Diagnostic tools validated by ICARDA's Germplasm Health Unit (GHU) for detection of cereal seed-borne pests

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Introduction

ICARDA's Germplasm Health unit (GHU) is responsible for the monitoring, clearance and documentation of safe germplasm movement at the Center's locations in Lebanon and Morocco. All incoming and outgoing genetic resources and breeding germplasm must go through a strict quarantine monitoring system, including seed health testing. To confirm the health status of seed samples, the GHU conducts different types of tests based on the nature of the pest to be identified. The following are the list and procedures of diagnostic tools validated by ICARDA's GHU for detection of cereal seed-borne pests:

a) List of the diagnostic tools

Test name	Pest type	Scientific pest name
Centrifuge washing test (CWT)	Fungi	*Tilletia controversa, Tilletia indica, Tilletia tritici, Tilletia laevis, Urocystis agropyri, Ustilago hordei, Ustilago tritici*
Freezing blotter test (FBT)	Fungi	*Alternaria* spp., *Cldosporium* spp., *Bipolaris sorokiniana* (= *Cochliobolus sativus*), *Drechslera graminea, Drechslera teres* f. sp. *Maculate, Fusarium avenaceum, Fusarium culmorum, Fusarium graminearum, Fusarium* spp., *Magnaporthe oryzae* (= *Pyricularia oryzae*), *Phaeosphaeria nodorum* (= *Stagonospora nodorum*), *Pyrenophora avenae, Pyrenophora tritici-repentis*
Agar plate test for fungi (APTF)	Fungi	*Alternaria* spp., *Cldosporium* spp., *Bipolaris sorokiniana* (= *Cochliobolus sativus*), *Fusarium avenaceum, Fusarium culmorum, Fusarium graminearum, Fusarium* spp., *Phaeosphaeria nodorum (= Stagonospora nodorum), Rhynchosporium commune* (= *R. secalis*)
Embryo test (ET)	Fungi	*Ustilago nuda, Ustilago avenae*
Agar plate test for bacteria (APTB) followed by slide agglutination test (SAT)	Bacteria	*Pseudomonas syringae* pv. *atrofaciens, Pseudomonas syringae* pv. *syringae, Xanthomonas translucens* pv. *undulosa*
Indirect Enzyme linked immunosorbent assay (I-ELISA)	Virus	Barley stripe mosaic virus, Wheat streak mosaic virus
Tissue blot immunoassay (TBIA)	Virus	Barley stripe mosaic virus, Wheat streak mosaic virus
Nematode extraction test (NET)	Nematode	*Anguina tritici*
Visual inspection	Insect	*Rhyzopertha dominica, Sitophilus granaries, Tribolium castaneum, Tribolium confusum, Trogoderma granarium*
	Fungi	*Claviceps purpurea*
Floating test	Insect	*Rhyzopertha dominica, Sitophilus granaries, Tribolium castaneum, Tribolium confusum, Trogoderma granarium*

b) Procedures of the diagnostic tools

1. Testing methods for detection of cereal seed-borne fungi

1.1. Centrifuge washing test (CWT)

Reference: (Shetty *et al.,* 1988; Begum and Mathur, 1989)

This method is applied for cereal seeds to detect seed-borne fungal pathogens.

Procedure

1. Distribute the working samples individually on clean working table in the seed preparation room.
2. Label the glass tube with sample number on both sides (or up and down), to make sure not to lose the number.
3. Place seeds (100 seeds minimum) in tubes and cover with 30 ml of water.
4. Shake the seeds vigorously or use a tube shaker for 30 sec.
5. Transfer the suspension into centrifuge tube and adjust the tube contents to become at the same level.
6. Centrifuge the tubes at 2000 rpm for 15 min.
7. Decant the water and add 0.5 ml water to the remaining sediment and scrape gently.
8. Examine few drops of the sediment solution under a compound microscope for identification and quantification of the Tiliospores.

Seed shaking by a vortex mixer to release fungal spores

Centrifugation of the washing suspension

Identification of fungal spores under trinocular microscope

1.2. Freezing blotter test (FBT)

*References: (*Hewett, 1979; ISTA, 2021; Punithalingam and Holliday, 1972)

Procedure

1. Label each plastic container with sample number and other related information in addition to the date of working day.
2. Divide the folded filter paper into two parts (25 lines in each).

Seed distribution on the folded filter paper inside the container

3. Place four seeds in the paper furrow (total#100 seeds per filter paper)
4. Add enough distilled water (35 ml) to keep the filter paper moist during the test period.
5. Incubate seeds for 24 h at 20˚C to imbibe water.
6. Place containers overnight into deep-freezer at -18˚C.
7. Incubate again at 20°C for 6-7 days, with near UV light, a one cycle of 12 h dark/12 h light to stimulate sporulation.
8. Evaluate directly under binocular microscope and identify developed colonies on the seeds and confirm identity under compound microscope examination, if necessary.

Seed incubation inside the incubation unit

Identification of fungal colonies under binocular microscope

1.3. Agar plate test for fungi (APTF)

References: (Hewett, 1979; ISTA, 2021; Punithalingam and Holliday, 1972)

- This method is applied for cereal seeds to detect seed-borne fungal pathogens.
- This method is not applicable for treated seeds with any type of seed dressings.
- The recommended medium is potato dextrose agar (PDA).
- The medium preparation is according to manufacturer's instructions.

Procedure

1. Seeds must be treated with freshly prepared sodium hypochlorite solution (0.5%) for 5 min to disinfect the seed surface, and then rinsed with disinfected water to wash off the sodium hypochlorite residue.
2. Dry the seeds on paper tissue and distribute them on the artificial medium.
3. The whole process must be carried out in the flow bench.
4. Distribute the seeds inside the Agar plates using disinfected forceps with sufficient distance (2-3 cm) between seeds (15-20 seeds/plate for wheats, barley, oat and their wild relatives) with many replicates depending on the size of the working sample. The plates are placed in the incubator for 7 days at 20±2°C with light adjustment at 12/12 hrs. (light/dark).
5. Check the plates during the incubation period to detect any potential contamination with saprophytic fungi.

Distribution of seeds in the agar plates

Incubation of plates

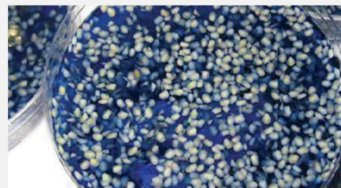
6. Examine the plates 7 days after incubation by the naked eye and check the colonies under a stereomicroscope. Identify the easily recognized colonies based on the most common growth characteristics such as color, fructification bodies, etc. and confirm the identity of doubtful pathogens under 10x or 40x magnification.
7. Record the number of identified infected seeds and calculate the seed infection rate.

1.4. Embryo test (ET)

References: (ISTA, 2021)

- The chemicals applied in this test are highly dangerous.
- Wearing lab coat and double gloves is obligatory at all steps.
- The applicants must implement all the steps in the cleaning sink at old kitchen.
- The soaked samples should be kept away from the staff and the labor in the cabinet above the sink.
- The applicant must follow the rules of chemical solutions preparation.

Procedure



The extracted embryos

Infected embryo to the left and healthy embryo to the right

1. Soak seeds (2000 seeds) in 1 liter of 5% fresh solution of NaOH with 0.2 g of aniline blue or trypan blue, at 20°C for 18 hr.
2. Add 1 liter of 5% fresh solution of NaCl and stir vigorously.
3. Wash with running warm water and stir constantly to separate embryos over a set of sieves (2.5 mm mesh to collect the endosperm and chaff, 1 mm mesh to collect embryos). Alternatively, a Fenwick can is very helpful as it speeds up the procedure.
4. Transfer the sample to a funnel closed with rubber tube and stopcock and cover with a mixture of lactophenol: water (3:1 v/v)
5. When specific gravity of the solution is correctly adjusted, the embryos will float and chaff sinks down, which can be removed by carefully releasing the stopcock.
6. Transfer the embryos to fresh lactophenol and boil for 30-60 sec.
7. Immerse the embryos in fresh glycerol and arrange them in rows.
8. Examine under binocular microscope with sub-stage illumination and count number of embryos with blue short hyphae, which vary from few to complete invasion of the scutellum tissue.

2. Testing methods for the detection of cereal seed-borne bacteria

2.1. Agar plate test for bacteria (APTB) followed by slide agglutination test (SAT)

Reference: (ISTA, 2021)

Procedure

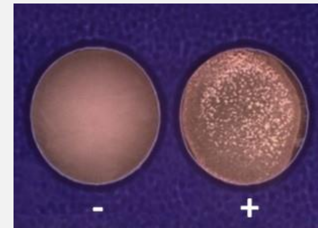
Seed extraction preparation

This test requires high accuracy to enhance reliability, ensure that it is contamination-free and avoid miss-reading. This is achieved by adhering to the following:

1. Before starting, autoclave all autoclavable items used in this test in a liquid cycle.

2. To extract bacteria from the seeds, grind the seeds in a seed mill at grade "0", then add around 2-5 g of seed powder to a glass tube, add sufficient volume of autoclaved cold saline solution (0.85 %), shake the tube vigorously and keep it at 4°C overnight.
3. On the next day, shake the tube for one hour at minimum speed (30 rpm).
4. Under the flow bench, take 100 µl from the suspension and add it to semi-selective agar plate (media type differs depending on the bacteria to be detected).
5. Using L-shape spreader, distribute the suspension on the whole agar surface in the plate.
6. Incubate the plates in an inverted position at 28 °C for up to 4 days.
7. Check the plates after 1-2 days for Pseudomonads; and up to 3-4 days for Xanthomonads.
8. For Pseudomonads identification, look for white to whitish-grey, circular, smooth, glistening, and raised colonies. On King's B medium, look for colonies, which have produced diffusible yellowish-green pigments that show florescence under UV light.

Identification of the bacterial colonies by agglutination test



Agglutination test results (+) infected, (-) healthy

9. For Xanthomondas identification, look for convex to domed, smooth, mucoid, creamy to yellow colonies. On KB medium, these bacteria do not produce diffusible pigments.
10. For more accurate and specific detection, the suspected colonies are subjected to the agglutination test by using specific commercial kit following the manufacturer's instructions.
11. For quantifying bacterial concentration, count the number of colonies in one plate (= CFU in 100 µl) then calculate the CFU based on the volume of the saline solution added to the seed powder.

Solution and media used in AP test for bacteria

1. *Saline solution (0.85%)*

8.5 g Sodium chloride (NaCl)
0.2 ml Tween 20
Make up the volume up to 1 liter with H_2O
Autoclave in a liquid cycle and store at 4°C

2. *King's medium B (KB)*

33 g King Agar B
10 ml Glycerol
Make the volume up to 1 liter H_2O
Adjust pH to 7.2 and sterilize by autoclaving at 121 ˚C for 15 min. (do not filter the medium)

Xanthomonas agar medium (XAM)

23 g Nutrient agar
5 g Glucose
Make the volume up to 1 liter with H_2O
Sterilize by autoclaving at 121 ˚C for 15 min.
Cool down and add the following antibiotics when the temperature around 45-45°C:
Cyclohxamide stock solution: 2 ml of 10% of Cyclohxamide in 75% ethanol
Gentamycine stock solution: 1 ml of 1% of Gentamycine in 75% ethanol
Cephalexine stock solution: 1 ml of 1% of Cephalexine in 75% ethanol

3. Testing methods for the detection of cereal seed-borne viruses

3.1. Indirect Enzyme-linked immunosorbent assay (I-ELISA)

Reference: (Lommel *et al.,* 1982)

Procedure

1. Samples preparation: This is applicable for seeds and fresh tissue testing

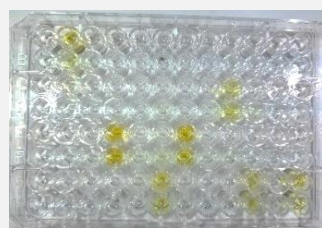
   Seed: grind the seed using the seed mill at grade "0", then add around 1 g of seed powder to the small glass tube, add sufficient volume of coating buffer, shake the tube vigorously and proceed to step 2.

   Tissue: after geminating the seeds in clean compost for 10-15 days, place around 2-3 leaflets in extraction bag, add 5 ml of coating buffer, pH 9.5, homogenize the samples in HOMEX XS homogenizer and proceed to step 2.

2. Add 200 µl aliquots of the test sample (extracted in coating buffer, pH 9.5) to duplicate wells.
3. Incubate at 37 °C for 4 hours.
4. Wash plate with PBST using wash bottle, let it sit for at least 5 min. then repeat washing four times.
5. Add 250 µl of blocking buffer (3% non-fat dry milk in PBST), and incubate for 1 hr at 37°C.
6. Wash as in step 4.
7. Add 200 µl specific antibody dilution (1/1000 in PBS buffer, cross absorbed with healthy tissues) to each well, and incubate overnight at 4°C.
8. Wash as in step 4.
9. Add goat anti-rabbit conjugate (dilution 1/4000 in conjugate buffer), and incubate at 37°C for 4 hours.
10. Wash as in step 4.
11. Add 200 µl aliquots of freshly prepared substrate (5 mg p-nitrophenyl phosphate dissolved in 10 ml substrate buffer) to each well. Incubate at room temperature for 30-60 min, or as long as necessary to observe reaction.
12. Stop reaction by adding 50 µl of 3 M NaOH to each well.
13. Assess results by measurement of absorbance at 405 nm.

Seed preparation for grinding by the mill

Transfer seed or plant extract to ELISA wells


ELISA reaction after adding the substrate buffer (yellow=positive reaction, colorless= negative reaction)

Measuring color intensity at 405 nm using ELISA microplate reader

Buffers used in ELISA

1. ***Coating buffer (pH 9.6)***

    1.59 g Sodium carbonate (Na_2CO_3)
    2.93 g Sodium bicarbonate ($NaHCO_3$)
    0.2 g Sodium azide (NaN_3)
    make up 1 liter H_2O

2. PBS (pH 7.4) phosphate buffer saline

    8.0 g Sodium chloride (NaCl)
    0.2 g Potassium phosphate (monobasic) (KH_2PO_4)
    1.15 g Sodium Phosphate (dibasic) (Na_2HPO_4)
    0.2 g Potassium chloride (KCl)
    0.2 g Sodium azide (NaN_3)
    make up to 1 liter with H_2O

3. ***PBS-tween (PBST)***

    PBS + 0.5 ml Tween 20 per liter

4. ***Conjugate buffer***

    PBST + 2% PVP (Sigma PVP-40 Polyvinyl pyrrolidone) + 0.2% egg albumin

5. Substrate buffer

    97 ml Diethanolamine
    800 ml H_2O
    0.2 g Sodium azide (NaN_3)
    add HCl to reach pH 9.8
    make up to 1 liter with H_2O

6. Cross absorption of polyclonal antiserum

To avoid any non-specific reaction (increase specificity), the polyclonal antibody is cross absorbed with healthy tissues as follows: extract healthy tissues from the crop that is being tested in PBS (pH 7.4) at 1/20 (W/V), then add to the extraction suspension antiserum to make a final antiserum dilution of 1/1000 and incubate overnight at 4 °C.

3.2. Tissue-blot Immunoassay (TBIA)

References: (Lin *et al.,* 1990; Hsu and Lawson, 1991; Makkouk and Kumari, 1996; Makkouk and Comeau, 1994.)

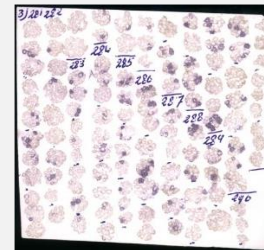
Procedure

1. After geminating the seeds in clean compost for 10-15 days, excise tissues (leaves, petioles, stems, … etc.).
2. For thin tissues such as leaves, roll them into a tight core.
3. Hold tissues (petiole, stem, rolled leaf) in one hand and cut with a new razor blade with the other hand in a steady motion to obtain a single plane cut surface.
4. Press (print), with a firm but gentle force, the newly cut surface onto a nitrocellulose membrane (NCM).

Preparation of germinated seeds for blotting

5. Wash 3 times with PBST at 5 min interval.
6. Block NCM with 1 μg/ml PVA (polyvinyl alcohol) in PBST solution and incubate for one min at room temperature.
7. Wash as in step 5.
8. Add antiserum (dilution 1/1000 in PBS, cross absorbed with healthy tissue) and incubate for one hour at room temperature.
9. Wash as in step 5.
10. Add goat anti-rabbit conjugate (dilution 1/4000 in conjugate buffer), and incubate for one hour at room temperature.
11. Wash as in step 5.
12. Add substrate solution (NBT/BCIP) for 20-30 min.
13. Wash with deionized H_2O to stop the reaction.

Nitrocellulose membrane shaking and incubation at room temperature



Detection of *Barley stripe mosaic virus* (BSMV) by TBIA in a group of 20 barley seedlings blotted on the nitrocellulose membrane as one sample

Buffers used in TBIA

1. *PBS (pH 7.4) phosphate buffer saline*

8.0 g Sodium chloride (NaCl)
0.2 g Potassium phosphate (monobasic) (KH_2PO_4)
1.15 g Sodium Phosphate (dibasic) (Na_2HPO_4)
0.2 g Potassium chloride (KCl)
0.2 g Sodium azide (NaN_3)
make up 1 liter H_2O

2. *PBS-tween (PBST)*

PBS + 0.5 ml Tween 20 per liter

3. *Conjugate buffer*

PBST + 2% PVP (Sigma PVP-40 polyvinyl pyrrolidone) + 0.2% egg albumin.

4. Substrate buffer: 0.1 M tris, pH 9.5 containing 0.1 M NaCl + 5 mM $MgCl_2$.

5. *Preparation of substrate solution:*

Tube 1. p-nitro blue tetrazolium (NBT), a stock solution of 25 mg/ml in 70% diemethylformamide. Store at -20°C.

Tube 2. 5-bromo-4-chloro-3-indolyl phosphate (BCIP), a stock solution of 50 mg/ml in undiluted diemethylformamide. Store at -20°C.

Then add 20 μl NBT solution (Tube 1) and 20 μl BCIP solution (Tube 2) to 5 ml substrate buffer and gently mix. The substrate solution should be freshly prepared just before use.

6. Cross absorption of polyclonal antiserum

To avoid any non-specific reaction (increase specificity) , the polyclonal antibody is cross absorbed with healthy tissues as follows: extract healthy tissues from the crop that is being tested in PBS (pH 7.4) at 1/20 (W/V), then add to the extraction suspension antiserum to make a final antiserum dilution of 1/1000 and incubate overnight at 4 °C.

4. Testing methods for detection of cereal seed-borne nematodes

4.1. Nematode Extraction test (NET)

Reference: (Asaad and Abang, 2009)

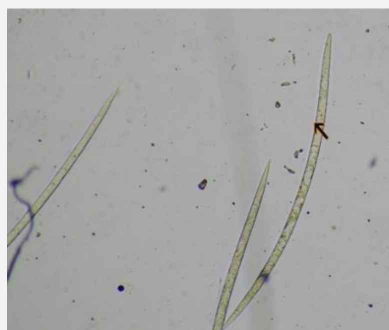
Procedure

Make sure that the tested samples are totally free from soil for accurate detection.

1. Place seeds in a funnel, which is closed with a rubber tube and pinchcock, cover with water, and incubate at cool or relatively cool conditions for 24 h. For better results, constant aeration is applied.
2. Open the pinchcock, collect the suspension, and examine under the stereomicroscope for larva, which has a slender body.

For quantitative information and for a low population of nematodes in the extract, it is advisable to concentrate the larvae in a known quantity of water as follows:

1. Centrifuge the total extraction at 500 rpm for 10 min., or
2. Let the extraction to stand in a beaker for 1 h. The larvae will concentrate at the bottom.
3. Remove water from the top with the help of a pipette and leave only 20ml in the beaker.
4. Transfer the water into examination dish.
5. Count numbers of larvae.



The second-stage larvae under the microscope

5. Testing methods for detection of cereal seed-borne insects

5.1. Visual inspection

Procedure

- This method is applied in the seed preparation room only to avoid any potential contamination in the testing laboratory by unexpected pests.
- Examine the seed sample visually or using a lens or stereo- microscope at a magnification of 40-60X. Determine and/or count the following unwanted impurities: Abnormal seeds, insects, weeds, soil particles… etc.

Seed examination under the magnifier lens

6. Testing for detection of cereal seed-borne insects

6.1. Floating test

Procedure

1. Mix the samples well and soak it in water.
2. Collect all floating abnormal seeds or bodies... etc.
3. Check them under binocular as in direct inspection test.

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