International Rules for Seed Testing 2024

Validated Seed Health Testing Methods

7-015: Detection of *Epichloë coenophiala* in *Festuca* spp. (fescue) and of *Neotyphodium lolii* in *Lolium* spp. (ryegrass) 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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7-015: Detection of *Epichloë coenophiala* in *Festuca* spp. (fescue) and of *Neotyphodium lolii* in *Lolium* spp. (ryegrass) seed

Host: *Festuca* spp., *Lolium* spp.
Pathogen(s): *Epichloë coenophiala* (Morgan-Jones & W. Gams) C.W. Bacon & Schardl; *Neotyphodium lolii* (Latch, M.J.Chr. & Samuels) Glenn, C.W.Bacon & Hanlin

Author: N. Hill, Dept. Crop and Soil Sciences, University of Georgia, Athens, GA, USA
E-mail: nhill@arches.uga.edu

Revision history

Version 1.0, 2003-01-01
Version 1.1, 2014-01-01: Reference material mandatory; common name of host added
Version 1.2, 2017-01-01: Reporting results revised
Version 1.3, 2018-01-01: Pathogen names changed
Version 1.4, 2021-01-01: Sample preparation changed to Sample size and paragraph revised
Version 1.5, 2024-01-01: Sample size revised

Background

This proprietary test kit (an immunological method) was compared with a microscopic method (Anon., 1998; Welty *et al.*,1985) in a comparative test conducted in 1999 on six samples (three tall fescue and three perennial ryegrass) in four laboratories with three blind replicates of each sample (Hill *et al.*, 2002). Mean infection levels were similar for both the immunoblot and microscopic methods and intra- and inter-laboratory variation were essentially the same. Thus, the immunoblot and microscopic methods for endophyte detection were considered to provide comparable results. The immunoblot method is more rapid than the microscope method and requires less operator training. Where large numbers of seed lots are tested this has the advantage of reducing the amount of time the analyst spends at the microscope and the potential for fatigue.

Safety precautions

The test kit contains small quantities of sodium azide to stabilise the reagents. While the concentrations in the kits are below established toxic levels, sodium azide can be toxic to humans. Therefore, care should be taken to prevent exposure to skin, eyes, and mucous membranes. Protective gloves should be used to minimise exposure to sodium azide. Reagents should never be ingested.

Sodium azide can react with lead or copper plumbing to form explosive metal halides. Flush drains with a large volume of water to prevent azide accumulation.

Treated seed

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

Sample size

The sample size (total number of seeds to be tested) depends on intended use, the maximum acceptable infection level and the analytical sensitivity of the method. The minimum sample size should be 100 seeds.

Materials

Reference material: reference cultures or other appropriate material (included in the test kit)
Incubator: operating at 45 °C
Oven: operating at 70 °C
Shaker: orbital set at 50 rpm
Plastic container: 9 × 15 cm with lid for incubating seeds
Cellulose sponge: 7.5 × 12 cm for incubating seeds
Aluminium foil: or other opaque wrap for Petri dish
Distilled/deionised water
Petri dish
Sodium hydroxide (5 % aq.)
Test kit: test kits can be purchased from: Agrinostics Ltd. Co., 2850 Elder Mill Rd., Watkinsville, GA, 30677, USA

Methods

Critical control points are indicated by CCP.

1. Read the instruction booklet provided with the test kit prior to carrying out the assay.
2. Allow reagents to reach room temperature and gently mix prior to use (CCP).
3. Remove ergot sclerotia from seed (CCP).
4. Surface sterilise seed by placing approx. 5 ml of seed into a 50 ml beaker. Add 40 ml of 5 % sodium hydroxide and stir thoroughly, making sure all seed are wet. Leave seed in the sodium hydroxide solution for 60 min, stirring every 15 min. Rinse seed with copious amounts of water to remove residual sodium hydroxide from the seed. The subsequent steps can be performed on the wet seed or the seed may be air dried.
5. Place a 7.5 × 12.0 cm cellulose sponge on the bottom of a 9.0 × 15.0 cm plastic container. Add EB solution to the container to wet (not cover) the sponge. Place one piece of the blotting paper on top of the sponge. Using forceps or tweezers, place the nitro-cellulose membrane on top of the blotting paper. Make sure no air bubbles are between the membrane and blotting paper. Use protective gloves and do not to touch the membrane with the naked hand (CCP).
6. Place seed close together on the membrane. The membrane is large enough for 100 seeds. Place cover on the container and incubate at 45 °C overnight (16 h).
7. Remove plastic container from incubator. Peel the nitro-cellulose membrane from the blotting paper and place it onto a dry piece of blotting paper. Gently remove the seeds or excess debris from the nitro-cellulose membrane with a soft brush.
8. Dry the membrane in an oven at 70 °C for 15 min or at room temperature for 1 h.
9. Place the membrane into the bottom of a Petri dish. Add 10 ml of BWW solution, cover the dish, and shake for 30 min on an orbital shaker set at 50 rpm (CCP).
10. Remove Petri dish from the shaker and gently pour off the solution.
11. Add 10 ml of BWW solution to the MAB tube. Pour MAB solution over the membrane in the Petri dish. Shake for 1 h on the orbital shaker.
12. Remove Petri dish from the shaker and gently pour off the antibody solution.
13. Add 10 ml of BWW solution and return Petri dish to the shaker for 6 min. Pour off BWW solution. Repeat this step one more time.
14. Add 10 ml BWW solution to the RAM tube. Pour RAM solution over the membrane in the Petri dish. Shake for 1 h on the orbital shaker.
15. Add 10 ml of BWW solution and return Petri dish to the shaker for 6 min. Pour off BWW solution. Repeat this step one more time.
16. Add BWW solution to the PA tube to reach the 10 ml mark, cap and mix by inverting. Pour PA solution over the membrane and cover the Petri dish. Shaker for 30 min on the orbital shaker.
17. Add 10 ml of BWW solution and return Petri dish to the shaker for 6 min. Pour off BWW solution. Repeat this step one more time.
18. Add BN chromogen, replace lid on Petri dish, return dish to the shaker and cover with aluminium foil (CCP).
19. Check colour reaction every 5 min (CCP). Stop the reaction when the positive control spot (marked '+' on the membrane) has a dark colour by pouring off the chromogen solution and rinsing the membrane twice in 20 ml distilled water.
20. Record the number of positive *Epichloë* (for fescue) or *Neotyphodium* (for ryegrass) seeds (see Fig. 1. and Limitations to Procedure in the instruction booklet).

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

The report must indicate the number of seeds tested.

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.

Quality assurance

- Cover or cap all reagents when not in use.
- Label all vessels containing solutions to avoid improper mixing.

Critical control points (CCP)

- Do not mix or use reagents from different kits.
- Keep kit components refrigerated when not in use. Warm reagents to room temperature prior to use (Step 2).
- Avoid ergot sclerotia at all times. Remove the sclerotia from seed prior to surface sterilisation. *Claviceps purpurea* has a soluble protein similar to that in *Neotyphodium* and can give false positives if seed is contaminated. Surface sterilisation and incubation temperature are critical to avoiding false positives from *Claviceps* mycelia found in seed. Be sure to follow procedures as stated to avoid false positive readings (Step 3).
- Always handle the nitro-cellulose membrane with extreme care. Nitro-cellulose is fragile and will rip or tear easily. DO NOT touch the nitro-cellulose membrane with bare skin and always wear protective gloves to prevent contamination by fingerprints (Step 5).
- It is important to keep the reagents agitated when reacting with the membrane (Step 9).
- The chromogen is light sensitive. It is important to cover the reaction vessels with aluminium foil or a suitable opaque wrap to prevent light from entering the reaction and causing chromogen coloration (Step 18).
- It is important to check the progress of the reaction and colour development every 5 min to avoid overexposure. The positive control ('+') spot should be a dark colour and the negative control ('-') spot should be colourless (Fig. 1), indicating that the test has been performed correctly (Step 19).
- It is important that reagents are prepared using distilled/deionised water only (Preparation of media and reagents).

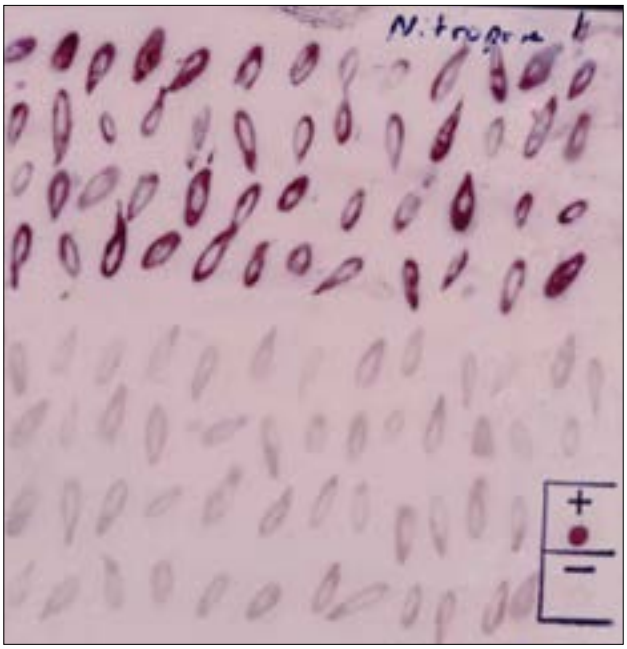

Figure 1. Membrane showing postive *Neotyphodium* (top 4 rows) and negative (bottom 4 rows) reactions and positive (+) and negative (-) controls.

Media and solutions

Preparation

1. Extraction solution should be diluted to 200 ml with distilled/deionised water only (CCP).
2. Dissolve the BWW reagents in 150 ml distilled/deionised water using a clean low density polyethylene plastic or glass container. The solution should be made immediately prior to assaying samples but can be stored at 4 °C for up to 3 d.
3. Dilute MAB, RAM, and PA with 10 ml of BWW solution just prior to each step using the respective reagents.
4. DO NOT dilute the chromogen reagent. Use it at full strength. Keep chromogen reagent out of light during storage and use.

References

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