SOP-IRRI-SHU-RSHT-400.00

Title: Routine Seed Health Testing (RSHT) Quality and Quantity Standards

Application: Terms of reference for evaluation of work performance by laboratory staff conducting various seed health testing activities

ACTIVITY	STANDARD QUANTITY (min qty/hr)	STANDARD QUALITY
1. Blotter Test		
a. Seeding	61-70 plates	according to ISTA rules & standards (25 seeds/plate; readable/ correct labels; w/ 2 layers of moist blotter paper; seeds equidistant from one another & properly oriented
b. Evaluation	Not Treated- 51-60 plates Treated -61-70 plates	Accurate identification of seedborne organisms - no missed/misidentified organism in 99% plates evaluated; timely evaluation – 5 to 7 days after incubation
2. Nematode Test		
a. Setting/extraction	81-90 samples	correct weight of samples, correct amount of water (seeds are covered) germinated seeds are properly spread over the mesh wire, no seeds in the tygon tubing, pinchcock properly placed-no leakage, spillage of water/ seeds
b. Evaluation	51-65 samples	accurate identification (+/-; saprophytic or parasitic) and actual count (if parasitic) evaluation conducted as soon as extracts are gathered
3. Macro Test/Tb cleaning	1.1-1.5 K	correct evaluation (+/-) of *Tilletia barclayana*
4. Documentation	181-200 samples	data/info complete & accurate; manner (no erasures, readable) in which data is recorded generally good; immediately done after evaluation (consistency - 99% of the time)
5. Washing	241-260 plates; 76-85 funnels; 101-120 wire mesh	no pencil marks, water marks, fungal growth; thoroughly dried

6.	Germination Test		
	a. Seeding	41-45 seedlots	correct number of seeds-100/tray; correct orientation of seeds, moisture of blotters, correct labelling;
	b. Evaluation	31-35 seedlots	timely with regards to evaluation (5 DAS, 7DAS, & 14 DAS); correct evaluation as to Normal/Abnormal seedlings, dead seeds (in accordance to ISTA Rules and Standards in Seedling Evaluation)
7.	Assigned tasks	n/a	met expectations; with very minimal supervision; output with minor correction; task done/output delivered on agreed schedule, consistent most of the time; very positive attitude

SOP-IRRI-SHU-RSHT-BT-401.00

Title: Blotter Test (please refer to SOP- IRRI-SHU Powerpoint Presentation No. as supplementary reference)

Application: Blotter Test is used for the detection of seedborne fungi that responds to sporulation

Background: The Blotter Method appears in Annexe 7.4.3.a.7 of the ISTA Rules (2008)

Material (s):

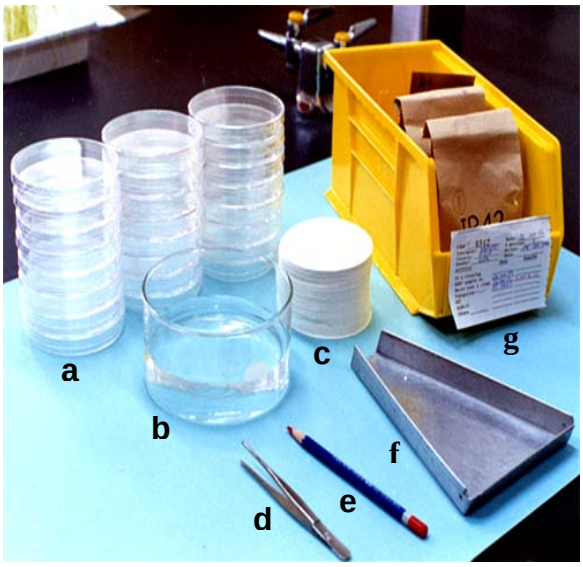


Fig.1. Materials used in the Blotter Test seeding.

Legend:

a. Plastic Petri Dish, 9.0 cm
b. Distilled water (placed in wide-mouthed glass container)
c. Round blotters (EDB No. 127.085, FRISENETTE ApS)
d. Pair of forceps
e. Marking pencil – water resistant but can be easily wiped off
f. Sampling pan
g. Seed samples

Not shown:

Incubator

Capable of operating in the range of 21ºC $\pm$ 1ºC. To stimulate sporulation, altering 12-h periods of darkness and near-ultraviolet light (NUV) during incubation are recommended.

The recommended source is the black light fluorescent lamp (320-400 nm, peak 360 nm) but daylight fluorescent tubes are satisfactory.

(SHU is using Philips TLD 36W/08)

Fig.2. Materials used for blotter test evaluation

Legend:

a. Tally counter
b. No ink blot marking pencil
c. Dissecting needles (2 pcs.)
d. Glass slides
e. Cover slips
f. Nail polish, clear (optional, for sealing semi permanent mounts)
g/i. Mounting Medium (distilled water, lactophenol-plain, with cotton blue)
h. Alcohol – for cleaning dissecting needles

Not shown:

Stereo microscope – for evaluation/identification (examination of habit character of seedborne fungi)

Compound microscope) – for confirmation of identification of seedborne fungi (examination of microscopic character)

Quality Assurance Check:

The designated lead person for blotter seeding shall:

1. check the incubation room regularly (everyday, early in the morning and before 5:00 PM) and ensure that the air-conditioning unit and thermostat are set accordingly to meet required/standard incubation temperature (21 ºC $\pm$ 1) and lighting requirement (alternating cycles 12 hrs NUV and 12 hrs darkness)
2. check/ensure that all materials to be used in this test are clean according to IRRI-SHU set standards (please refer to SOP-IRRI-SHU-RSHT-BT-401.01)
3. The designated lead person shall ensure that the seeded plates are set properly (F0080005-1, F0080005-2, F0080005-3, and so on) on the assigned shelves inside the incubation room (please refer to SOP-IRRI-SHU-RSHT-BT-401.02: Guidelines for setting seeded plates on shelves inside incubation room)

4. ensure that non-SHU staff who shall conduct the test are properly oriented with regards to the procedure, rules, and standards.

The person conducting the test is knowledgeable with accurate identification of the different seedborne fungi and shall:

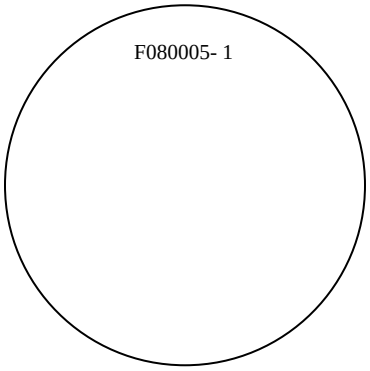
1. counter check that the labels of the seedlots are consistent with the information in the seedlist
2. ensure that the number of plates and blotters set on the working table corresponds to the number of seedlots and recommended working sample to be seeded.
3. ensure that the plates are labeled accordingly
4. ensure that the samples are seeded on the corresponding plates.
5. ensure that the blotter papers are not over soaked to eliminate the “wet blotter effect” which prevents the growth of seedborne fungi.
6. ensure that the corresponding data sheets have been prepared.
7. ensure that the corresponding data are recorded and computed accurately/accordingly.

Procedure:

Pre-treatment – none

Sampling – please refer to SOP-IRRI-SHU-DSI-SAMPLE-303.00

A. Seeding
    1. Labelling
       Label using a marking pencil. The label (which includes the assigned SHU Reference Number-Seedlot Number) must be placed at one side of the plastic petri dish cover



    2. Place 2-3 pieces of moistened round blotter paper in the labeled petri dishes.
    3. Place 25 seeds equidistantly in each petri dish following recommended pattern (Please refer to SOP-IRRI-SHU-RSHT-BT-401.03: Guidelines for Blotter test seeding)

B. Incubation
   1. Bring seeded plates inside incubation room. Arrange plates following SOP-IRRI-SHU-RSHT-BT-401.02: Guidelines for setting/retrieval of seeded plates on shelves inside near ultra violet light (UNV) incubation room.
   2. Incubate seeded plates for 5-7 days under alternating cycles 12 hrs Near Ultraviolet Light (NUV) and 12 hrs darkness at ±1 21°C

C. Evaluation
   1. After the required incubation period, retrieve the plates from the incubation room
   2. Examine each seed under a binocular stereo microscope at 12-50X magnification for the different seedborne fungi
   3. Identify and quantify the different seedborne fungi accordingly (Please refer to SOP-IRRI-SHU-RSHT-BT-401.04: Identification of seedborne fungi)
   4. Collate/record data
      Note: Germination of seeds is also taken into account/recorded.
   5. Compute for % detection level and % Germination.

Calculation: N/A
Solution: N/A
Data entry: Refer to IRRI-SHU Powerpoint No. ___ and IRRI-SHU-RSHT-BT-4001.05: Data sheet for Blotter test results

1. All relevant information regarding the batch of seeds must be provided/recorded (reference/batch number, seedlot number, date of seeding, date of evaluation number of seeds used, type of seeds – treated or non treated, discard or sample)
2. Total quantity of each seedborne fungi (or total number of seeds with x fungi) per plate is recorded in the data sheet. In the data sheet, one column represents one plate (with 25 seeds)
3. Final data (% detection) is calculated following this formula:

   $$\textbf{\%}\ \text{Detection} = \frac{\text{Total number of seeds with X Fungi}}{\text{Total number of seeds}} \times 100$$

4. In addition, germination of seeds are also included and calculated following this formula:

   $$\%\ \text{Germination} = \frac{\text{Total number of seeds that germinated}}{\text{Total number of seeds}} \times 100$$

(Refer to Chapter 5.1 of ISTA Rules for Seed testing on Germination and ISTA Handbook on Germination)

Safety:

This procedure shall be conducted in the seed health testing laboratory by persons with working knowledge of this method and familiar with the principles of Good Laboratory Practice (please refer to IRRI-SHU Policies and Guidelines)

Seeds and blotters after evaluation shall be disposed following Disposal Protocol (Please refer to SOP-IRRI-SHU Doc._____.)

Reference(s):

ISTA. 2005. International Rules for Seed Testing. CH-Switzerland

ISTA. 2005. International Rules for Seed Testing. Annexe to Chapter 7 Seed Health Testing. Seed Health Testing Methods. CH-Switzerland

MEW, T. W. and P.G. GONZALES. 2002. A Handbook of Rice Seedborne Fungi. Los Baños (Philippines): International Rice Research Institute, and Enfield, N.H. (USA): Science Publishers, Inc. 83 p.

MEW, T.W. and J.K. MISRA. 1994. A Manual of Rice Seed Health Testing. . Los Baños (Philippines): International Rice Research Institute. 113 p.

NEERGARD, P. and A. SAAD. 1962. Seed Health Testing of Rice. A contribution to development of laboratory routine test methods. Indian Phytopathology. 15:85-111.

Attachment(s):

IRRI-SHU Policies and Guidelines
IRRI-SHU Powerpoint Presentation No. RSHT 1a. Blotter Test

SOP-IRRI-SHU-RSHT-BT-401.01:	SHU standards for cleanliness and orderliness
SOP-IRRI-SHU-RSHT-BT-401.02:	Guidelines for setting seeded plates on shelves inside incubation room
SOP-IRRI-SHU-RSHT-BT-401.03:	Guidelines for blotter test seeding
SOP-IRRI-SHU-RSHT-BT-401.04:	Identification of seedborne fungi
SOP-IRRI-SHU-RSHT-BT-401.04a:	Illustration Sheet for Identification of seedborne fungi
SOP-IRRI-SHU-RSHT-BT-401.04b:	
SOP-IRRI-SHU-RSHT-BT-401.05:	Data sheet for blotter test results.

SOP-IRRI-SHU Doc._____.

SOP REVISION HISTORY

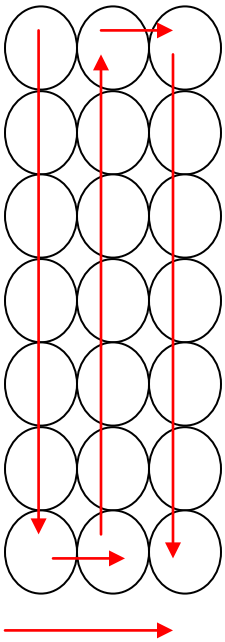
Revision History	Prepared by Name & Signature	Date	Approved by Name & Signature	Date	Remarks
0	Patria G. Gonzales	11 March 2008			Original
1					
2					
3					
4					

SOP-IRRI-SHU-RSHT-BT-401.02: Guidelines for setting/retrieval of seeded plates on shelves inside near ultra violet light (UNV) incubation room.

For safety purposes, the general switch for the NUV lights should be switched off before entering the incubation room (then switched on after putting the seeded plates and the main door has been closed).

1. As a general policy,
   a) care should be observed when “transporting” seeded plates from the seeding room to the incubation room and when arranging the seeded plates on the shelves so as to maintain the quality of seeding and
   b) seeded plates should be placed in the designated space/shelf inside the incubation room

2. The seed plates should be arranged following this pattern.



3. Seeded samples with the same reference number should be placed in the same shelf if possible.

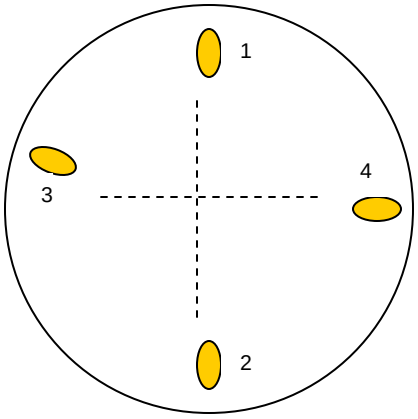
4. The reference number and date of evaluation of the seed batch should be indicated in the shelf.

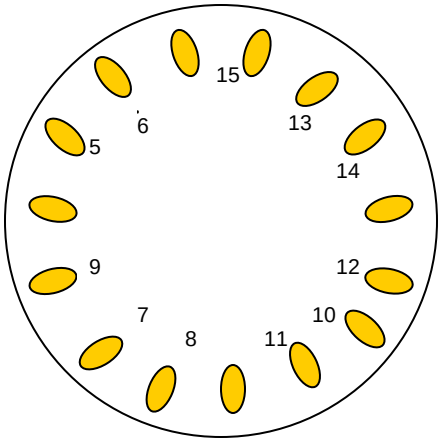
5. In retrieving, care should be observed so as not to disturb the seeds and plates with the same reference number should be stacked altogether, in groups of eight or 10.

SOP-IRRI-SHU-RSHT-BT-401.03: Guidelines for blotter test seeding

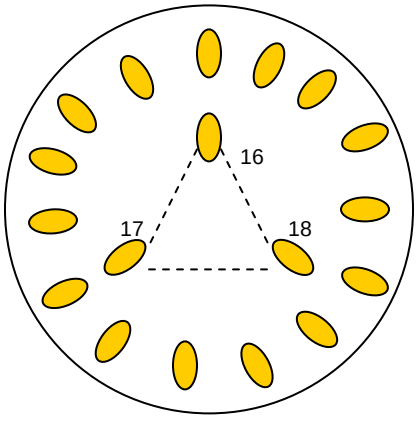
Aspects to observe when doing blotter seeding

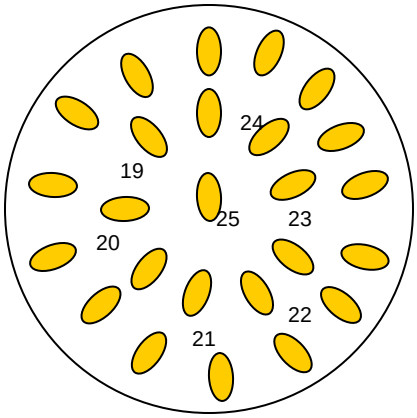
1. Seeds should be randomly chosen, irregardless of quality.
2. Seeds must be placed equidistantly from each other and not too near the edge of the plate.
3. Suggested manner of seeding:
   a. For the "outer circle", put the 1st four (4) seeds in an imaginary quadrangle; on the upper left space, put two (2) seeds; then on the three (3) spaces, put three seeds each.





4. For the inner circle, put three (3) seeds in an imaginary triangle; then put two (2) seeds between each space





5. Put the 25th seed at the middle

SOP-IRRI-SHU-RSHT-BT-401.04: Laboratory Identification of seedborne fungi

In seed health testing, identification of seedborne fungi is based on their:

A. **Habit character -** refers to their morphological character on natural substrate (i.e. rice seed) as observed under the stereo microscope. The morphological character includes presence or absence of aerial mycelia, presence of special structures like pycnidia, conidia appear as irregular mass known as pionnotes, and other features like presence of conidial appendage, and/or false heads.

B. **Microscopic character-** refers to the microscopic features which include the size, shape, and color of mycelia, conidiophore, and conidia as observed under the compound microscope. Conidiophores may be present or absent. If present, they are either distinct or reduced, occur singly, in loose clusters or form a sporodochium or cushion-like structures. They also vary in length and amount of branching. Conidia of fungi come in various sizes, shapes, and color.

   Other features such as presence or absence of septations and/or reticulations, manner in which conidia are borne or attached to the conidiophores, and presence of appendages are also included. Septations can be oblique, transverse, or both. Cell walls are either smooth or rough. Conidia are either borne singly or in groups; or they are contained in special structures called pycnidia or ascus. If borne in groups, they are catenulate (in chains) or acropleurogenous (in whorls). Some conidia have sterile appendages, while others do not have.

   Each fungi, displays a unique set of habit and morphological characters, thereby distinguishing it from other fungi. In seed health testing, a seed health analyst must acquire this skill-identification of the different fungi based on habit and microscopic characters. Accurate identification relies on the ability/skill of the seed health analyst. However, the success of seed health testing is also influenced by other factors such as detection methods and tools/instruments used.

SOP-IRRI-SHU-RSHT-BT-401.04a

Title: Guide in how to use/calibrate stereo and compound microscope.

Application: For evaluation/examination of seeds or other plant parts suspected to be infected with microorganisms or for identification of microorganisms and/or insect pests

Parts of a Stereo Microscope
Parts of a compound Microscope

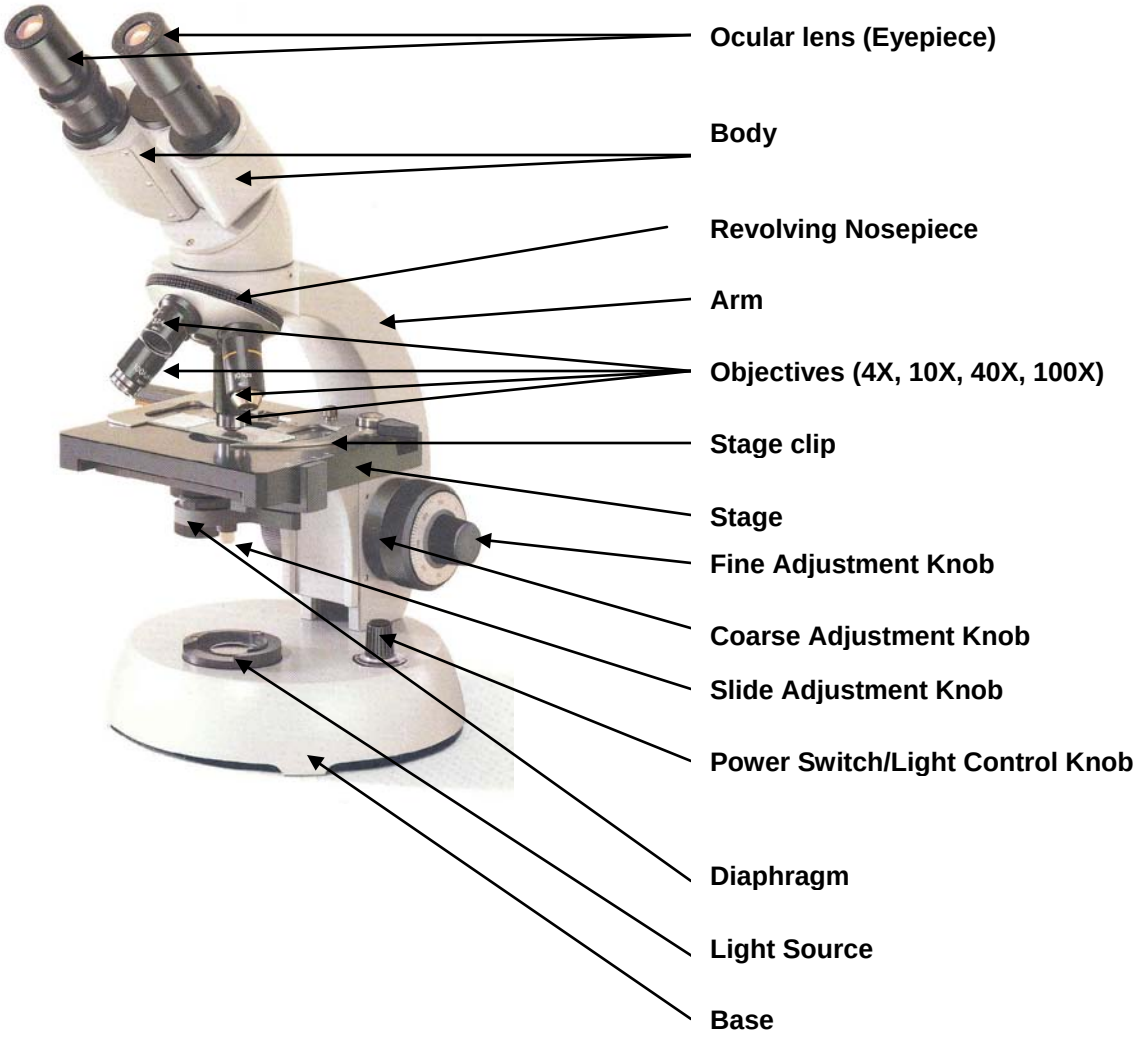


Fig. 1. PARTS OF A COMPOUND MICROSCOPE

General Guidelines:

- Set the microscope on a flat sturdy surface and plug the microscope's power cord into an outlet, making sure that the excess cord is out of the way so no one can trip over it or pull it off of the table.
- When moving a microscope from one place to another, always carry it with both hands. Grasp the arm with one hand and place the other hand under the base for support.

Maintenance/Cleaning

- When cleaning the exterior side of lenses, use only special lens paper and a non-solvent solution designed for cleaning optics or eyeglases. Do not touch lenses with fingers. (With a moist lens paper, clean the lenses in a circular motion. Repeat procedure with a dry lens paper until the lens is clean and dry.)
- When cleaning interior side of lenses, use a bulb-type duster or a compressed gas canister designed for cameral and other optical instruments)
- When cleaning the body of the microscope, use a soft dry or damp cloth.
- When not in use, always keep the microscope covered.

Procedure:

1. Ensure that the lowest power objective is into position.

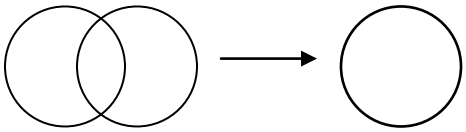
2. Turn on light source with the power switch (light control knob).
   If you will be looking at a microscope slide or other transparent object, bottom lighting will work best. If the specimen you are viewing is opaque or solid (light cannot pass through it from below), use top lighting so that the light can reflect off the specimen's surface.

3. Put the prepared slide mount/specimen on the stage and fasten it with the stage clips (if needed).
   If your specimen is thin and flat, or if its edges curl up easily, try using the stage clips to hold it in place. To do this, pull up the pointed end of one stage clip and slide it over one end of the specimen, then do the same with the stage clip on the other side. If your specimen is larger than the stage plate, you might need to turn the stage clips out so that they are hanging off the stage or removing them completely so that there is more room to work.

4. Look through the ocular lenses (eyepiece) and adjust the interpupillary distance* of the eyepieces. While doing the adjustments, the eyes should focus on the specimen, and only use the peripheral vision as one proceeds. Then, pull the eyes back from the eyepieces at about 1". You will see two field views overlapping each other. Open or close the distance between the

eyepieces by pulling them apart or pushing them together until the two circles merge together and appear as one circle. The interpupillary distance is set correctly when you see just one field view circle.

Photo of eyepieces



*Refers to the distance between the pupils of your eyes and varies from person to person. Thus, each observer should make this adjustment before using a binocular or stereo head microscope for the greatest viewing comfort and best image quality.

5. Adjust the diopter* by rotating the diopter ring and aligning its edge with the reference groove. Cover your left eye with your hand, and with the right eye look into the right eyepiece and adjust the right diopter ring until the specimen comes into focus. After this, cover the right eye with you hand, and with the left eye look into the left eyepiece and adjust the left diopter adjustment ring until the specimen comes into focus.

*This is used to make up for focusing differences between your eyes. This adjustment will also vary from person to person, thus, should be done by every observer/user for clearer image.

6. Set the desired magnification level by rotating the objective turret (a “clicking” sound is heard when the magnification is in its proper setting).

   To determine the magnification of your microscope, multiply the magnification level of the eyepiece lens by that of the objective lens. For example, on the microscope in the diagram above, the total magnification at the 2x objective is 20x (2 x 10 = 20).

7. Adjust the distance between the objective and the specimen (up/down movement) by rotating the coarse adjustment knob until the specimen you are looking at comes in clearly.

   While looking through the eyepiece(s), slowly turn the focus knob until the specimen comes into view. Once you can see the outline of the specimen, turn even more slowly to focus as sharply as possible. If you aren't able to see anything, try moving the specimen around slightly on the stage plate to make sure it is directly below the objective lens and then try focusing again. Once you have focused on the specimen, you can move it around to see its other parts. You may have to refocus slightly on each new area.

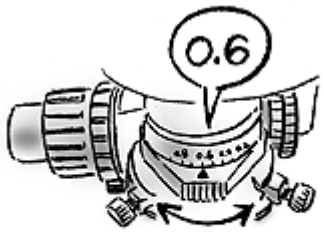
Take note that with a stereo microscope you will often be viewing three-dimensional specimens that have many different levels. You will not be able to focus every feature clearly at the same time.

8. Then, adjust the fine adjustment knob for clearer image of the specimen.

   Note: You will know that your specimen is focused correctly when you can look through them without straining your eyes. (or feeling dizzy after one or 2 seconds)

9. Adjust the aperture diaphragm

   Normally, if the aperture diaphragm is closed to 70-80% of the numerical aperture of the objective, then a clear image with reasonable contrast will be obtained. The scale on the condenser aperture diaphragm ring shows numerical aperture (N.A.), so adjust the condenser aperture diaphragm ring in accordance with the scale



In the case of the objective N A = 0.75
0.75 x 0.7-0.8 = 0.525-0.6
Adjust to this.

   40X/0.65X magnification 40X, numerical aperture 0.65

   The numerical aperture of every objective is indicated on the side of the objective.

10. Adjusting the field diaphragm

    The field diaphragm restricts the illumination light to the observation area of the specimen. If it is opened more than necessary it will result in reduced contrast due to stray light.
    Whenever the objective is changed, rotate the field diaphragm ring and adjust the field diaphragm image so that it is circumscribed by the surrounding field of view

11. Adjusting the illumination

    If the magnification of the objective is increased then the field of view becomes darker. Adjust the illumination with the brightness adjuster as necessary. Note that when the illumination changes the color tone also changes at the same time. If the voltage is turned down, the illumination

darkened red tones increase. If the voltage is turned up and illumination increased, then blue tones increase. In order to maintain a specific tone an ND filter is used to adjust the illumination.

SOP-IRRI-SHU-RSHT-BT-401.04b:

Title: How to prepare a good slide mount of fungi (please refer to SOP- IRRI-SHU Powerpoint Presentation No. as supplementary reference)

Application: Slide mounts are necessary for the examination of microscopic characteristics of fungi. These characteristics aide in identification, thus a good slide mount shall enable the analyst to make accurate identification.

Quality Assurance Checks:

The person preparing the slide mount should ensure that:

1. Cover glass and cover slips are clean (free from smudges/water marks).
2. The appropriate mounting medium is used in the preparation.
3. Mounting medium is freshly prepared.
4. Dissecting needles are clean; otherwise, dip the tips in alcohol to ensure that tips are free from any mycelial growth

When preparing slide mounts, consider the following aspects:

1. Age of the specimen
   Choose fungal growths which are not more than 7 days. Conidia, conidiophores, and mycelia from "fresh" specimens will provide clear microscopic details.
2. Abundance/density of the fungal growth
   Choose fungal growths which are not overcrowded
3. Number of fungi present in the seed.
   If possible, choose seeds with one genus of fungi only.

Materials:

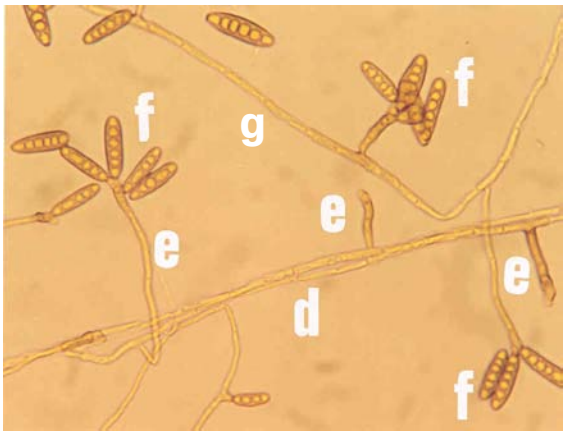
1. Dissecting needle (2 pcs)
2. Glass slides and cover slips
3. Mounting medium and stains (plain lactophenol; lactophenol with cotton blue)
4. Alcohol
5. Nail polish (colorless)
6. Paper towel
7. Stereo Microscope, binocular
8. Compound Microscope, binocular

Procedure:

1. Put a small drop of mounting medium on the (center of) glass slide
2. Focus on the specimen under the binocular stereo microscope.
3. With the aid of a dissecting needle "hold the seed"; then, with the other dissecting needle, get a small portion of the fungal growth from the specimen
4. While holding the dissecting needle with the fungal growth on one hand, carefully remove the specimen under the microscope and replace it with the glass slide with a mounting medium. Focus on the drop of mounting medium; then carefully dislodge/transfer the fungal growth on the mounting medium

Note: Steps 3 and 4 should be done under the stereo binocular microscope preferably at 6X or 12X

5. Remove the glass slide under the stereo microscope
6. Put the cover slip on the mounting medium with the fungal growth
   Note: this step should be done carefully/slowly (setting down) so as to avoid bubbles in the mount.
7. Examine under the prepared slide mount under the compound microscope



A good slide mount shows clearly the details of the e) condiophore, f) conidia, and g) mycelia of the fungi. In this slide mount the manner of attachment of the conidia to the conidiophore is clearly seen.

8. Seal the sides of the cover slip with a clear nail polish.
9. Put appropriate labels: name of the fungi, date of slide preparation
   Note: labels should be clearly written, correct spelling, etc.
10. Put/store prepared slides in designated slide folders

SOP-IRRI-SHU-RSHT-BT-401.04c: Guide (re: making illustrations/descriptions in the identification of seedborne fungi)

Habit character:

When doing Illustration/Drawing-

1. Use pencil
2. Make line illustration/drawing only. No shadings except when emphasizing color difference.
3. Size of organism should be realistic in relation to size of seed
4. Illustration should be as seen in specimen
5. Do not forget to specify magnification

When describing, take note of the following aspects:

1. Aerial mycelia — Present or Absent
   - If present:
     - Abundance (Abundant, Moderate, Scanty)
     - Appearance (Hairy, Cottony, others –specify)
     - Color (Colorless, brown, white, cream, others-specify)
     - Location on the seed (Sterile glumes, awn, all over the seed surface, others-specify)
     - Other remarks – abundant branching (loose or compact)
     - conidia collected together as false heads (dry or wet)
   - If absent:
     - Conidiophore and conidia only; mass of spores (pionnotes)
     - Conidia contained in specialized structures, i.e. pycnidia (describe shape, color, other features)
     - Location on the seed (sterile glumes, awn, scattered all over the seed surface)
     - Other remarks – relative length of conidiophore

Microscopic Character:

When doing Illustration/Drawing-

1. Use pencil
2. Make line illustration/drawing only. No shadings except when emphasizing color difference.
3. Draw at least 5 illustrations for big conidia (i.e. *Bipolaris oryzae*) and 10 illustrations for small conidia (i.e. *Sarocladium oryzae*)
4. Orientation should be in one direction only
5. Do not forget to specify magnification

When describing conidia or spores, take note of the following aspects:

1. Color – hyaline, slightly pigmented, brown, others-specify
2. Shape – globose, fusiform, clavate, sickle, others-specify
3. Septation – present or absent; if present –how many
4. Cell Wall – rough, smooth

5. Other features – shiny, opaque, with sterile appendage, others-specify

When describing conidiophores, take note of the following aspects:

1. Simple, branched-how many
2. Color – hyaline, brown, slightly pigmented, others-specify
3. Cell Wall –smooth, rough
4. Septation-present or absent
5. Other features – is it tapering towards the top; becoming lighter in color towards the top

SOP-IRRI-SHU-RSHT-BT-401.05: Data sheet for blotter test results.

SOP-IRRI-SHU-RSHT-TFN-402.00

Title: Modified Baermann Funnel Method /Sedimentation Test (please refer to IRRI-SHU Powerpoint Presentation No. as supplementary reference)

Application: This method is used for the detection and extraction of seedborne nematodes in rice seeds

Background:

Material(s):

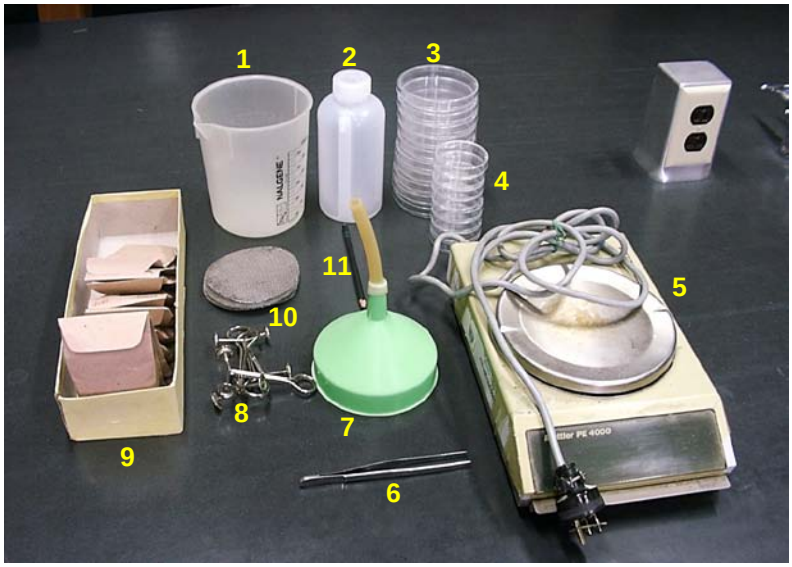


Fig. 1. Materials used in Modified Baermann Funnel Method – setting up

Legend:

1 – Plastic beaker
2 – Wash bottle
3 – Petri dish, plastic ( )
4 – Petri dish, plastic ( )
5 – Weighing balance
6 – Forceps
7 – Funnel, plastic with tygon tubing
8 – Pinchcock
9 – Seed samples
10 – Wire mesh (round)
11 – Marking Pencil

Quality Assurance Check:

The designated lead person for NematodeTest shall:

1. check the germination room regularly (everyday, at 8:00 AM and before 5:00 PM) and ensure that the air-conditioning unit and thermostat are set accordingly to meet the required/standard incubation temperature (28 ºC) and lighting requirement of the germination shelves (alternating cycles 12 hrs fluorescent light and 12 hrs darkness)
2. check/ensure that all materials to be used in this test are clean according to IRRI-SHU set standards (please refer to SOP-IRRI-SHU-RSHT-BT-401.01)
3. ensure that non-SHU staff who shall conduct the test are properly oriented with regards to the procedure, rules, and standards.

The person conducting the test shall:

1. ensure that the number of plates set on the working table corresponds to the number of seedlots and recommended working sample to be seeded.
2. ensure that the plates are labeled accordingly
3. ensure that the required amount of sample is accurately weighed and samples placed in the corresponding plates.
4. ensure that the required volume of water is added to the seed samples.
5. ensure that the samples are properly set on the pre-germination shelves.
6. ensure that the test and evaluation are conducted according to set standards.
7. ensure that the results are recorded accurately and accordingly.

Procedure:

Pre-treatment: none

Sampling – please refer to SOP-IRRI-SHU-DSI-SAMPLE-303.00

A. Pre-germination of samples
    1. Labelling
       Label plates using a marking pencil. The label (which includes the assigned SHU Reference Number-Seedlot Number) must be placed at one side of the plastic petri dish cover
    2. Weigh the samples (standard weight for INGER seedlots – 12.5 grms; GRC accessions- 200 seeds)
    3. Arrange the plates in the germination shelves accordingly
    4. Add enough tap water to cover the seeds
    5. Pre-germinate the seeds for 2-3 days at 28ºC and alternating cycles of 12 hrs fluorescent light and 12 hrs darkness

B. Setting-up of funnels/transferring of samples
   In setting up of funnels and transferring of samples, start at the top/uppermost level of the shelf, one level at a time until reaching the lowest shelf. For each level, it would be best if the setting up at each level is done in one direction (i.e. right to left)
    1. Put the wire mesh in the funnels.
    2. Attach the pinchcock to the tygon tubing.
    3. Put enough amount of water into the funnel (using the “neck: of the funnel as guide).
       Note: Leakage at this point is indicative that the pinchcock is loose and should be immediately addressed – change the pinchcock.
    4. Plates with pre germinated seeds are placed near the funnels.
    5. Transfer all of the contents of the plate into the funnel (1 plate = 1 funnel)
       Note: Extra precaution should be observed so as to avoid spillage6. Spread the seeds making sure that the seeds do not go beyond the sphere of the wire mesh; otherwise the seeds shall go down the tygon tubing which is not acceptable.
    6. Make sure that the seeds are covered with water/seeds are submerged
       Note: Add some more water if there is a need to i.e. the seeds are not covered with water.

7. Put the cover of the plate to the funnel – to serve as label.
8. “Incubate”/set aside overnight.
9. Extract the following day.

C. Extraction

In extraction, start at the lowest shelf, working one level at a time until reaching the top/uppermost shelf

1. Extract at least 10 ml.
2. The plate cover should be used to cover the extract.

   Note: In the event that the amount of extract exceeded the required amount, return the extract; then set aside overnight. Do not extract immediately.

D. Evaluation

Examine the extract under the stereo microscope for the presence/absence of seedborne nematodes. In the event that nematodes are observed in the extract, a sample of the nematode shall be mounted so that details of the mouthparts can be examined to determine whether the nematode is saprophytic or parasitic. Refer to existing literatures for confirmation/ validation.

(In rice, there is only one (1) seedborne nematode, *Aphelenchoides besseyi.)*

Do actual count, if the nematodes present in the extract are *Aphelenchoides besseyi.*

If the nematodes present in the extract are saprophytes, there is no need to do actual count and identify the genus and species,

Calculation: N/A

Solution: N/A

Data entry: Refer to IRRI-SHU Powerpoint No. ___ and IRRI-SHU-RSHT-BT-4001.05: Data sheet for Blotter test results

Results are recorded in the Data sheet for Blotter Test (specifically at the space for *Aphelenchoides besseyi*) as follows:

1. If extract is negative for the presence of nematodes, data is reflected as zero (0).
2. If extract is positive for the presence of *Aphelenchoides besseyi (Ab)*, result is reflected as actual count.
3. If extract is positive but the nematode is saprophytic, the result is reflected as zero (0) indicating the absence of *Ab* and to indicate the presence of saprophytic nematodes, a letter S is written.
4. If the extract is positive for both Ab and saprophytic nematode, result is reflected as actual count of *Ab* and S.

Safety: This procedure shall be conducted in the seed health testing laboratory by persons with working knowledge of this method and familiar with the principles of Good Laboratory Practice (please refer to IRRI-SHU Policies and Guidelines)

Seeds and blotters after evaluation shall be disposed following Disposal Protocol (Please refer to SOP-IRRI-SHU Doc._____.)

Reference(s):
Attachment(s): Recording Sheet No.
Prepared by: Patria G. Gonzales
Date:
Approved by: **Date:**
Issued by: **Date:**

SOP-IRRI-SHU-RSHT-MAC-403.00

Title: Macro examination for *Tilletia barclayana* (please refer to IRRI-SHU Powerpoint Presentation No. as supplementary reference)

Application: Macro examination is used to detect and examine seeds that are infected and/or contaminated with *Tilletia barclayana* (Tb).

Material (s):

Photo

Quality Assurance Check:

The lead person shall ensure that

1. the physical samples are consistent with the seedlist in terms number and corresponding labels.
2. safety precautions are observed while doing the test
3. the person conducting the test is working knowledge of the procedure and has the skill to identify the teliospores of *Tilletia barclayana*

Pre-treatment: N/A

Procedure:

1. Put seed sample on petri dish (at least one layer only)
2. Scan seeds under the stereo microscope for the absence (–) or presence (+) of *Tilletia barclayana*
3. If negative for the presence of Tilletia barclayana, indicate (-).
   For seedlots weighing 500 gms or less, ensure that all seeds have been scanned before declaring a negative observation.
   For seedlots weighing more than 500gms, ensure that 50% of the seeds have been scanned before declaring a negative observation
4. If positive for the presence of *Tilletia barclayana*, get 400 seeds at random and separate seeds and with and without Tb. Calculate detection %.
   If the detection % is <10%, scan all seeds and manually remove seeds with Tb.
   If the detection % is >10%, do not proceed in scanning. Indicate in the seedlist the detection percentage.

Calculation: The % detection is calculated as follows:

$$\% \text{ detection} = \frac{\text{Total number of seeds with } \textit{Tilletia barclayana}}{400} \times 100$$

Solution: N/A

Data entry: Results are indicated in the seedlist beside the SHU reference number as - **,** + cleaned (if detection is <10%), or + % detection (if detection is >10%)

Safety: Staff doing the test should wear a face mask to avoid possible inhalation of dust particles brought about by transferring the seeds from the bags to the sampling pans.

Reference(s):
Attachment(s):
Prepared by: Patria G. Gonzales **Date:** 12 December 2008
Approved by: **Date:**
Issued by: **Date:**

SOP-IRRI-SHU-RSHT-BAC-404.00

Title: Seed Wash Assay Test using Semi selective medium (please refer to IRRI- SHU Powerpoint Presenation No. as supplementary reference)

Application: To extract, isolate, and purify the following bacteria in rice seeds.

a. *Acidovorax avenae* subsp*. Avenae*
b. *Burkholderia glumae*
c. *Pseudomonas fuscovaginae*
d. *Xanthomonas oryzae* pv*. oryzae*
e. *Xanthomonas oryzae* pv*. oryzicola*

Material(s):

a. Mortar and pestle
b. L-shaped glass rods
c. Pipettor and pipette tips (10 ml, 1 ml, .01 ml)
d. Burners/alcohol lamp
e. Igniter/Lighter/match
f. Petri Dish, glass, 9mm
g. Test tubes and test tube racks
h. Erlenmeyer flasks
i. Nylon mesh
j. Green ties
k. Beaker, plastic/equivalent plastic container
l. Spatula (plastic/metal)
m. Paper towel
n. Wireloops/inoculating sticks
o. Vortex mixer
p. Laminar flow with Ultraviolet (UV) light
q. Orbital shaker – capable of holding at least 15 Erlenmeyer flasks and run 100 rpm
r. Incubator –capable of operating under varying ranges in temperature (28-30°C)
s. Laminar flow hood
t. Solutions/culture medium
    i. Sterile distilled water , 9 ml in test tubes
    ii. Cycloheximide (0.01%) - antifungal
    iii. Phosphate buffer saline (PBS) with 0.25% Tween
    iv. King's Medium B (KMB) for fluorescent *Pseudomonas*
    v. Tryptic Soy Agar (TSA) for non fluorescent *Pseudomonas*
    vi. Suwa's Medium/ Wakimoto's Medium (WF-P) for *Xanthomonas* spp.

Quality Assurance Check:
The designated lead person in the preparation of materials, solutions, and culture medium to be used in the isolation, extraction, and purification shall:

1. ensure that all materials are prepared according to set standards and prepared at least 2 days before the scheduled run date(s).
2. ensure that the isolation room is clean and "sterilized".
3. ensure that the temperature of the incubator has been calibrated according to required temperature (Note: must be done at least 2 days before the extraction/isolation)
4. ensure that persons who will assists in conducting the tests are properly oriented with regards to the procedure, rules, and standards.
5. ensure that the tests and evaluation shall be done according to set standards.

Procedure:
Pre-treatment- N/A
Sampling - please refer to SOP-IRRI-SHU-DSI-SAMPLE-303.00
A. Extraction

1. Preparation of samples

1. Put working sample in nylon mesh (individually).

2. Tie the nylon mesh with green tie (or equivalent) so as to secure the seeds.

3. Put the samples in a container (plastic or glass).
   The container must allow samples to "move about"

4. Cover the container with nylon mesh and secure with rubber band (or equivalent) to secure the samples inside.

5. Wash the seed samples in running water for 30 minutes.

6. Blot dry seed samples by putting each seedlot on a sterile paper towel

7. Crush seed samples in a sterile mortar and pestle until 80% of the seeds are crushed.

8. Transfer crushed seed samples in an Erlenmeyer flask with Phosphate Buffer Saline (PBS) solution with 0.25% Tween

Standard:

Vol. of seeds	Vol. of PBS
25 seeds	5 ml
5 gms	25 ml
25 gms	50 ml
50 gms	100 ml

9. Allow set up to stand for 1 hr

10. Shake samples for 2 hrs in an orbital shaker at 100rpm

B. Isolation

From the seed extract, prepare a ten-fold dilution series up to 10^{-5} in sterile Saline Solution (0.85% NaCl)

1. Transfer 1 ml of seed extract into 9 m of sterile Saline Solution

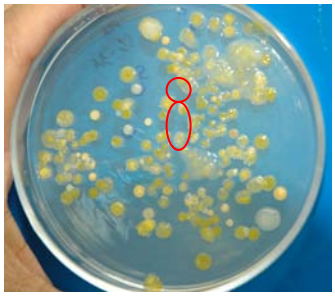
2. Pipette 0.1 ml. from each serial dilution of 10^{-3}, 10^{-4}, and 10^{-5} on plated semi selective medium*

*King's Medium B (KMB)- for fluorescent Pseudomonas, Tryptic Soy Agar (TSA), Suwa's Medium/Modified Wakimoto Medium. Cycloheximide (0.1%) is added to the medium prior to plating to reduce fungal contamination.

3. Spread bacterial suspension on the surface of the agar using sterile L-shaped glass rods.

4. Incubate KMB/TSA plates for 72 hrs, while Suwa's/Wakimoto Medium for 6 days at 28-30°C.

5. Observe plates after incubation period.

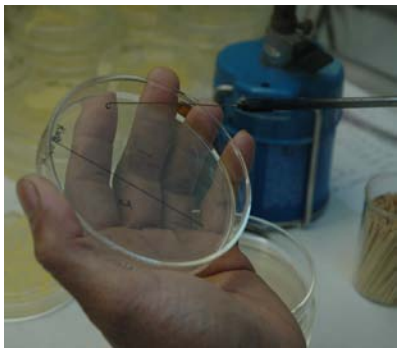


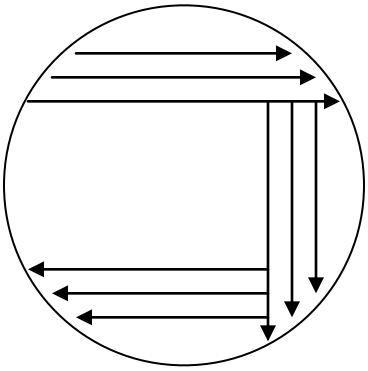
6. Compare colonies with reference Strain Isolates for identification. Mark suspected colonies

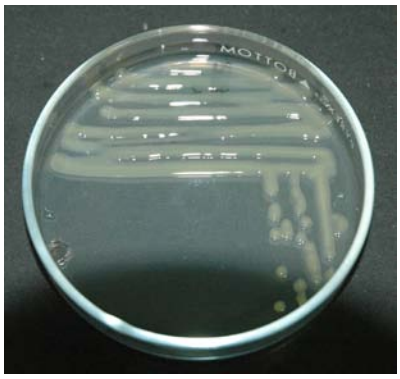
7. Record observations (use Forms SHU-BAC-404.01 and 404.02).

C. Purification

1. With a sterile wireloop or sterile inoculating stick, pick a single distinct colony and streak in duplicated media. Incubate at 28-30°C for 48 hrs.







2. Single, distinct colonies are then streaked in test tube slants.

Calculation: N/A
Solution: N/A
Data entry: Following the data sheet SOP-IRRI-SHU-BAC-404.01, observations are indicated as + (for presence of target genus of bacteria) or – (for absence of target genus of bacteria). If +, the isolate number and dilution are indicated.
Following the data sheet SHU-BAC-404.02, the morphology of the bacteria are described following appropriate descriptions (refer to SOP-IRRI SHU-BAC-404.03 and 404.04)
Safety: This procedure shall be conducted in the isolation room (under the laminar flow hood) of the seed health testing laboratory by persons with working knowledge of this method and familiar with the principles of Good Laboratory Practice (please refer to IRRI-SHU Policies and Guidelines)
After extraction, isolation, and purification, seeds, and agar media used in this test shall be disposed following Disposal Protocol (Please refer to SOP-IRRI-SHU Doc._____.)

Reference(s):

Cottyn, Bart. 2002-2003. Bacteria Associated with Rice Seed from Philippines Farmer's Field. IRRI 236 pp.

Kyin Kyin Win. 2008. Studies on the transmission of *Xanthomonas oryzae* pv. *oryzae* (Ishiyama) Swings et al through rice (Oryza sativa L.) seeds from bacterial blight infected fields

Bacterial Pathogens. Lecture presented by Ms. Isabelita Ona during the Training Workshop on the Diagnostics of Seed-borne Rice Diseases, 30 August- 05 September 2008, IRRI

Unpublished Final Reports of Ms. Helen Barrios (former AS I) and Ms. Jocelyn Guevarra (former Researcher) who conducted research and test runs re: seed wash assay test using semi selective medium for bacterial testing

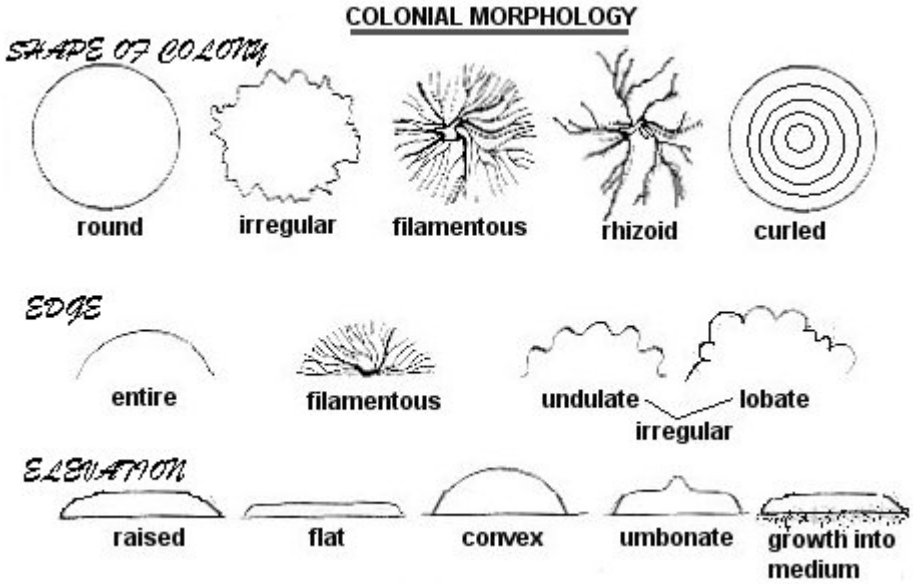
Seed Health Unit
International Rice Research Institute
Form RSHT-BAC-404.01 **Page ___ of ___**

Reference No. _____ **Date of Extraction/Isolation** _____

Name and Signature of Analyst _____**Volume of seeds used** _____

	For *Pseudomonas* spp.			**For *Xanthomonas* spp.**			
	Media used _____			**Media used** _____			
	Date of Evaluation _____			**Date of evaluation** _____			
	E V A L U A T I O N						
SEEDLOT NO.	10^{-3}	10^{-4}	10^{-5}	10^{-3}	10^{-4}	10^{-5}	REMARKS

SOP-IRRI-SHU BAC-404.03. Guide in describing morphology of bacterial colonies.



Source: 8/2005, Jackie Reynolds, Richland College

Prepared by: Patria G. Gonzales
Date: 24 September 2008
Approved by: **Date:**
Issued by: **Date**

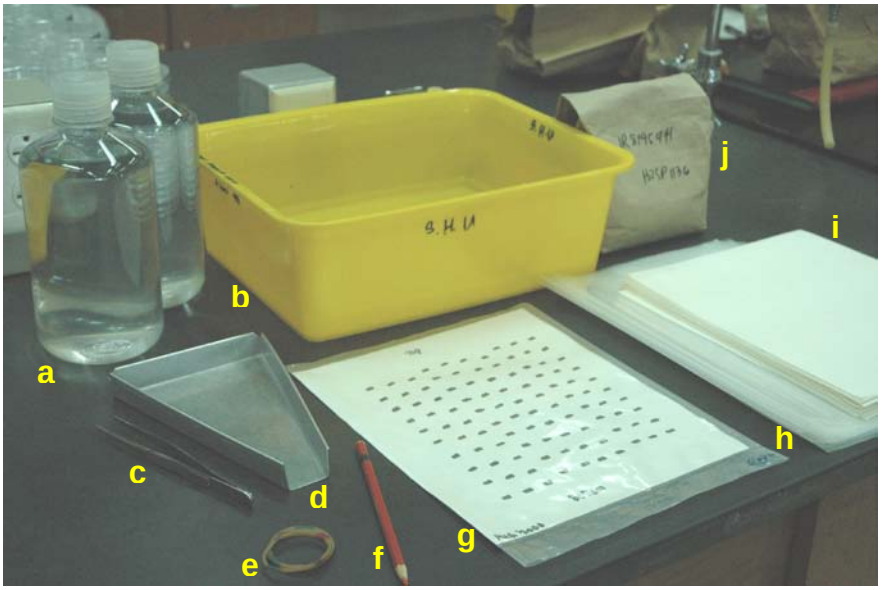
SOP-IRRI-SHU-RSHT-BP-405.00

Title: Between–paper (BP) test (please refer to IRRI-SHU Powerpoint Presentation No. as supplementary reference)

Application: To evaluate germination and vigour of rice seeds. Evaluation for normal and abnormal seedlings and ungerminated seeds are included in this test.

Background: BP test appears in Chapter 5 of the International Seed Testing Association (ISTA) Rules for Seed Testing, 2008 edition

Materials:



a) Distilled water
b) Tray, plastic, rectangular
c) Forceps
d) Sampling pan
e) Rubber bands
f) Marking Pencil-water proof
g) Template-for seeding
h) Plastic bags
i) Germination blotters-rectangular, 8”x11.5”
j) seed samples

Not shown:
Incubator/Incubation room (not shown)- capable of operating at 28°C and alternating cycles of 12 hrs darkness and 12 hrs light (from an artificial source).

Quality Assurance Check:

The designated lead person for the between paper test shall:

1. check the incubation room regularly (everyday, early in the morning and before 5:00 PM) and ensure that the air-conditioning unit and thermostat are set accordingly to meet required/standard incubation temperature (28 ºC $\pm$ 1) and lighting requirement (alternating cycles 12 hrs NUV and 12 hrs darkness)
2. check/ensure that all materials to be used in this test are clean according to IRRI-SHU set standards (please refer to SOP-IRRI-SHU-RSHT-BT-401.01)

3. The designated lead person shall ensure that the set up are set properly on the assigned shelves inside the incubation room.
4. ensure that non-SHU staff who shall conduct the test are properly oriented with regards to the procedure, rules, and standards.

The person conducting the test shall:
1. counter check that the labels of the seedlots are consistent with the information in the seedlist
2. ensure that the number of blotters, plastics, etc. set on the working table corresponds to the number of seedlots and recommended working sample to be seeded.
3. ensure that the blotters are labeled accordingly
4. ensure that the samples are seeded on the corresponding germination blotters.
5. ensure that the seeding and evaluation is done according to set standards.
6. ensure that the corresponding data sheets have been prepared

Procedure:
Pre-treatment: N/A
Sampling: 400 seeds/seedlot are used as working sample, taken randomly.

A. Seeding
   Use 2 pcs of germination blotters for 100 seeds
   1. With a water proof marking pen, put label on the upper left hand corner of the germination blotter
   2. Dip the germination blotters in distilled water
   3. Lay down the moist germination blotters on top of the seeding template making sure that the label is at the back
   4. Put/place 100 seeds making sure that the seeds are equidistant from each other.
   5. After seeding, roll the germination blotters carefully making sure that the set up is not too tight not loose.
   6. Put the rolled germination blotters inside a plastic bag in an upright position using the label as the point of reference.
   7. Secure the plastic bag with rubber bands.
   8. Incubate under alternating cycles of 12 hrs light and 12 hrs darkness and 28°C.

B. Evaluation
   Evaluate on the 5^{th}, 7^{th}, and 15^{th} day after seeding and seedlings must be evaluated in accordance to the general principles stated in Sections 5.2.3 and 5.2.4 of the ISTA Rules.
   When evaluating, care should be taken so as not to “disturb” other seedlings.
   1. On the 1^{st} and 2^{nd} evaluation, remove and count all normal seedlings and badly decayed seeds or seedlings so as to reduce the risk of secondary

infection. Seedlings that can not be evaluated as normal should be retained until the final evaluation.

2. Roll back the germination blotters and put inside the plastic bags again for re-incubation.
3. On the final evaluation, record the final observations and compute accordingly.
4. Seeds which failed to germinate and abnormal seedlings with lesions can be examined under the microscope for presence of microorganisms; or can be blotter tested to allow growth of microorganisms which can be the possible cause of failure in germination or abnormality.

Calculation: $\frac{\text{Total Number of type of seed/seedling}}{\text{Total Number of seeds}} \times 100 = \%$

Solution: N/A

Data entry: Data are recorded in Data Sheet for Germination BP (Document or Form No.)

For each evaluation day, data should reflect the number of Normal (N) seedlings, Abnormal (Ab) seedlings, Dead/Ungerminated seeds. Other observations should be indicated - if there are fungal/bacterial growth.

On the final day of evaluation, the total number of N/ Ab seedlings and D seeds are collated

Safety: This procedure shall be conducted in the seed health testing laboratory by SHU staff with working knowledge of this method and familiar with the principles of Good Laboratory Practice (please refer to IRRI-SHU Policies and Guidelines)

After the test, all materials shall be disposed following Disposal Protocol (Please refer to SOP-IRRI-SHU Doc._____.)

Reference(s): International Rules for Seed Testing, ISTA, 2008 Edition
Attachment(s): Recording Sheet 405.1
Prepared by: Patria G. Gonzales
Date: 25 September 2008
Approved by: **Date:**
Issued by:

SOP-IRRI-SHU-RSHT-TP-406.00

Title: Top of the paper

Application: To determine seedling vigour and germination of seeds at the same time determine the cause of seedling abnormality and failure in germination.

Material:

plastic trays with cover
germination blotters (rectangular)
marking pencil
distilled water

Quality Assurance Check:

The person conducting the test has a working knowledge in conducting the test and evaluation based on set standards

The person conducting the test should ensure that all materials are prepared before conducting the test

Procedure:

A. Setting Up

1. Label the plastic trays accordingly
2. Put 1 layer of moistened germination blotter paper
3. Put the seeds
   Note: make sure that there are 10 rows and each row should include 10 seeds; seeds should have the same orientation and equidistant with one another)
4. Put the transparent plastic cover
5. Incubate seeded trays under 28 °C with alternating cycles of 12 hrs darkness and 12 hrs light*

B. Evaluation

Evaluate on the 5^{th}, 7^{th}, and 15^{th} day after seeding and seedlings must be evaluated in accordance to the general principles stated in Sections 5.2.3 and 5.2.4 of the ISTA Rules.

When evaluating, care should be taken so as not to “disturb” other seedlings.

1. On the 1^{st} and 2^{nd} evaluation, count all normal and abnormal seedlings.
2. Mark (preferably with toothpick) spots wherein seeds failed to germinate.
3. Put back plastic cover and re incubate.
4. On the final evaluation, record the final observations and compute accordingly.
5. Examine under the stereo microscope seeds which failed to germinate/abnormal seedlings for the presence of microorganisms; or incubate following the blotter test method to allow microorganisms to grow which can be the possible cause of failure in germination or abnormality.

Setting Up:

1. Label the plastic trays accordingly
2. Put 1 layer of moistened germination blotter paper
3. Put the seeds
   Note: make sure that there are 10 rows and each row should include 10 seeds; seeds should have the same orientation and equidistant with one another)
4. Put the transparent plastic cover /
5. Incubate under 28 °C with alternating cycles of 12 hrs darkness and 12 hrs light*
6. Evaluate at 5, 7, and 15 days after seeding.

Calculation: $\frac{\text{Total Number of type of seed/seedling}}{\text{Total Number of seeds}} \times 100 = \%$

Solution: N/A

Data entry: Data are recorded in Data Sheet for Germination BP (Document or Form No.)
For each evaluation day, data should reflect the number of Normal (N) seedlings, Abnormal (Ab) seedlings, Dead/Ungerminated seeds. Other observations should be indicated - if there are fungal/bacterial growths.
On the final day of evaluation, the total number of N/ Ab seedlings and D seeds are collated

Safety: This procedure shall be conducted in the seed health testing laboratory by SHU staff with working knowledge of this method and familiar with the principles of Good Laboratory Practice (please refer to IRRI-SHU Policies and Guidelines)
After the test, all materials shall be disposed following Disposal Protocol (Please refer to SOP-IRRI-SHU Doc._____.)

Reference(s): International Rules for Seed Testing, ISTA, 2008 Edition
Attachment(s): Recording Sheet 407.1
Prepared by: Patria G. Gonzales
Date: 06 August 2009
Approved by: **Date:**
Issued by:

Approved by: **Date:**
Issued by: **Date:**

SOP-IRRI-SHU-RSHT-SAND/SOIL-407.00

Title: In sand/soil test

Application: To determine seedling vigour and germination of seeds at the same time determine the cause of seedling abnormality and failure in germination.

Material:

Photo

Quality Assurance Check:

The person conducting the test has a working knowledge in conducting the test and evaluation.

The person conducting the test should ensure that all materials are prepared before conducting the test

Procedure:

A. Seeding

1. Label the bema trays accordingly
2. Put appropriate amount of soil/sand (500gms/bema tray)
   Note: initially put about 400 grms; the remaining 100 grms shall be used to cover the seeds
3. Create furrows (4) longitudinally with the help of a pot label.
4. Each tray should accommodate 100 seeds; 25 seeds per furrow
   (For each furrow, put 25 seeds equidistantly, making sure that the seeds are in one orientation)
5. Cover the seeds with the remaining 100 grms of the soil/sand.
6. Water the set up then put the cover (approximately ------ ml/ tray)
7. Put the transparent plastic cover of the bema tray.
8. Incubate under 28 °C with alternating cycles of 12 hrs darkness and 12 hrs light*

B. Evaluate on the 5^{th}, 7^{th}, and 15^{th} day after seeding and seedlings must be evaluated in accordance to the general principles stated in Sections 5.2.3 and 5.2.4 of the ISTA Rules.

When evaluating, care should be taken so as not to “disturb” other seedlings.

1. On the 1^{st} and 2^{nd} evaluation, count all normal and abnormal seedlings and mark spots wherein seeds failed to germinate.
2. Put plastic cover back and re-incubate.
3. On the final evaluation, record the final observations and compute accordingly.
4. Seeds which failed to germinate and abnormal seedlings with lesions can be examined under the microscope for presence of microorganisms; or can be blotter tested to allow growth of microorganisms which can be the possible cause of failure in germination or abnormality.

Calculation: $\frac{\text{Total Number of type of seed/seedling}}{\text{Total Number of seeds}} \times 100 = \%$

Solution: N/A

Data entry: Data are recorded in Data Sheet for Germination BP (Document or Form No.)

For each evaluation day, data should reflect the number of Normal (N) seedlings, Abnormal (Ab) seedlings, Dead/Ungerminated seeds. Other observations should be indicated - if there are fungal/bacterial growth.

On the final day of evaluation, the total number of N/ Ab seedlings and D seeds are collated

Safety: This procedure shall be conducted in the seed health testing laboratory by SHU staff with working knowledge of this method and familiar with the principles of Good Laboratory Practice (please refer to IRRI-SHU Policies and Guidelines)

After the test, all materials shall be disposed following Disposal Protocol (Please refer to SOP-IRRI-SHU Doc._____.)

Reference(s): International Rules for Seed Testing, ISTA, 2008 Edition
Attachment(s): Recording Sheet 4007.1
Prepared by: Patria G. Gonzales
Date: 06 August 2009
Approved by: **Date:**
Issued by: