CCP).
1.3 Remove the tea strainer from the 1.2 % NaOCl solution, shake off the excess liquid, and immerse the tea strainer and seeds in sterilised, deionised/or distilled water in a small glass beaker for 30 s with constant agitation. Use enough water to fully immerse the tea strainer and seeds (CCP).
1.4 Repeat the rinse step twice more, using a new batch of sterilised, deionised/distilled water for each rinse.
1.5 Using aseptic technique, spread the seeds onto dry, sterilised paper towel in a laminar flow hood or biological safety cabinet to dry thoroughly for at least 60 min.
1.6 Using aseptic techniques, place the surface-sterilised, dried seeds in a sterilised, disposable Petri dish or other sterilised container. Seeds should be stored at room temperature (approximately 22–25 °C) for no longer than 24 h before plating.
2. Positive control (reference material)
   For the blotter method, plate aseptically, seeds of a known *V. dahliae*-infected spinach seed lot on a blotter in sterilised boxes or Petri dishes as a reference seed lot for obtaining isolate(s) of *V. dahliae*. The number of seeds to be plated in boxes or plates will depend on the infection level of the positive control seed lot. For the NP-10 agar method, subculture a reference isolate of *V. dahliae* onto NP-10 agar. Incubate the reference

isolate or boxes as for the rest of the boxes or Petri dishes for the seed lot(s) being tested.

NP-10 Method

1. Plating
1.1 Use sterilised acrylic boxes and lids or sterilised Petri dishes containing NP-10 agar medium (CCP).
1.2 Aseptically, under a laminar flow hood, place a maximum of 34 seeds evenly spaced in each acrylic box of 10 cm × 10 cm onto the NP-10 agar medium (Fig 1a). To plate 100 seeds per subsample, use three boxes, with 34 seeds in each of the first two boxes, and 32 seeds in the third box (CCP).
1.3 Press each seed into the agar medium slightly to prevent seeds from rolling around when the boxes are moved. Close each box with a sterilised lid.
2. Incubation (CCP)
Incubate the seeds at 20–24 °C under a day/night cycle of 12 h of light (near-ultraviolet [NUV] and cool white fluorescent light)/12 h of dark.
3. Examination (CCP)
3.1 At 5, 9 and 14 d after plating, remove the lid of each box or Petri dish and examine the seeds for *V. dahliae* using a dissecting microscope (8–100× magnification).
3.2 Compare any suspect isolates of *V. dahliae* to the positive control isolate or seed lot.
3.3 Record the number of *V. dahliae*-infected seeds per box at each reading day.

Blotter method

1. Plating
1.1 Use sterilised acrylic boxes and lids or sterilised Petri dishes (CCP).
1.2 Aseptically, in a laminar flow hood for each subsample of 100 seeds, soak the sterilised blotter paper with sterilised water and place the blotter in a sterilised acrylic box or Petri dish (CCP).
1.3 Drain off any excess water from the blotter paper and place the appropriate number of seeds on the moistened blotter (maximum of 100 seeds on the blotter in each 20 cm × 14 cm acrylic box, maximum of 34 seeds in each 10 cm × 10 cm acrylic box, and maximum of 25 seeds in each 9 cm-diameter Petri dish) (Fig 1a, b) (CCP).
1.4 Close each box or Petri dish with a sterilised lid.
2. Incubation (CCP)
Incubate the seeds in an incubator at 20–24 °C for 24–25 h in the dark to imbibe water from the blotters. Larger seeds may need to imbibe for the longer duration to ensure adequate imbibition prior to the freezing step.
3. Freezing
Transfer the seeds to a freezer at –18 °C to –22 °C for 24–25 h to kill the embryos. Only embryos that have imbibed adequate water will be killed by this freezing step. Larger seeds may need the longer duration of freezing to kill embryos of the maximum number of seeds.
4. Post-freezing
After freezing, incubate the seeds in an incubator at 20–24 °C under a day/night cycle of 12 h of light (near-ultraviolet [NUV] and cool white fluorescent light)/12 h of dark.
5. Examination (CCP)
5.1 Non-treated seed is examined at 5, 9 and 14 d after plating. Treated seed is also examined 21 d after plating as seed treatments might slow down the development of *V. dahliae*. (du Toit *et al*., 2005; du Toit and Hernandez-Perez, 2005; du Toit *et al*., 2007; du Toit and Derie, 2008; du Toit *et al*., 2009; du Toit *et al*., 2010).
5.2 Compare any suspect isolates of *V. dahliae* to the positive control isolate or seed lot.
5.3 Record the number of *V. dahliae*-infected seeds per box at each reading day.

Identification criteria

***Verticillium dahliae*:** Examine plates for miscrosclerotia, conidiophores and conidia typical of *V. dahliae*.

Microsclerotia: Black survival structures (Figs 2a, b and 3a, b) that are masses of melanised fungal cells ranging from 10–230 µm in diameter (Qin *et al*., 2008; Sorensen *et al*., 1991).

Conidiophores and conidia: Verticillate, tree-like structures with phialides borne in whorls on the conidiophores, and clumps of hyaline, single-celled conidia borne at the end of each phialide (du Toit *et al*., 2005; du Toit and Hernandez-Perez, 2005) (Figs 3b, c).

To determine the potential effect of seed treatments on *V. dahliae*, it is important to distinguish between viable and dead microsclerotia on the seeds by observing development of typical verticillate conidiophores on the same seed as those on which microsclerotia are observed (Fig 4). The presence of microsclerotia on a seed without conidiophores, or without the presence of newly developed microsclerotia in the blotter paper is an indication that the *Verticillium* sp. on that seed is not viable (Fig 4b) (du Toit *et al*., 2009; du Toit *et al*., 2010; du Toit and Hernandez-Perez, 2005).

Verticillium spp. can be misidentified with *Acremonium* spp. which form conidiophores that resemble those of *V. dahliae*. However, the mycelium of *Acremonium* spp. tends to develop into 'rope-like strands' by the 9 and 14 d readings, from which individual conidiophores branch off

at right angles, and which do not normally form phialides in distinct verticillate whorls (i.e., the phialides are not verticillate) (Fig 3d). Also, *Acremonium* spp. do not form microsclerotia.

Other species of *Verticillium* have been found associated with spinach seed, e.g. *V. nigrescens*, which has been re-named *Gibellulopsis nigrescens* (Iglesias-Garcia *et al*., 2013; Villarroel-Zeballos *et al*., 2012). This fungus forms conidiophores that resemble those of *V. dahliae*, but does not form microsclerotia. Also, *G. nigrescens* forms tiny, black chlamydospores in NP-10 agar medium that are much smaller than microsclerotia and uniformly round, but does not form chlamydospores on blotter paper.

Verticillium tricorpus also has been observed on spinach seed, and tends to form a yellow pigment in NP-10 agar (unlike *V. dahliae*), larger microsclerotia than those of *V. dahliae*, and the microsclerotia tend to be scattered in a random pattern in and on the NP-10 agar medium whereas those of *V. dahliae* tend to form in concentric rings (Fig 2a).

General methods

Checking tolerances: Tolerances provide a means of assessing whether or not the variation in results within or among tests is sufficiently wide to raise doubts about the accuracy of the assay. 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 results 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'.

The report must indicate the size of the sample tested. In 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 such training.

Critical control points (CCP)

- Opened bottles of concentrated NaOCl should be stored in a refrigerator, and the 1.2 % NaOCl solution be prepared just prior to treating the seed sub-samples (<1 h prior). It is the responsibility of the user to demonstrate the level of activity of the chlorine solution used. Chlorine activity is quickly reduced in the presence of oxygen and organic material. Therefore, do not use the 1.2 % solution more than once. (Preparation of spinach seeds, steps 1.2 and 1.3).
- For the NP-10 method, the 10 cm × 10 cm acrylic boxes (e.g. Hoffman Manufacturing, Inc.) are included in the description; however, other size acrylic boxes or Petri dishes can be used. It is the responsibility of the user to show equivalence in performance (Materials and NP-10 method step 1.1).
- For the blotter method, acrylic boxes (20 cm × 14 cm) are included in the description; however, other size acrylic boxes (e.g. 10 cm × 10 cm acrylic boxes, Hoffman Manufacturing, Inc.) or Petri dishes (9 cm diameter) can be used. It is the responsibility of the user to show equivalence in performance (Materials and Blotter method step 1.1).
- Acrylic boxes and lids can be sterilised by spraying with 70 % isopropyl alcohol or equivalent and then air-dried in a biological safety cabinet or laminar flow hood. Optionally, if the biological safety cabinet or laminar flow hood has ultraviolet light, boxes and lids can be exposed to UV light for 10–15 min after the alcohol has dried for an additional sterilisation step; however, this step can be omitted if UV light is not available (NP-10 agar medium step 1.1 and Blotter method step 1.1).
- For the blotter method, Steel blue germination blotter paper (Anchor Paper Co.) is recommended; however,

other germination blotter paper can be used. Different weights and types of blotter paper differ in ability to absorb and release water, i.e. the type of blotter paper determines the amount of water that should be added for adequate seed imbibition as this affects the ability to kill the seed during the freezing step. It is the responsibility of the user to show equivalence in performance for the type and weight of blotter paper used. (Materials and Blotter method step 1.2).

- Sterilise the blotter papers by autoclaving them twice for at least 60 min at 121 °C, with a 24 h interval between the two autoclavings; or soaking the blotters in 70 % isopropyl alcohol or equivalent sterilant, and then drying the blotters under sterile conditions (e.g. in a laminar flow hood). (Blotter method step 1.2).
- Results of ring tests (du Toit, 2011) with a constant incubation temperature of 20 °C or constant 24 °C did not differ significantly in the incidence of seeds on which *V. dahliae* was detected (NP-10 method step 2 and Blotter method step 2).
- The ability to detect *V. dahliae* with this test can be influenced by the presence of other fungi, actinomycetes, or bacteria on the seed or in biological seed treatments. These microorganisms can influence the reliability of the test (NP-10 method step 3 and Blotter method step 5).
- It is recommended as a confirmation step that each seed on which *V. dahliae* has not yet been detected, be flipped over on the final reading to check for the presence of *V. dahliae* where the seed is in contact with the blotter (Blotter method step 5).
- The amount of water added to the blotter will affect the ability for the seed to imbibe adequately and, therefore, be killed by the freezing step. If the seed does not imbibe enough water because the blotters are too dry, the seed will not be killed during the freeze step, and will continue to germinate over the duration of the assay, making it difficult to examine the seed microscopically over multiple readings. If too much water is added to the blotters, growth of bacteria present in/on the seed will impede the development of *V. dahliae*. (Blotter method step 1.3).

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.

NP-10 agar medium

(Modified from Sorenson's NP-10 agar medium, Kabir *et al*., 2004; Sorensen *et al*., 1991.)

Bottle A components	Quantity
Polygalacturonic acid, Na salt from orange, SIGMA Grade (P-3889)	5.0 g
NaOH Pellets (0.025 N) [a]	1.2 g
Distilled or deionised water up to	500 ml

[a] NaOH concentration in bottle A becomes approximately 0.025 N.

Bottle B components	Quantity
Agar (Difco Bacto)	15.0 g
KNO_3	1.0 g
KH_2PO_4	1.0 g
KCl	0.5 g
$MgSO_4 . H_20$	0.5 g
Tergitol NP-10	0.5 ml
Distilled or deionised water up to	500 ml
Chloramphenicol stock solution [a]	0.5 ml
Streptomycin sulphate stock solution [b]	2.0 ml
Chlortetracycline hydrochloride stock solution [c]	3.3 ml

[a, b, c] Add after autoclaving.

Bottle A + Bottle B (once cooled to 50 °C)	Quantity
Bottle A	500 ml
Bottle B	500 ml
Total volume	1000 ml

Preparation

1. Prepare and autoclave (120 °C for 20 min) the contents of Bottle A and Bottle B separately.
2. Cool both bottles to 50 °C slowly (e.g. by placing the bottles in a hot water bath set at 50 °C).
3. Prepare a stock solution of chlortetracycline (15 mg/ml methanol), chloramphenicol (100 mg/ml methanol), and streptomycin sulphate (25 mg/ml deionised or distilled, sterilised water); and filter-sterilise each. Store the stock solutions in a refrigerator.
4. Add the appropriate amount of each antibiotic stock solution to Bottle B and mix well.
5. Add the contents of Bottle A to Bottle B.
6. Mix thoroughly using a magnetic stir plate and stir bar.
7. Dispense 35 ml of molten NP-10 agar medium into each sterilised acrylic box (10 cm × 10 cm) or 25 ml into each 9 cm diameter Petri dish (40 plates/l of NP-10 agar medium).

Storage

Store prepared acrylic boxes with NP-10 agar medium at 4–10 °C in the dark for no more than 4 weeks.

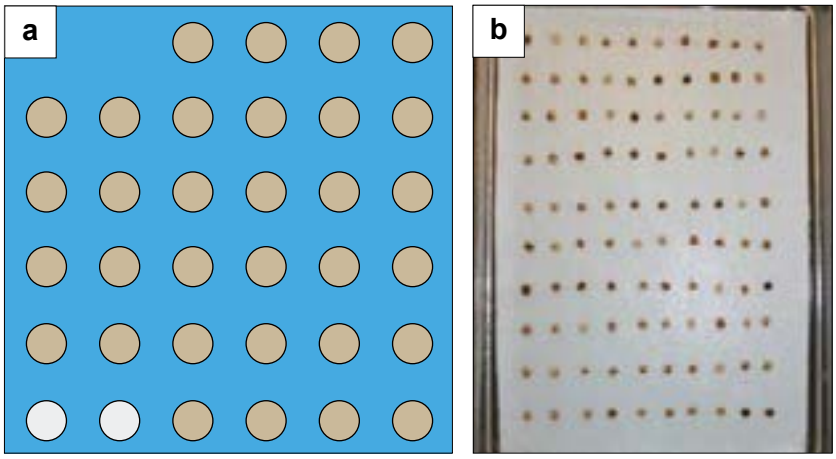

Figure 1. a Drawing (not to scale) showing an example of the layout of 34 spinach seeds on a Steel blue blotter or NP-10 agar in a 10 cm × 10 cm Hoffman acrylic box. **b** Photograph showing 100 spinach seeds on blotter paper in a 20 cm × 14 cm acrylic box. Ultimately, size and shape of the box compel seed arrangement.

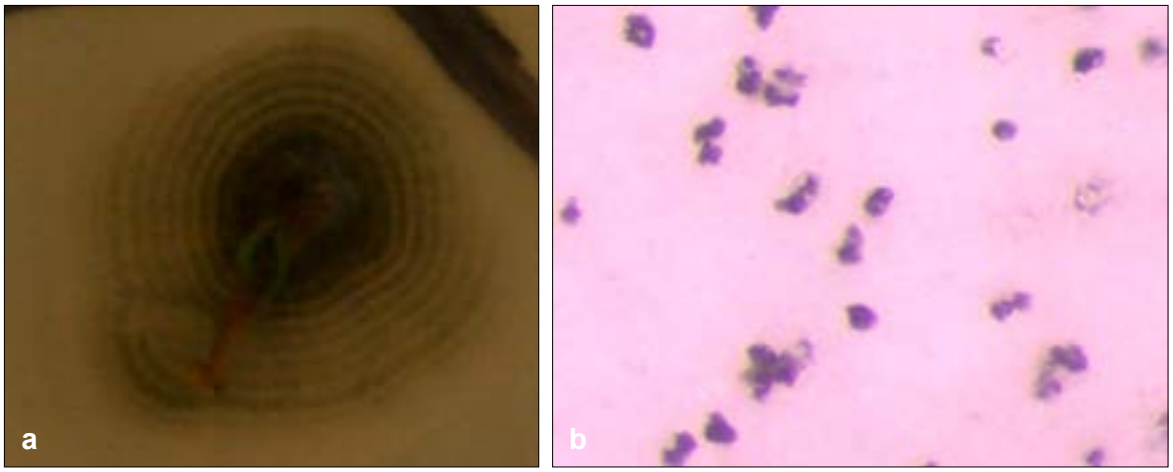

Figure 2. a Microsclerotia of *Verticillium dahliae* formed in concentric rings on NP-10 agar medium (no magnification, photograph courtesy of B. Brenner). **b** Microsclerotia of *Verticillium dahliae* on a blotter (60× magnification, photograph courtesy of G. Hiddink).

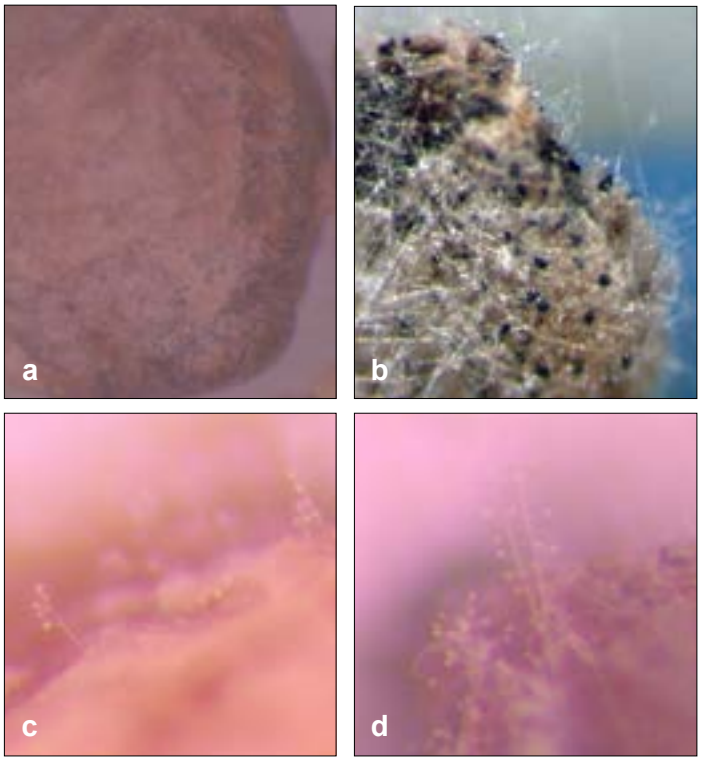

Figure 3. Typical *Verticillium dahliae* structures on spinach seed. **a** Microsclerotia of *V. dahliae* (photograph courtesy of G. Hiddink). **b** Mycelium and microsclerotia of *V. dahliae* (photograph courtesy of L. du Toit). **c** Conidiophores of *V. dahliae* (photograph courtesy of G.Hiddink). **d** Conidiophores and mycelium of an *Acremonium* spp. (photograph courtesy of G.Hiddink).

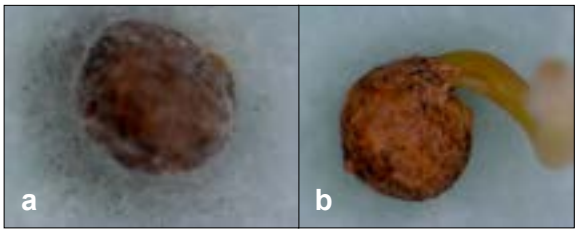

Figure 4. a Viable microsclerotia of *Verticillium dahliae* growing on a blotter versus, **b** non-viable microsclerotia on a spinach seed, based on the lack of microsclerotia forming on the blotter plus lack of conidiophores observed on the pericarp after incubating the plated seed for 14 to 21 d (photographs courtesy of E. Gilijamse).

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