Methods for the Detection of *Verticillium dahliae* on Spinach seed

Crop: Spinach (*Spinacia oleracea*)
Pathogen: *Verticillium dahliae*
Revision history: Version 1.0, July 2017

Sample and sub-sample size

The test is done on a minimum sample size of 400 seeds with a maximum sub-sample size of 100 seeds.

Principle

- o Detection of *Verticillium dahliae* (*V. dahliae*) by incubation of non-treated seeds on NP-10 agar medium (9, 12) or of non-treated seeds and fungicide-treated seeds on blotter paper (1, 2, 3, 4, 5, 9, 10, 16, 17).
- o Identification of the fungus by microsclerotia and conidial morphology.

Restrictions on Use

General

- o Both test methods, NP-10 and blotter, are 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.
- o The ability to detect *Verticillium dahliae* with this test can be influenced by the presence of other fungi, actinomycetes, or bacteria in or on the seed. These microorganisms can influence the reliability of the test.

NP-10 method

- o This method is **not** recommended for fungicide-treated seeds or seeds treated with biological control agents **if** the objective of the assay is to assess how well a treatment on the seed works against seed borne *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 seed borne *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

Note: The section **Validation** has been updated.

of the treatment against any seed borne *V. dahliae* and resulting in increased levels of detection.

Blotter method

- Apart from non-treated seeds and seeds treated with physical (e.g. hot water) or chemical (e.g. chlorine) processes (10), the blotter method is suitable for fungicide-treated seeds (6, 7, 8, 9) and seeds treated with biological control agents (2).

Validation

Two comparative tests organized by ISHI-Veg evaluate the efficacy of NP-10 agar medium and blotter paper methods for the detection of *Verticillium dahliae* on non-treated and fungicide-treated spinach seed.

Results of the comparative test were approved by ISTA in 2017 and the ISTA Rule 7-032 will come into effect in January 2018.

Materials

Blotter method

- Acrylic boxes of 20 cm x 14 cm (e.g. DBP Plastics) or
- Acrylic boxes of 10 cm x 10 cm (e.g. Hoffman Manufacturing, Inc.) or
- Petri dishes of 9 cm diameter (e.g. Greiner)
- Steel blue germination blotter paper (Anchor Paper Co.; www.anchorpaper.com) or All Paper T 10 D 550 gr/m2 (All Paper B.V., www.allpaper.nl) or equivalent

NP-10 method

- Acrylic boxes of 10 cm x 10 cm (Hoffman Manufacturing, Inc.)

NOTE: Acrylic boxes and Petri dishes of other sizes can also be used. Blotting paper of different weights can also be used. However, different weights and types of blotter paper differ in their ability to absorb and release water. The type of blotter paper used determines the amount of water that should be added for adequate seed imbibition and killing the embryos during freezing.

It is the responsibility of the user to show equivalence in performance for different sized containers and the type and weight of blotter paper used.

Method description

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-sterilized 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 seconds with constant manual agitation of the strainer to keep the seeds swirling throughout the 60 seconds. Make sure that the volume of 1.2% NaOCl solution covers the tea strainer fully

NOTE: Opened bottles of concentrated NaOCl should be stored in a refrigerator, and the 1.2% NaOCl solution should be prepared just prior to treating the subsamples of seed (<1 hour prior to seed sterilization). 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% NaOCl solution more than once.

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 sterilized, de-ionized/or distilled water in a small glass beaker for 30 seconds with constant agitation. Use enough water to fully immerse the tea-strainer and seeds.

1.4. Repeat the rinse step two more times, using a new batch of sterilized, deionized/distilled water for each rinse.

1.5. Using aseptic technique, spread the seeds onto dry, sterilized paper towel in a laminar flow hood or biological safety cabinet to dry thoroughly for at least 60 minutes.

1.6. Using aseptic techniques, place the surface-sterilized, dried seeds in a sterilized, disposable Petri dish or other sterilized container. Seeds should be stored at room temperature (approximately 22-25^{o}C) for no longer than 24 hours before plating.

2. Seed plating and incubation

<u>NP-10 method</u>

2.1. Use sterilized acrylic boxes and lids or sterilized Petri dishes to which NP-10 agar has been added.

NOTE: Acrylic boxes and lids can be sterilized by spraying with 70% isopropyl alcohol or equivalent and then air-drying the boxes and lids 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 minutes after the alcohol has dried for an additional sterilization step; however, this step can be omitted if UV light is not available.

2.2. Use flame-sterilized forceps and aseptic techniques in a laminar flow hood to plate spinach seeds onto NP-10 agar medium in each box (maximum of 34 seeds in each 10 cm x 10 cm acrylic box as shown in Fig. 1A). To plate 100 seeds per subsample, use three boxes, with 34 seeds in each of two boxes and 32 seeds in the third box.

2.3. Press each seed into the agar medium slightly to prevent seeds from rolling around when the boxes are moved. Close each box with a sterilized lid.

2.4. Incubate seeds at 20-24^{o}C under a day/night cycle of 12 hours light (with near-ultraviolet and cool white fluorescent light) and 12 hours dark.

<u>Blotter method</u>

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2.2. Sterilize the blotter papers by autoclaving the blotters twice for at least 60 minutes, at 121^{o}C with a 24-hour-interval between the two autoclavings; or soaking the blotters in 70% isopropyl alcohol or equivalent sterilant. Then dry the blotters under sterilized conditions (e.g. in a laminar flow hood).

2.3. Aseptically, in a laminar flow hood for each subsample of 100 seeds, soak the blotter paper with sterilized water and place the blotter in a sterilized acrylic box or Petri dish.

2.4. Drain off any excess water from the blotter paper and place the sub-sample of seeds on the moistened blotter paper, with enough boxes or Petri dishes to plate 100 seeds per subsample (maximum of 100 seeds on the blotter in each 20 cm x 14 cm acrylic box or a maximum of 34 seeds in each 10 cm x 10 cm acrylic box or a maximum of 25 seeds in each 9 cm-diameter Petri dish). Place a sterilized lid on each container (Fig. 1A-1B).

NOTE: The amount of water added to the blotter will affect the ability of 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 or on the seed will impede the development of *V. dahliae*.

2.5. Incubate the seeds in an incubator at 20-24°C for 24-25 hours in the dark to imbibe water from the blotters. Larger spinach seeds may need to imbibe for the longer duration to ensure adequate imbibition prior to the freezing step.

2.6. Transfer the seeds to a freezer at -18 to -22°C for 24-25 hours 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.

2.7. After freezing, incubate the seeds in an incubator at 20-24°C under a day/night cycle of 12 hours light (with near-ultraviolet and cool white fluorescent light)/12 hours dark. A constant incubation temperature of 20ºC or 24ºC gave similar results in comparative tests.

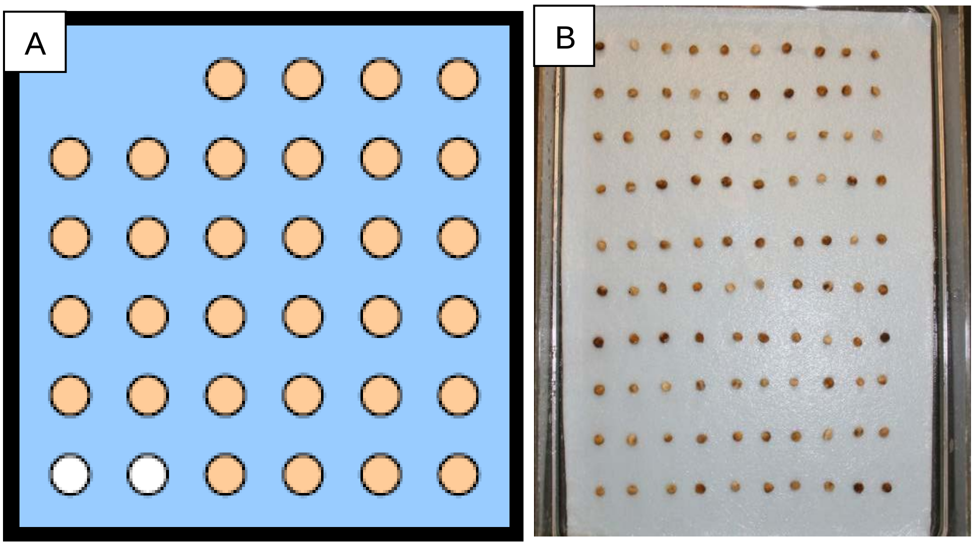

Fig. 1A. Drawing (not to scale) representing the layout of 34 spinach seeds on a Steel blue blotter or NP-10 agar medium in a 10 cm x 10 cm Hoffman acrylic box, and **1B.** Photo showing 100 spinach seeds on a blotter paper in a 20 cm x 14 cm acrylic box.

3. Positive control isolate of *V. dahliae*

For the blotter method, plate aseptically seeds of a known *V. dahliae*-infected spinach seed lot on a blotter in sterilized 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.

4. Identification

<u>NP-10 method</u>

4.1. Examine the seeds for development of *V. dahliae* using a dissecting microscope (i.e. 8 - 100x magnification) at 5, 9, and 14 days after plating.

4.2. The lid of each box or Petri dish must be removed for detailed examination of the seeds microscopically.

4.3. Compare any fungal growth to the positive control isolate or seed lot, and count the number of *V. dahliae*-infected seeds on each of the three reading days.

4.4. Typical structures are:

4.4.1. *V. dahliae* microsclerotia: Black survival structures that are masses of melanized fungal cells, and that range from 10 to 230 µm in diameter (Fig. 2A, Fig. 3A-3B) (14, 15).

4.4.2. *V. dahliae* 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 (Fig. 3B-3C). *Verticillium* spp. can readily 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 day readings, from which individual conidiophores branch off at right angles, and which do not normally form phialides in distinct verticillate whorls (Fig. 3D). Also, *Acremonium* spp. do not form microsclerotia (9, 10).

4.5. Other species of *Verticillium* can also be detected on spinach seed.

NOTE: Other species of *Verticillium* have been found associated with spinach seed, such as *Verticillium nigrescens*, now re-named *Gibellulopsis nigrescens* (12, 17). This fungus forms conidiophores that resemble those of *V. dahliae*, but does not form microsclerotia. *G. nigrescens* also forms tiny, black chlamydospores in NP-10 agar medium that are much smaller than microsclerotia of *V. dahliae* and uniformly round, but chlamydospores do not form readily on blotter paper.

V. tricorpus also has been observed on spinach seed, and tends to form a yellow pigment in NP-10 agar medium, 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). Chlamydospore of *Gibellulopsis nigrescens*: a black, thick-walled, asexual, resistant spore produced by the fungus.

4.6. To be able 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 agar medium paper is an indication that the *Verticillium* on that seed is not viable.

<u>Blotter method</u>

4.1. Examine the seeds for development of *V. dahliae* using a dissecting microscope (e.g. 8 - 100x magnification) at 5, 9, and 14 days after plating non-treated seeds; and 5, 9, 14 and 21 days after plating treated seeds (5, 6, 7, 8, 9, 10). The fourth reading at 21 days is needed if any seed treatments slow the development of fungi like *V. dahliae*.

4.2. The lid of each box or Petri dish must be removed for detailed examination of the seeds microscopically.

4.3. Compare fungal structures on the seed to the positive control seed or isolates of *V. dahliae*, and count the number of *V. dahliae*-infected seeds at each reading.

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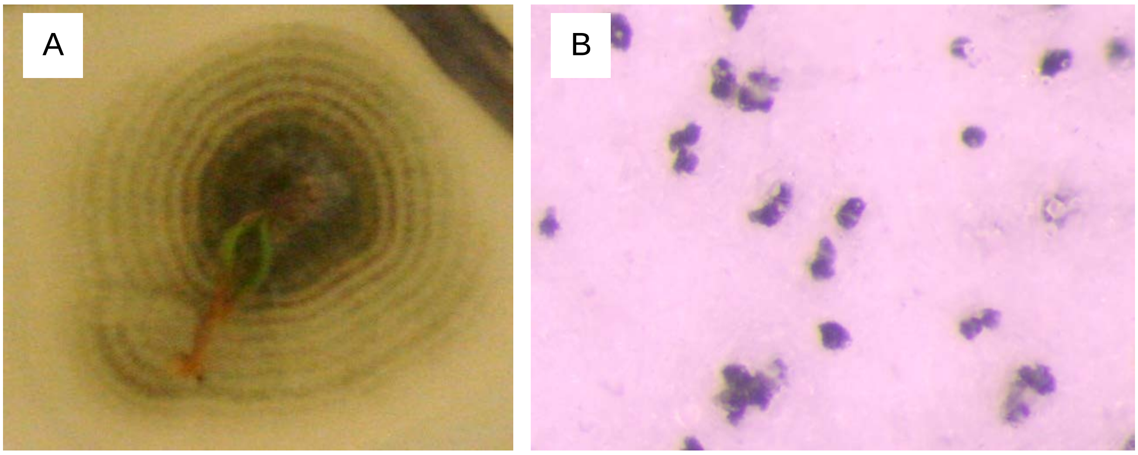

Fig. 2A. Microsclerotia of *V. dahliae* formed in concentric rings on NP-10 agar medium (no magnification provided, photo courtesy of B. Brenner), and **2B**. Microsclerotia of *V. dahliae* on a blotter (60x magnification, photo courtesy of G. Hiddink).

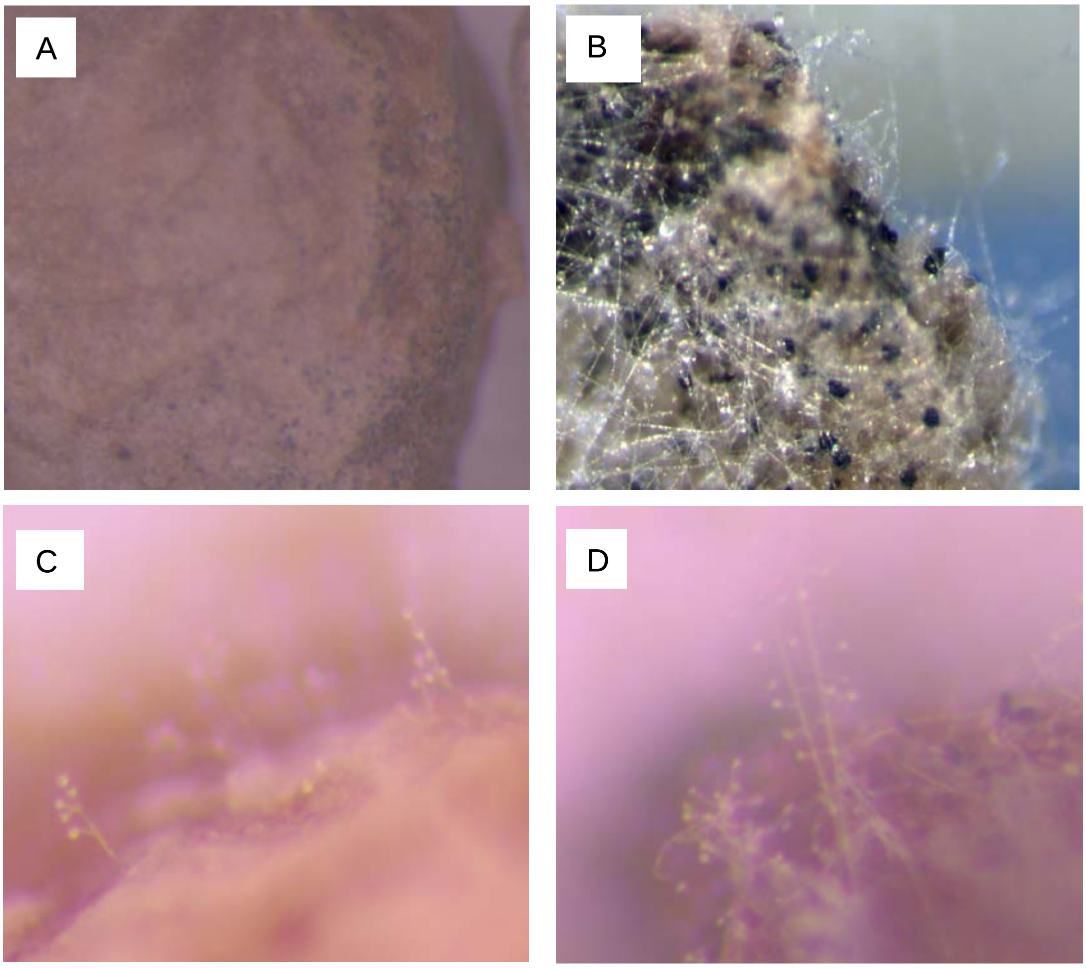

Fig. 3. Typical *V. dahliae* structures on spinach seed. **A**. Microsclerotia of *V. dahliae* (photo courtesy of G. Hiddink). **B.** Mycelium and microsclerotia of *V. dahliae* (photo courtesy of L. du Toit). **C.** Conidiophores of *V. dahliae* (photo courtesy of G. Hiddink). **D.** Conidiophores and mycelium of an *Acremonium* spp. (photo courtesy of G. Hiddink).

Fig. 4A. Viable microsclerotia of *Verticillium dahliae* growing on a blotter versus and **B**. non-viable microsclerotia on a spinach seed based on the lack of microsclerotia forming on the blotter plus the lack of conidiophores observed on the pericarp after incubating the plated seed for at least 14 days (photos courtesy of E. Gilijamse).

Buffers and media

NP-10 agar medium (13, 15)

Bottle A components	Quantity (in ml or g/500 ml)
Polygalacturonic acid, Na salt from orange, SIGMA Grade (P-3889)	5.0 g
NaOH pellets [a] (0.025N)	1.2 g
Add distilled or de-ionized water up to	500 ml

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

Bottle B components	Quantity (in ml or g/500 ml)
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
Add Distilled or de-ionized water up to	500 ml
Chloramphenicol stock solution [a]	0.5 ml
Streptomycin sulfate stock solution [b]	2.0 ml
Chlortetracycline hydrochloride stock solution [c]	3.3 ml

[a, b, c] Add after autoclaving

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

1. Preparation of NP-10 agar medium (modified Sorenson's NP-10 agar medium (13, 15). Prepare and autoclave (120°C for 20 min) the contents of Bottle A and Bottle B separately.
2. Cool both bottles to 50ºC slowly (e.g. by placing the bottles in a hot water bath set at 50ºC).
3. Prepare a stock solution of chlortetracycline (15 mg/ml methanol), chloramphenicol (100 mg/ml methanol), and streptomycin sulfate (25 mg/ml deionized or distilled, sterilized water); and filter-sterilize 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 sterilized acrylic box (10 cm x 10 cm) or 25 ml into each 9 cm-diameter Petri dish (40 plates/liter of NP-10 agar medium).

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