International Rules for Seed Testing 2022

Validated Seed Health Testing Methods

7-013a: Detection of *Ustilago nuda* in *Hordeum vulgare* subsp. *vulgare* (barley) seed by embryo extraction

Including changes and editorial corrections adopted at the online Ordinary General Meeting 2021

Effective from 1 January 2022

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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7-013a: Detection of *Ustilago nuda* in *Hordeum vulgare* subsp. *vulgare* (barley) seed by embryo extraction

Host: *Hordeum vulgare* L. subsp. *vulgare*
Pathogen(s): *Ustilago nuda* (Jens.) Rostr.

Prepared by: ISTA-PDC Method Validation Sub-committee

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Revision history

Version 1.0, 2001-11-20
Revised 2001-11-20 J. Sheppard, V. Cockerell
Reprinted 2003
Version 1.1, 2008-01-01: Treated seed revised; Reporting results revised
Version 1.2, 2012-01-01: Renumbered 7-013a following introduction of method 7-013b
Version 1.3, 2013-01-01: Definition of sample preparation
Version 1.4, 2014-01-01: Examination: comparison with positive control added
Version 1.5, 2017-01-01: Reporting results revised
Version 1.6, 2021-01-01: Sample preparation changed to Sample size and paragraph revised; Methods revised
Version 1.7, 2022-01-01: Background, Materials, Methods, Media and solutions, and Validation references revised

Background

This method was originally published in the *ISTA Handbook of Seed Health Testing* in November 1964 as S.3. No. 25 and revised in 1988 by W J Rennie, Agricultural Scientific Services, East Craigs, Edinburgh, Scotland. The method was incorporated into the newly revised *Annexe to Chapter 7* in 2002 from the 1999 edition of the ISTA Rules. The method was reviewed by the ISTA Seed Health Committee (SHC) in 2006 (Cockerell & Koenraadt, 2007) with the recommendation to accept for a further five years.

Based on a method validation study carried out by Science and Advice for Scottish Agriculture (SASA) for the ISTA Seed Health Committee, coupled to a Proficiency Test (PT) and a report 'Using methyl blue to stain *Ustilago nuda* hyphae in ISTA Methods 7-013a and 7-013b', an option of methyl blue stain was added and could help laboratories that have difficulty identifying hyphae in the scutellum.

Safety precautions

When preparing the aqueous sodium hydroxide solution, it is essential that the operation is carried out in a well-ventilated room; the analyst should wear full protective clothing.

This procedure may involve the handling of chemically treated seed. Analysts should familiarise themselves with the risks attributed to chemical treatments by reference to the material safety data sheets. Contact by inhalation, skin absorption or ingestion must be avoided.

Treated seed

This method can be used to detect *Ustilago nuda* in untreated seed and in seed where a chemical seed treatment has been applied. (Definition of treatment: any process, physical, biological or chemical, to which a seed lot is submitted. See 7.2.3.)

Sample size

The sample (total number of seeds tested) and subsample size to be tested depend on the desired tolerance standard (maximum acceptable percentage of seeds infested) and detection limit (theoretical minimum number of pathogen propagules per seed which can be detected). The working sample consists of 100–120 g containing 2000–4000 seeds depending on TSW. A minimum of 1000 embryos are examined.

Materials

Reference material: seed known to be infected or other appropriate material
Incubator: operating at 20 ±2 °C
Fume cupboard
Brass sieves: 1 mm mesh (2 additional sieves of larger mesh size can be useful; see point 2.3)
Microscope: with substage illumination, ×25 and ×50 magnification
5 % sodium hydroxide: see below
Lactic acid solution: see below
Optional methyl blue: see below
Glycerol (glycerine)

Sample preparation

This test can be carried out on both treated and untreated seed. Further sample replicates can be tested if required.

Methods

1. Extraction and clearing of embryos

1.1 Place the seeds in 1 l of a freshly prepared 5 % aqueous solution of sodium hydroxide (NaOH) and maintain at 20 °C for 24 h.
CCP A weaker solution of NaOH or a lower temperature makes extraction difficult.

1.2 After soaking, the entire sample should be transferred to a suitable container and washed in warm water to separate the embryos, which appear through the softened pericarps.

1.3 Collect the embryos in a sieve of 1 mm mesh. Additional sieves of larger mesh can be used to collect pieces of endosperm and chaff.

1.4 Transfer the embryos to a mixture of equal quantities of glycerol and water in which further separation of the embryos and chaff can be made.

1.5.1 Transfer the embryos to a beaker containing 50 ml of lactic acid solution and clear them by maintaining the lactic acid solution at boiling point for up to 5 min in a fume cupboard; or

1.5.2 Transfer the embryos to a beaker containing 50 ml of lactic acid solution with methyl blue and clear them by maintaining the lactic acid solution at boiling point for up to 5 min in a fume cupboard.

1.6 Transfer the embryos to fresh glycerol for examination. The scutellum becomes more transparent when embryos are left in glycerol for 1–2 h, making examination easier.

2. Examination

2.1 Examine embryos at ×16–25 magnification with adequate substage illumination for the characteristic golden brown mycelium of *U. nuda*.

2.2 Mycelium is approximately 3 µm thick, is golden brown in colour and visible without a stain (Fig. 1). Infection may vary from a few strands of short hyphae to complete invasion of the scutellum tissues. Occasionally fungi other than *U. nuda* occur in the scutellum but are usually darker in colour and quite distinct. When cell walls become discoloured they may be confused with mycelium of *U. nuda*, but this can be checked by examination at ×50 or higher magnification (Fig. 2). Compare with positive control (reference material).

General methods

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

Critical control points (CCP)

Where the wording of the original Working Sheet suggests that an action is critical this has been marked with CCP.

Media and solutions

5 % sodium hydroxide solution – option A

The exact concentration of the sodium hydroxide solution is not critical.

Sodium hydroxide pellets: 50 g
Cold tap water: 1 l

Preparation

1. Weigh 50 g sodium hydroxide pellets.
2. Dissolve sodium hydroxide pellets in 1 l of cold tap water. It is important to ensure that all the pellets are completely dissolved and this necessitates constant stirring with a metal rod.

5 % sodium hydroxide solution – option B

Sodium hydroxide can be purchased as 50 % stock solution.

Sodium hydroxide 50 %solution: 100 ml
Cold tap water: 900 ml

Preparation

Add 100 ml of 50 % stock solution to 900 ml cold tap water.

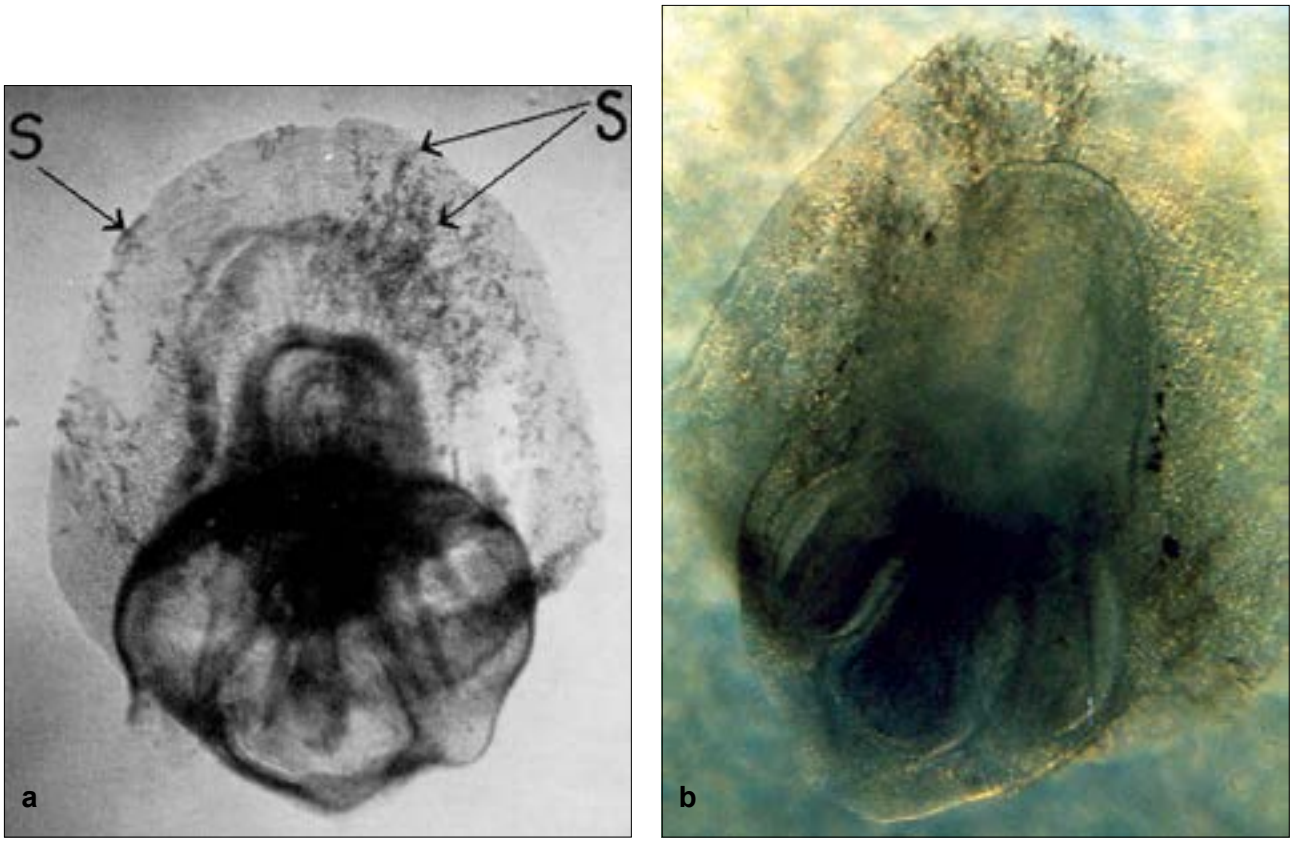

Figure 1. Infected embryo, smut mycelium at S in scutellum.

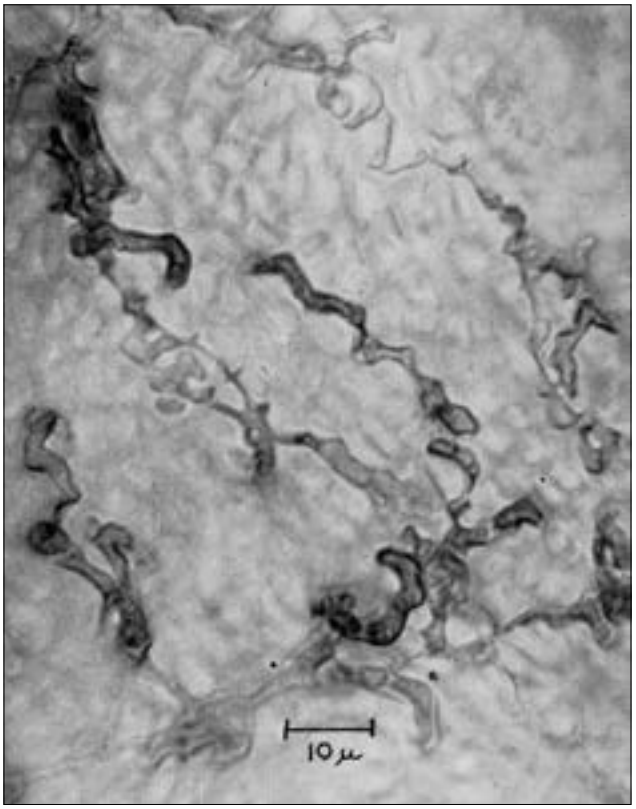

Figure 2. Smut mycelium in scutellum.

Lactic acid solution

Glycerine: 333.3 ml
Lactic acid (90 % pure, minimum assay 88 % purity): 333.3 ml
Water: 333.3 ml

Preparation

1. Add equal parts of glycerine, lactic acid and water. Mix thoroughly.
2. Final solution should be clear and almost colourless. The solution will turn yellow with age and exposure to light. Store in amber bottle and avoid exposure to light.

Option – Lactic acid solution with methyl blue: Add 0.16 g/l of methyl blue to lactic acid solution. Mix thoroughly.

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The following references are extracted from the *ISTA Handbook of Seed Health Testing*, Working Sheet No. 25, W. J. Rennie, 1988.

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Validation references

Studied in international comparative testing: 1960, 1963, 1964, 1979.

Comparative tests organised by the ISTA Plant Diseases Committee (now Seed Health Committee) gave reasonable agreement between stations when samples with more than 1.0 % infection were tested by stations experienced in the test procedure (Rennie, 1978; Tempe, 1976).

ISTA (2021). Using methyl blue to stain *Ustilago nuda* hyphae in ISTA Methods 7-013a and 7-013b. Method Validation Reports. International Seed Testing Association, Bassersdorf, Switzerland.