

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 legume seed-borne pests:

a) List of the diagnostic tools

Test name	Pest type	Scientific pest name
Agar plate test for fungi (APTF)	Fungi	<i>Alternaria alternata</i> , <i>Ascochyta fabae</i> (= <i>Didymella fabae</i>), <i>Ascochyta lentis</i> (= <i>Didymella lentis</i>), <i>Ascochyta pinodes</i> (= <i>Didymella pinodes</i>), <i>Ascochyta pisi</i> , <i>Ascochyta rabiei</i> (= <i>Didymella rabiei</i>), <i>Ascochyta</i> spp., <i>Botrytis cinerea</i> , <i>Botrytis fabae</i> , <i>Botrytis fabiopsis</i> , <i>Colletotrichum lindemuthianum</i> , <i>Colletotrichum truncatum</i> , <i>Fusarium oxysporum</i> f. sp. <i>cicero</i> , <i>Fusarium oxysporum</i> f. sp. <i>lentis</i> , <i>Fusarium oxysporum</i> f. sp. <i>pisii</i> , <i>Fusarium oxysporum</i> , <i>Fusarium</i> spp., <i>Phoma medicaginis</i> var. <i>medicaginis</i> , <i>Phoma</i> spp., <i>Stemphylium botryosum</i>
Freezing blotter test (FBT)	Fungi	<i>Alternaria alternata</i> , <i>Fusarium oxysporum</i> f. sp. <i>cicero</i> , <i>Fusarium oxysporum</i> f. sp. <i>lentis</i> , <i>Fusarium oxysporum</i> f. sp. <i>pisii</i> , <i>Fusarium oxysporum</i> , <i>Fusarium</i> spp.
Agar plate test for bacteria (APTb) followed by slide agglutination test (SAT)	Bacteria	<i>Curtobacterium flaccumfaciens</i> pv. <i>flaccumfaciens</i> , <i>Pseudomonas syringae</i> pv. <i>phaseolicola</i> , <i>Pseudomonas syringae</i> pv. <i>pisii</i> , <i>Xanthomonas axonopodis</i> pv. <i>phaseoli</i>
Indirect Enzyme linked immunosorbent assay (I-ELISA)	Virus	Alfalfa mosaic virus, Bean yellow mosaic virus, Broad bean mottle virus, Broad bean stain virus, Cucumber mosaic virus, Pea seed-borne mosaic virus
Tissue blot immunoassay (TBIA)	Virus	Alfalfa mosaic virus, Bean yellow mosaic virus, Broad bean mottle virus, Broad bean stain virus, Cucumber mosaic virus, Pea seed-borne mosaic virus
Nematode extraction test (NET)	Nematode	<i>Ditylenchus dipsaci</i> , <i>Ditylenchus gigas</i>
Visual inspection	Parasitic weed	<i>Cuscuta</i> spp., <i>Orobancha</i> spp., <i>Phelipanche</i> spp.
	Insect	<i>Acanthoscelides</i> spp., <i>Bruchus ervi</i> , <i>Bruchus lentis</i> , <i>Bruchus pisorum</i> , <i>Bruchus</i> spp., <i>Callosobruchus chinensis</i> , <i>Callosobruchus maculatus</i> , <i>Callosobruchus</i> spp.
Floating test	Parasitic weed	<i>Cuscuta</i> spp., <i>Orobancha</i> spp., <i>Phelipanche</i> spp.
	Insect	<i>Acanthoscelides</i> spp., <i>Bruchus ervi</i> , <i>Bruchus lentis</i> , <i>Bruchus pisorum</i> , <i>Bruchus</i> spp., <i>Callosobruchus chinensis</i> , <i>Callosobruchus maculatus</i> , <i>Callosobruchus</i> spp.

b) Procedures of the diagnostic tools

1. Testing methods for the detection of legume seed-borne fungi

1.1. Agar plate test for fungi (APTF)

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

- This method is applied for food legume seeds to detect seed-borne fungal pathogens.
- This method is not applicable for treated seeds with any type of seed dressings.
- The standard medium is malt extract agar (MEA) or potato dextrose agar (PDA) according to ISTA validated methods.
- The medium is prepared 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 (2-5 cm) between seeds (5-7 seeds/plate for faba bean and its wild relatives, 10-12 seeds/plate for chickpea and its wild relatives, 15-20 seeds/plate for lentil, grasspea 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 $20\pm 2^{\circ}\text{C}$ 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.
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.



Distribution of seeds in the agar plates



Incubation of plates



Identification of fungal colonies under trinocular microscope

1.2. Freezing blotter test (FBT)

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

Procedure

1. Label each petri dish with sample number and other specific information in addition to the date of working day.
2. Place seeds at a fixed distance (2-3 cm distance between each) on filter paper (circle 90 mm) in petri dishes (you can use plastic germination boxes (14 x 17 cm) in case the quantity of working sample is big).
3. Add enough distilled water to keep the filter paper moist during the test period.
4. Incubate seeds for 24 h at 20°C to imbibe water.
5. Place containers overnight into deep-freezer at -18°C.
6. Incubate again at 20°C for 6-7 days, with lighting system, a one cycle of 12 h dark/12 h light to stimulate sporulation.
7. Evaluate directly under binocular microscope and identify developed colonies on the seeds and confirm identity under compound microscope examination, if necessary.



Labeling Petri dishes with samples information



Seed distribution inside a germination box

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

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

Reference: (ISTA, 2021)

- This test requires high accuracy to enhance reliability, ensure that it is contamination-free and avoid miss-reading.
- Before starting, autoclave all autoclavable items used in this test in a liquid cycle.

Procedure

The test is achieved by adhering to the following:

1. 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.
2. On the next day, shake the tube for one hour at minimum speed (30 rpm).
3. Under the flow bench, take 100 µl from the suspension and distribute it on semi-selective agar plate (KB medium for Pseudomonads and XTS for Xanthomonads)
4. Using L-shape spreader, distribute the suspension on the whole agar surface in the plate.

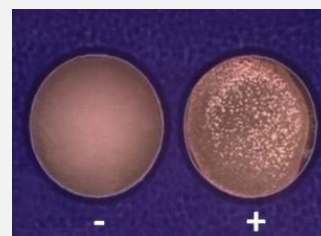


Seed extraction preparation

5. Incubate the plates in an inverted position at 28 °C for up to 4 days.
6. Check the plates after 1-2 days for Pseudomonads; and up to 3-4 days for Xanthomonads.
7. 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 fluorescence under UV light.
8. For Xanthomonads identification, look for convex to domed, smooth, mucoid, creamy to yellow colonies.
9. 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.
10. 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 (0.85%) added to the seed powder.



Identification of bacterial colonies by agglutination test



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

Solution and media used in APTB

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 H₂O
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 H₂O
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 H₂O
Sterilize by autoclaving at 121 °C for 15 min.
Cool down and add the following antibiotics when the temperature around 45-45°C:
Cyclohexamide stock solution: 2 ml of 10% of Cyclohexamide 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 legume seed-borne viruses

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

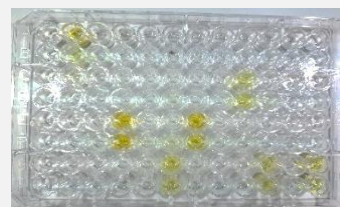
Reference: (Lommel *et al.*, 1982)

Procedure

1. After germinating the seeds in clean compost for 10-15 days, place about 2-3 leaflets in extraction bag and add 5 ml coating buffer (pH 9.5), extract the samples in HOMEX XS homogenizer and proceed to step 2.
2. Add 200 µl aliquots of the test sample (extracted in coating buffer) 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 h at 37°C.
6. Wash as in step 4.
7. Add 200 µl specific polyclonal 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



Samples extraction using HOMEX XS homogenizer



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 I-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)

Procedure

1. After germinating 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.

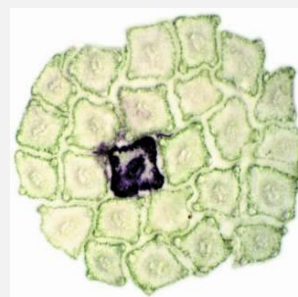


View of the blotted lentil stem cut surfaces on nitrocellulose membrane

4. Press (print), with a firm but gentle force, the newly cut surface onto a nitrocellulose membrane (NCM).
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 polyclonal 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 H₂O to stop the reaction



Membrane shaking and incubation at room temperature.



Detection of broad bean strain virus (BBSV) by TBIA in an infected lentil seedling in a group of 25 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) (KH₂PO₄)
- 1.15 g Sodium Phosphate (dibasic) (Na₂HPO₄)
- 0.2 g Potassium chloride (KCl)
- 0.2 g Sodium azide (NaN₃)
- make up 1 liter H₂O

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 MgCl₂.

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 legume seed-borne nematodes

4.1. Nematode extraction test (NET)

Reference: (FAO, 2015)

Procedure

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

1. Place seeds in funnel, which is closed with a rubber hose and pinchcock, cover with water, and incubate at cool or relatively cool conditions for 24 hr. For better results, constant aeration is applied.
2. Open the pinchcock, collect the suspension, and examine under the stereomicroscope for juveniles (fourth stage), 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 hr. The larvae will concentrate at the bottom.
3. Remove water from the top with the help of a pipette and leave only 20 ml in the beaker.
4. Transfer the water into examination dish.
5. Count numbers of juveniles (fourth stage).



Seed soaking in a funnel



Ditylenchus dipsaci juveniles (4th stage) under microscope

5. Testing methods for detection of legume seed-borne insects and weeds

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, weed seeds, soil particles... etc.



Seed examination under the magnifying lens

6. Testing for parasitic weeds (dodder and broomrape)

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.

References

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