International Rules for Seed Testing 2024

Validated Seed Health Testing Methods

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

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

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

Prepared by: ISTA Seed Health Committee, *Ustilago nuda* Working Group and Nordic *Ustilago* Working Group

Submitted by: Nordic Seed Pathology Working Group and ISTA Seed Health Committee

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Revision history

Version 1.0, 2010-10-01
Version 1.1, 2014-01-01: Addition of positive control
Version 1.2, 2017-01-01: Reporting results revised
Version 1.3, 2021-01-01: Sample preparation changed to Sample size and paragraph revised
Version 1.4, 2022-01-01: Background, Materials, Methods, Media and solutions, and Validation references revised
Version 1.5, 2024-01-01: Sample size and Figures revised

Background

Since the 1970s, the Nordic laboratories have used a modification of ISTA method 7-013a described by Joelson (1968). The method described here differs from 7-013a in the embryo extraction technique and the procedure used to clear embryos for examination of the *Ustilago* mycelium. A validation study comparing the two methods was carried out. Three seed lots with infection levels between 1 % and 4 % were tested by three laboratories using both the current method 7-013a and the 'Nordic' method (7-013b). The validation study shows that the two methods produce equivalent results (Sperlingsson, 2011). The Nordic method offers an alternative method for laboratories that do not have access to plentiful warm water, nor a fume hood. The alternative embryo-clearing process adds a day to the duration of the test, so may not be suitable where a quicker turnaround is required. It does, however, offer an alternative clearing procedure which could be used in combination with the existing method to provide flexibility of resources within laboratories during busy periods.

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

Great care must be taken when working with sulphuric acid and sodium hydroxide; the analyst should wear full protective clothing.

Treated seed

This method has not been validated for the determination of *Ustilago nuda* 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 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 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
Oven: capable of operating at 75 ±5 °C
Electric hand mixer: at low speed

Brass sieves: 1 mm mesh, with one additional sieve of larger mesh (approx. 2.4 mm) and an additional fine sieve with mesh smaller than 1 mm
Microscope: with substage illumination
Sulphuric acid (H_2SO_4): concentration 25–37 % by weight
Sodium hydroxide + sodium chloride: 10–15 % NaOH plus 110–175 g salt per litre of solution
Lactic acid: more than 70 %
Optional methyl blue: see below
Glycerol (glycerine)
95 % ethanol
Glycerol-ethanol solution: one part glycerol to two parts ethanol

Methods

Dehulling

1. Place the working sample in a glass beaker with 25–37 % H_2SO_4 until the seeds are covered.
2. Incubate in an oven at 75 °C for 50 min or until the seeds turn a medium-brown colour.
3. Carefully pour off the H_2SO_4 solution. Rinse seeds by pouring water into the beaker, gently mix and pour out the water. Add new water and remove the loosened hulls by stirring robustly with a rod. Remove hulls by carefully removing the water. If hulls remain, add new water, and either use an electric hand mixer at low speed (maximum 3 min) or continue stirring. Repeat procedure until all hulls are removed. Be careful not to lose any kernels (seed without hulls).

Embryo extraction

4. Place drained kernels in a container with the NaOH-NaCl solution.
5. Incubate overnight (approximately 15 hours) in 22 ±3 °C.
6. Stir mixture gently to loosen the embryos from the kernels. Pour the loosened embryos which float to the top of the liquid into a beaker.
7. Repeat the procedure until all embryos are released.
8. To ensure that there are no remaining embryos, place the dissolved kernels on top of a coarse sieve combined with a fine sieve. The coarse sieve must have a mesh of approximately 2.4 mm, enough to let the embryos pass but retain the remains of the kernels. The fine sieve should have a mesh of 1 mm. If there are any embryos in the bottom sieve, add these to the beaker.
9. Using a fine sieve, drain the NaOH-NaCl solution from the embryos and rinse in running water for approximately 10 s.
10. If there is a large amount of chaff with the embryos, add water and remove the floating chaff.

11.1 Drain the embryos, place in a beaker and cover with lactic acid; or
11.2 Drain the embryos, place in a beaker and cover with lactic acid solution with methyl blue.

12. Incubate overnight in an oven at 75 ±5 °C
13. Using a fine sieve, drain the lactic acid from the embryos. The embryos can be made more transparent by being washed in ethanol or covered for a few minutes in 95 % ethanol. Cover the embryos with glycerol-ethanol (95 %)(1:2) solution or pure glycerol.
14. Examine the embryos under the microscope according to ISTA method 7-013a. 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)

None listed.

Media and solutions

Sulphuric acid

For safety reasons, ready-made sulphuric acid 25–37 % (by weight) is preferable.

If ready-made 25–37 % sulphuric acid is not available, add concentrated sulphuric acid to water. **Not the reverse, never add water to acid!** The density of H_2SO_4 is 1.8356 g/ml. The quantity is calculated depending on the required concentration.

Weight/density = volume.

Figure 1. *Ustilago nuda* in an embryo.

Sodium hydroxide

For safety reasons, ready-made NaOH solution 10–15 % or ready-made NaOH-NaCl is preferable.

If these are not available, dissolve 130–175 g sodium hydroxide pellets and 110–150 g sodium chloride in 1 l of cold tap water.

Lactic acid solution with methyl blue

Lactic acid: 1000 ml
Methyl blue: 0.16 g

Preparation

Mix thoroughly.

References

Joelson, G. (1968). Laboratoriemetod för bestämning av naket sot hos korn. Årsredogörelse 1967–1968, Lokala Frökontrollen I Skara.

Joelson, G. (no date). Analys av naket sot enligt metod utarbetad I Skara av G.Joelson (available from the ISTA Secretariat).

Validation references

ISTA (2011). Alternative embryo extraction procedure to 7-013b *Ustilago nuda/Hordeum vulgare*. *Method Validation Reports*. International Seed Testing Association, Bassersdorf, Switzerland.

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.