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

7-022: Agar method for the detection of *Microdochium nivale* on *Triticum* spp.

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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: *Triticum* spp.

Pathogen: *Microdochium nivale*

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Sponsored by: ISTA Seed Health Committee, *M. nivale* Working Group.

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Background

A method for *Microdochium nivale* was originally published in the *ISTA Handbook of Seed Health Testing* in November 1964 as S.3. No. 33 (Anon.,1964), and was never revised for inclusion in the second edition of the Handbook. The agar plate test proposed is based on the comparative test organized by the ISTA Seed Health Committee and experience of routine testing in a number of laboratories. Summary of changes to original working sheet: Either potato dextrose agar (PDA) or malt agar (MA) can be used; incubation temperature reduced to 20 °C; incubation in dark followed by 3-4 h in daylight or under near ultraviolet (NUV); changes to format and layout.

Validation studies

Cockerell, V. and Roberts, A.M.I. (2007)
Copies are available by e-mail from ista.office@ista.ch, or by mail from the ISTA Secretariat.

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 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. autoclaving or disinfection) and in accordance with local health, environmental and safety regulations.

Treated seed

This method has not been validated for the determination of *Microdochium nivale* on treated seed. Seed treatments may affect the performance of the method.
(Definition of treatment: any process, physical, biological or chemical, to which a seed lot is subjected, including seed coatings. See 7.2.3)

Sample size

The total number of seeds to be tested depends on the desired tolerance standard (maximum acceptable percentage of seeds infected).

Materials

Reference material: the use of reference cultures or other appropriate material is recommended.
PDA or MA plates with streptomycin sulphate: 9.0 cm Petri dishes (one per five seeds).
Incubator: capable of operating at 20 ± 2 °C.
1% NaOCl: see page 9.

Sample preparation

The test is carried out on a working sample as described in section 7.4.1 of the *International Rules for Seed Testing*.

Methods

Critical control points are indicated by “CCP”.

1. Pretreatment

Immerse seeds in NaOCl solution (1% available chlorine) for 10 min, then drain.

2. Plating

Aseptically place a maximum of 5 seeds, evenly spaced around the perimeter of the plate, onto the agar surface of each PDA or MA plate (CCP).

3. Incubation

Incubate plates for 7 d at 20 °C in the dark.

4. Reference culture

Subculture a reference culture to a PDA or MA plate at the same time the seeds are plated and incubate with the test plates. Alternatively, a sample of seed known to be infected may be surface sterilized, plated on PDA or MA and incubated under the same conditions as the test samples.

5. Examination

Microdochium nivale

Examine the plates after 7 d incubation. *M. nivale* colonies from seed are relatively fast growing; aerial mycelium is white to very pale pink. Spores may be present, usually at colony margins, occurring as orange masses (Nelson et al., 1983). From the reverse view, the colony colour is salmon pink (CMI mycological chart, sheet 1 No. 41, Rayner, 1970) or colourless to light orange (Nelson et al., 1983). The colour is uniform throughout the entire colony. After incubation in the dark, the reverse of the colony may be white. If the suspect colonies are left in the light for a few hours, the salmon pink/orange colour should develop if *M. nivale*. Further confirmation can be made by examining the conidia. Conidia are curved, with blunt terminal cells that are difficult to distinguish from one another; the basal cell may be notched but is never foot shaped; 1-3 septate; conidia 10–30 × 2.5–5 μm; and chlamydospores are not observed (Booth, 1971; Nelson et al., 1983).

Record the number of seeds infected with *M. nivale*.

General methods

(common to many test procedures)

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. Suitable tolerances, which can be applied to most direct seed health tests, can be found in Tables 5B of Chapter 5 of the ISTA Rules, or in the *Handbook of Tolerances and Measures of Precision for Seed Testing* by S.R. Miles (*Proceedings of the International Seed Testing Association* 28 (1963) No 3, p 644).
2. **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 International Seed Analysis 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 (for example 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 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.

Critical control points

(identified in the methods by "CCP")

- Method section 2: *M. nivale*, some *Fusarium* spp and many saprophytic fungi present on *Triticum* spp are comparatively fast growing; with more than 5 seeds per plate, there is a high risk of the plates being unreadable.
- Preparation of PDA or MA plates: The source of agar may influence the results. The level of available nutrients may vary from manufacturer to manufacturer. Both PDA and MA can be bought as a powdered medium, or MA can be made up as per recipe (see below). Suitable products used in the comparative test include PDA (Oxoid, Basingstoke, UK), Cristomalt (DIFAL, Seysses, France); agar-agar (VWR, West Chester, Pa., USA) and streptomycin (Sigma-Aldrich, Mo., USA). Any equivalent products should be suitable. Whenever a new batch of agar is used, a check on the quality should be made, using a reference lot with a known infection level, or a reference isolate and sustainability of isolate measured. Pay particular attention to the growth characteristics of reference isolates.

Preparation of PDA + streptomycin

PDA (CCP): make according to specification of supplier.

Distilled/de-ionized water: 1000 mL.

Streptomycin sulphate*: may be used between 50 mg and 130 mg, depending on the level of saprophytic bacterial contamination commonly encountered.

* Added after autoclaving

Preparation

1. Weigh out ingredients into a suitable autoclavable container.
2. Add 1000 mL of distilled/de-ionized water.
3. Dissolve powdered PDA in the water by stirring.
4. Autoclave at 121 °C and 15 p.s.i. for 15 min.
5. Allow agar to cool to approximately 50 °C and add streptomycin sulphate dissolved in 70% ethanol.
6. Pour 20–22 mL of molten agar into 9.0 cm Petri dishes and allow to solidify before use.

Streptomycin sulphate

Streptomycin sulphate can be dissolved in either 70% ethanol or water. Filter sterilization is required if dissolved in water.

Storage

Prepared plates may be stored at 4 °C for up to 6 weeks.

Preparation of MA + streptomycin

Agar-agar: 20 g
Malt: 10 g
Distilled/de-ionized water: 1000 mL
Streptomycin sulphate*: may be used between 50 mg and 130 mg, depending on the level of saprophytic bacterial contamination commonly encountered.
* Added after autoclaving

Preparation

1. Weigh out the malt into a suitable container. Add two volumes of the distilled/de-ionized water per volume of malt, and bring to boiling point.
2. Add the agar-agar and the remainder of the water, and dissolve completely before autoclaving.
3. Autoclave at 121 °C and 15 p.s.i. for 15 min.
4. Allow agar to cool to approximately 50 °C and add streptomycin sulphate dissolved in 70% ethanol.
5. Pour 20–22 mL of the molten agar into 9.0 cm Petri dishes and allow to solidify before use.

Streptomycin sulphate
Streptomycin sulphate can be dissolved in either 70% ethanol or water. Filter sterilization is required if dissolved in water.

Storage

Prepared plates may be stored at 4 °C for up to 6 weeks.

Preparation of sodium hypochlorite solution

Sodium hypochlorite for pretreatment of seed can be prepared from fresh 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 in %) to calculate the volume of commercial bleach stock solution required to prepare sodium hypochlorite solutions for use in seed pretreatment.

Example

To prepare 1000 mL of a solution of sodium hypochlorite containing 1% chlorine from a stock of commercial bleach containing 12% available chlorine:

$V_{stock} = 1000 \times 1/12 = 83$

Hence, add 83 mL of the 12% stock to 917 mL water.

Alternatively, sodium hypochlorite solutions can be prepared by using sodium dichloroisocyanurate tablets (e.g. Presept, Johnson and Johnson, Skillmann, NJ, USA) according to the manufacturer's instructions.

References

Anonymous (1964). Working Sheet S3 No. 3. In *International Seed Testing Association Handbook on Seed Health Testing,* International Seed Testing Association, Basserdorf, Switzerland.

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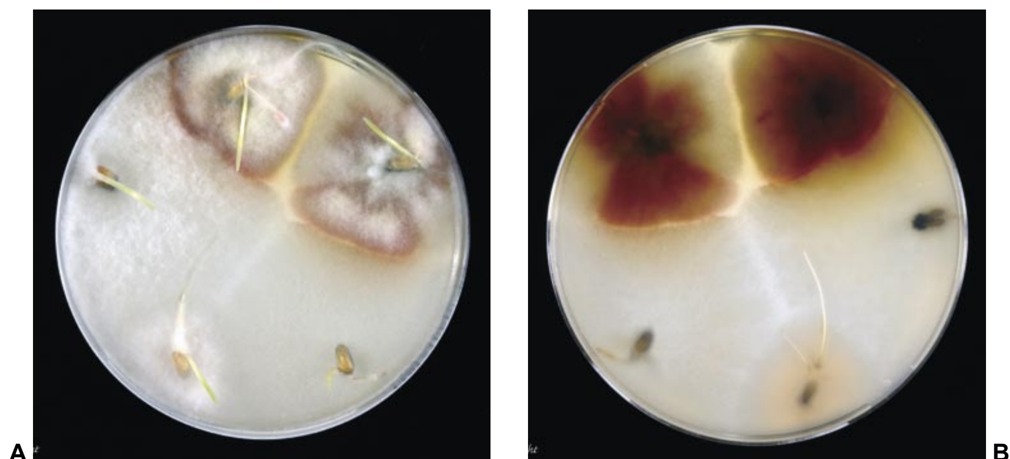

Fig. 1. Colonies of *Microdochium nivale* on PDA after 7 days incubation in the dark. **A** Top surface of plate. **B** Bottom surface of plate.

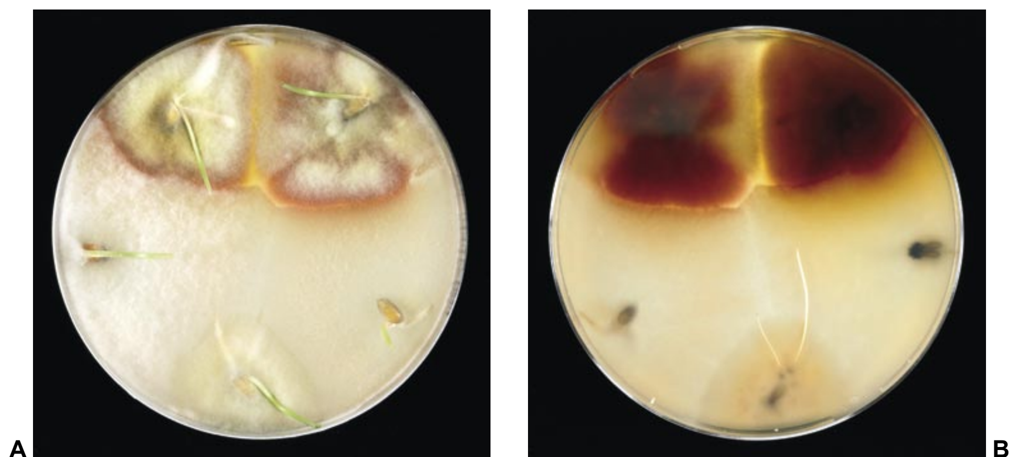

Fig. 2. Colonies of *Microdochium nivale* on PDA after 7 days incubation in the dark plus 4 h under NUV. **A** Top surface of plate. **B** Bottom surface of plate.