United States Department of Agriculture
Agricultural Marketing Service
Federal Grain Inspection Service

Directive

9180.47 April 23, 2021

EXAMINATION OF GRAIN FOR THE PRESENCE OF TCK SPORES

Contents

1. PURPOSE

This directive transmits sample handling, testing, and certification procedures for grain examined for the presence of Tilletia controversa Kühn (TCK) smut spores.

2. REPLACEMENT HIGHLIGHTS

This directive replaces Program Directive 9180.47, Examination of Grain for the Presence of TCK Smut Spores, dated July 24, 2012. It updates the background; responsibilities; sample size; testing procedures; and certification information. Additionally, it adds FGIS as a TCK service provider; sections on Authorizing Official Personnel; Review Inspections; Proficiency Verification; and Fees. An attachment titled Official Procedure for Identifying and Enumerating Tilletia controversa Kühn (TCK) Teliospores in Grain Samples Using Light Microscopy was added.

3. POLICY

The Administrator has determined that the Federal Grain Inspection Service (FGIS) will, upon request, provide for analysis of grain samples for the presence of TCK smut spores under authority of the U.S. Grain Standards Act.

4. BACKGROUND

TCK, also called Dwarf Bunt, is a smut fungus found in the soil or on the surface of wheat kernels. The organism only infects plants that grow during the winter. High levels of the disease are usually found after early and persistent snow cover. Wheat plants infected with TCK are obviously dwarfed in contrast to healthy plants, and kernels are replaced with brown masses of spores known as smut balls. The spores are not harmful to humans or livestock.

TCK testing is offered as Official Criteria under the United States Grain Standards Act. Using microscopy techniques, it is possible to ascertain the presence of spores. Differentiation of TCK from common bunt is based on identifying structural differences in the teliospores of TCK versus other smut species. Upon request, analysis of grain samples other than wheat for presence of TCK smut spores is permissible.

5. REQUEST FOR SERVICES

Based on an agreement between the United States and the Peoples Republic of China (PRC), FGIS has arranged for TCK smut testing service (quantitative) to be provided through the Wheat Marketing Center (WMC) laboratory in Portland, Oregon for testing samples. Additionally, FGIS (League City (Texas) Field Office (LCFO) and Technology and Science Division (TSD)) may provide qualitative or quantitative TCK testing service to customers. Shipments to PRC are restricted to quantitative analysis only.

TCK testing may be performed on shiplot composite samples, unit train composite samples, bargelots, carlots, trucklots, and submitted samples. Interested parties wishing to receive TCK testing should contact their Official Agency or Field Office (hereafter OSP, official service provider) for service.

6. RESPONSIBILITIES

a. Applicant for Service. The applicant will request TCK testing through their OSP. The request must include the applicant's name and telephone number and the destination of the lot, if known.

b. Official Service Provider. The OSP will forward the sample to WMC or LCFO at the applicant's expense with the required information. The OSP will issue the certificate and bill the applicant for service for samples tested by WMC. For samples derived outside the Pacific Northwest and Texas, FGIS will advise the OSP where to send the sample.

c. WMC. For TCK requests in the Pacific Northwest, WMC is responsible for performing an original test, and reinspection analysis if requested; maintaining a file sample for 90 days; reporting the results to the OSP in a timely manner; and billing the OSP for the specified per sample analysis rate. The WMC will maintain all test records and documentation and provide them to FGIS upon request.

d. FGIS. For TCK tests not performed by WMC, FGIS is responsible for performing an original test and review (reinspection/appeal) analysis/Board appeal, if requested; maintaining a file sample for 90 days; reporting the results to the applicant for service in a timely manner; and billing the applicant for the specified per sample analysis rate. A quantitative analysis (actual number of spores per 50 grams) is provided unless a qualitative analysis (a screening for the presence of TCK) is requested. The qualitative analysis will cease when the first teliospore is detected. FGIS will issue the certificate for testing performed by FGIS.

7. GENERAL SAMPLING AND ANALYTICAL PROCEDURES

The TCK analysis requires a 650-gram minimum sample. Original service points must maintain an additional 650-gram sample as a backup file sample. Upon completion of official sampling and/or inspection, the service point will forward the official sample to WMC or the LCFO. The WMC or LCFO will maintain a file sample on each original and appeal inspection for 90 days.

The OSP will use a Boerner divider to obtain a 650-gram minimum sample from a representative sample (vessel or unit-train composite) and place it in an airtight container (e.g., double walled, sealed plastic bag) for shipment to the lab. The sample bag will be marked with the carrier identification name or number and type of test requested, and be accompanied by documentation identifying the carrier, lot number (if applicable), original inspection certificate number (if available), Field Office or Official Agency address and phone number, the date, destination (mandatory if going to the

PRC), and a legible copy of the application for inspection. Retain a file sample of at least 650 grams in an airtight container.

For samples to be tested at WMC, send the sample(s) and documentation by overnight delivery (if appropriate), at applicant's expense, to:

Wheat Marketing Center, Inc.
1200 N.W. Naito Parkway, Suite 230
Portland, OR 97209-2800
ATTN: TCK TESTING

For samples to be tested by FGIS, send the sample(s) and documentation by overnight delivery (if appropriate), at applicant's expense, to:

USDA-AMS-FGIS.
1025 E. MAIN STREET, #104
LEAGUE CITY, TX 77573- 2483
281-338-2787
ATTN: TCK TESTING

Note: Sample analysis will take hours and possibly days. The lab will notify the OSP of the result(s) promptly upon completion of testing.

8. REVIEW INSPECTIONS

Sections 800.125 and 800.135 of the regulations permit a review inspection on either official grade/factors or official criteria. When requested, a review inspection for official grade/factors and official criteria may be handled separately even though both sets of results are reported on the same certificate.

Applicants may request a reinspection, appeal, and Board appeal. WMC or LCFO will perform reinspections on the basis of the file sample. The reinspection analysis should be performed with a different analyst if possible, using the file sample.

The appeal must be performed at or under the supervision of the field office on a new extraction. The board appeal must be performed by the FGIS Technology and Science Division on the extraction from the appeal analysis, in the 15-ml tube containing the sample suspended in Shearer's solution.

Applicants may request either qualitative or quantitative review analysis unless the original or a review analysis was quantitative. Then, only a quantitative analysis is available. All review inspection results replace previous results.

9. CERTIFICATION

All shipments to the PRC shall be analyzed on a quantitative basis. Certify the results in the results section on the appropriate official certificate. The results must be certified on the official grade certificate unless separate certification is requested by the applicant. The applicant for service must make this request in advance of the lot being certified. Submitted sample results must be certified on a submitted sample certificate.

Quantitative TCK testing:

The certification procedure/remark is based on the export destination.

The following factor remarks must be used to describe the factor result:

Quantitative TCK testing:

Export shipments to the PRC:

Tilletia controversa Kühn spores (exceed/do not exceed) 30,000 per 50 grams of sample.

For all export (other than PRC) and domestic analyses:

(number of spores) Tilletia controversa Kühn spores per 50 grams of sample.

Qualitative TCK testing:

Tilletia controversa Kühn spores (present/not present).

10. FEES

FGIS will use billing code G2TCK, as stated in FGIS Program Directive 9180.74, found on the AMS website: https://www.ams.usda.gov/about-ams/fgis-program-directives

11. AUTHORIZING FGIS PERSONNEL

FGIS will authorize personnel to perform TCK testing. For Federal personnel, authorization requires a written test in FOL and a proctored practical test, to ensure procedures are followed according to the Official Procedure document. Authorization records will be housed in the FOL module of FGISonline, consistent with established practices for licensing official personnel to perform official duties.

12. PROFICIENCY VERIFICATION

FGIS will establish and maintain a proficiency verification program to evaluate the proficiency of personnel performing TCK testing at both the WMC and FGIS sites. Proficiency will be evaluated on at least an annual basis. FGIS subject matter experts will randomly choose 10 percent of samples processed per service location for testing. Evaluation of proficiency will follow the statistical model of Whitaker et al (2001, Plant Pathology 50:755-760).

13. QUESTIONS

Any questions should be directed to the Policies, Procedures, and Market Analysis Branch (PPMAB) at (816) 659-8403.

14. ATTACHMENT

ATTACHMENT

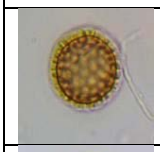
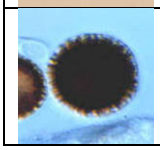
Official Procedure for Identifying and Enumerating *Tilletia controversa* Kühn (TCK) Teliospores in Grain Samples Using Light Microscopy.

AMS-FGIS gratefully acknowledges the contributions of ARS, and in particular Dr. Gary Peterson of the Foreign Disease-Weed Science Research Unit for his generous contributions in training FGIS personnel on the procedures of TCK analysis and the preparation of these instructions.

Table of Contents

1. *Tilletia* spp Infecting Wheat

There are four *Tilletia* spp that infect wheat however two (*T. laevis* Kühn and *T. indica* Mitra) are morphologically distinct and not relevant to this testing procedure.

	Species	Other Names	Disease
	*T. controversa* Kühn	TCK	Dwarf bunt
	*T. tritici* Winter	*T. caries* Tul., TCT	Common bunt
	*T. laevis* Kühn	*T. foetida* Liro.	Common bunt
	*T. indica* Mitra	*Neovossia indica,* Mundkur., KB	Karnal bunt

2. Protocol for Isolation, Enumeration and Identification of *Tilletia* Teliospores from Grain Samples

a. Sample Extraction

Step 1

If processing more than one sample, label a 600 ml beaker, a 500ml flask, two 50 ml centrifuge tubes and one 15 ml centrifuge tube with the sample number.

Step 2

Mix the sample by gently rotating kernels in a plastic bag. The bag should have sufficient air space for the kernels to roll around freely. **Do not** shake vigorously (see Appendix VIII).

Step 3

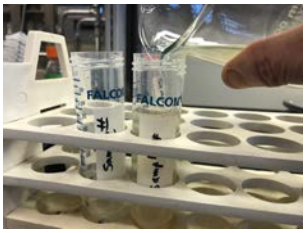
Weigh 50 g sample into a weighing dish or 3-oz paper cup and transfer to a 500-ml Erlenmeyer flask.

Step 4
When ready to begin the extraction, add 100 ml of water containing Tween-20 water (Appendix III). Do not allow the wheat to sit in the solution for extended periods of time.

Step 5
Swirl the flask by hand or on an orbital shaker at 200 rpm for 5 min.

Step 6
Immediately pour the contents of the flask into a 52-µm nylon micromesh pore-size sieve set on an angle over the beaker. Rinse the contents of the flask with 10 ml of water, pouring the rinse into the sieve. There may be some wheat kernels remaining in the flask, but this is not of concern. Allow the sieve with grain to sit on the beaker until it has stopped dripping.


Step 7
Swirl the beaker to keep the contents in suspension then pour equal amounts into each of the two 50-ml centrifuge tubes.



Step 8
Centrifuge at 600 x *g* for 5 min in a horizonal bucket rotor or 800 X *g* for 15 min in a fixed angle rotor. After the centrifuge comes to a stop, gently remove the centrifuge tubes and slowly pour off the supernatant into a 250 ml beaker. If the pellet dislodges from the centrifuge tube during decanting, the contents of the 250 ml beaker can be centrifuged again.



Step 9
With a pipette, add 1.0 ml Shear's mounting medium (see Appendix III) to each of the 50-mL conical centrifuge tubes. Using a disposable Pasteur pipet to aspirate the sample in and out of the pipette to resuspend the pellet and the transfer both suspensions to a single 15-ml centrifuge tube. If only one sample is being processed, add an equal amount of water to a second 15 ml centrifuge tube to balance the centrifugation.

Step 10
Centrifuge the sample in the 15 ml centrifuge tube at the same speed and time as before. Carefully pour off the supernatant into a 250 ml beaker.

Step 11
After pouring off the supernatant, there will be a small amount of supernatant that adheres to the inner wall of the centrifuge tube and settles back down on top of the pellet. Re-suspend the pellet using a Pasteur pipette.

Step 12
Draw the entire suspension into the Pasteur pipette then slowly return the suspension back into the 15-ml tube counting the individual drops. If there are less than 12 drops, add additional drops of Shears with a clean pipette to raise the count to 12 drops and mix. If the sample suspension is very viscus and opaque due to the amount of debris in the sample, add an additional 12 drops of Shears with a clean pipette and resuspend. If the debris pellet is very large, additional Shears can be added and additional drops included. **Record** the total number of drops in the tube on the data sheet.

Note: *Once the sample is suspended in Shears Medium, it can be stored at room temperature for more than a year with no changes. This is useful if you want to examine the sample at a later time, use for training or shipping to another laboratory or authority for results confirmation.*

Step 13
Clean-up: The extracted 50g wheat sample can be dumped in the trash or alternatively, first dump it into a fine-holed colander or colander lined with cheese cloth set in the sink. Grains left behind in the flask can rinsed into the colander. This prevents grain from lodging in the sink trap and germinating.

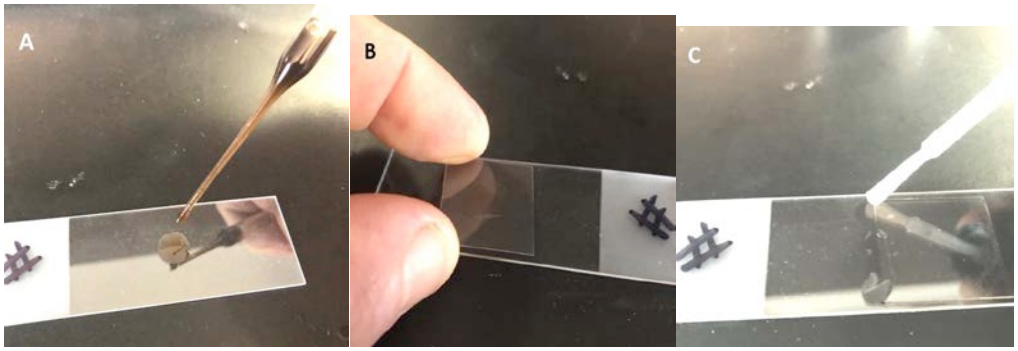
Sieves need to be back-washed with water, dipped in a dish washing detergent solution and transferred to a plastic box with lid containing a 33% Clorox solution for 15 minutes, rinsed and set out to dry. **Do not** leave the sieves in the Clorox solution past 15 minutes. The Clorox destroys or decolorizes the teliospores that may be left behind in the sieve, so they are not detected in future extractions.

b. Microscopic Evaluation

Step 1
Label a microscope slide with sample number.

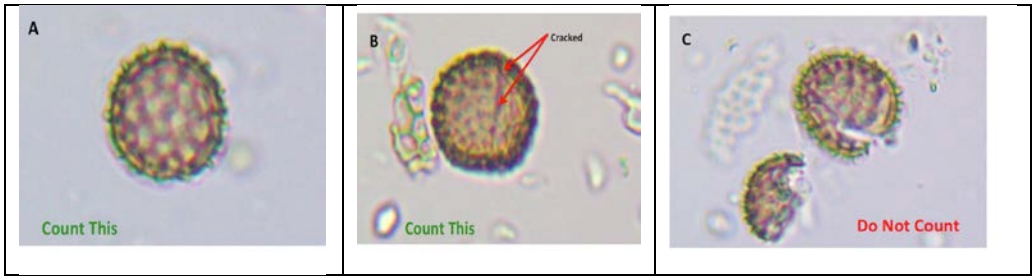
Step 2
Use a disposable Pasteur pipet to thoroughly aspirate to re-suspend the pellet.

Step 3
Using the Pasteur pipette, place one drop of the sample suspension on the slide and add a 22 x 22 mm cover slip. Seal edges of cover slips with clear nail polish to prevent drying out or movement of the coverslip when subjected to examination under oil immersion.



Apply of one drop (A); Apply cover slip (B); Seal with clear nail polish (C)

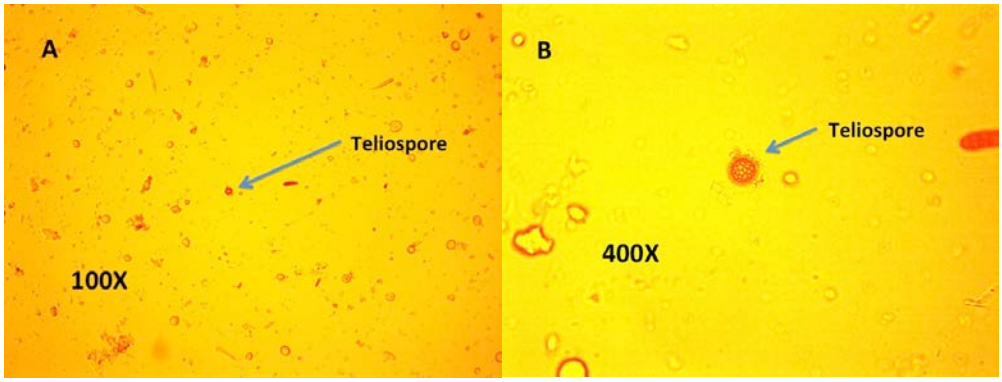
Notes: *You will be counting all of the whole* Tilletia *teliospores encountered on the slide in addition to recording measurements for up to 30 teliospores. You may notice that many of the teliospores are cracked. As long as there is not a noticeable piece missing, it is counted and measured. For training purposes, slides with specimens of pure* T. controversa *and* T. tritici *teliospores suspended in Shears Medium are helpful. Before you begin examining slides at 100X, it is helpful to examine a slide with a drop from one of these pure specimens to train your eyes to recognize the size and color of teliospores. If you seal the specimen slide well, it can be kept and used many times.*



(A) Count and measure whole teliospore and (B) those with line cracks
but (C) not those with pieces missing pieces

Step 4

Starting at a corner of the cover slip, scan the entire slide under the microscope, side to side, in slightly overlapping fields, at 100X or 200X. If you observe something that looks like a teliospore, center it in the field and switch the objective to 400X magnification to confirm that the surface reticulations are typical of the two *Tilletia* species.

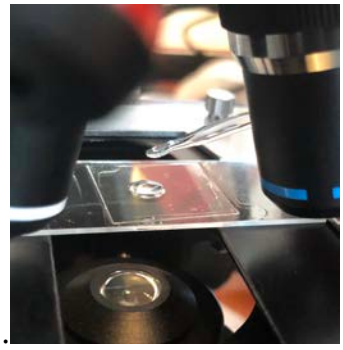


Teliospore in extraction drop at 100X (A) and 400X (B) magnification

Note: If the debris on the slide is very dense, you can add additional Shears medium to the sample, mix and make a new slide. Just keep track of the number of additional drops added.

At the 400X magnification, center the teliospore in the view field. Rotate the 400X objective away from the slide and place one drop of immersion oil on the slide cover slip. Rotate the 100X objective into place (in contact with the oil) and gingerly focus on the teliospore. Usually, you do not need addition immersion oil each time you switch to 1000X magnification on that slide. Never try to switch magnification directly from 100X or 200X to 1000X; you may miss the 1000X focal plane and drive the objective into the slide and break it.

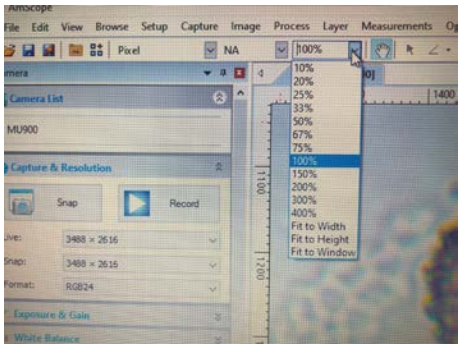
*Note: When finished for the day, clean the oil objective with microscope lens cleaner. **No not clean with alcohol**, it will dissolve the adhesive that holds the lens in the objective.*



Adding oil to coverslip for 1000X magnification

Step 5

On the video monitor, with the microscope magnification at 1000X (oil immersion), focus on the periphery of the spore. If you are using an AmScope microscope/camera combination with a laptop, make sure the magnification tab in the menu bar is set to 100%. What you want to see will be a dark, obtuse line (endospore wall) with what appears to be, depending on species, obtuse spikes or short projections arising from it.

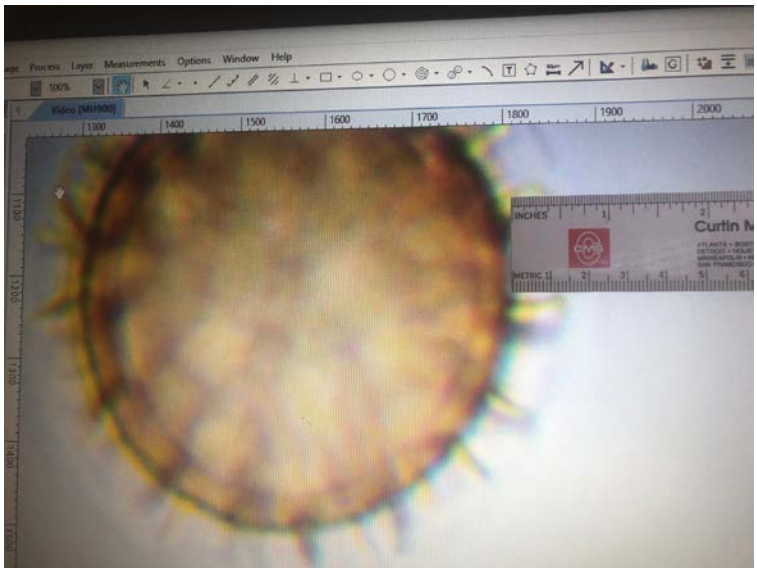


AmScope software screenshot; select 100% for measuring at 1000X oil immersion lens

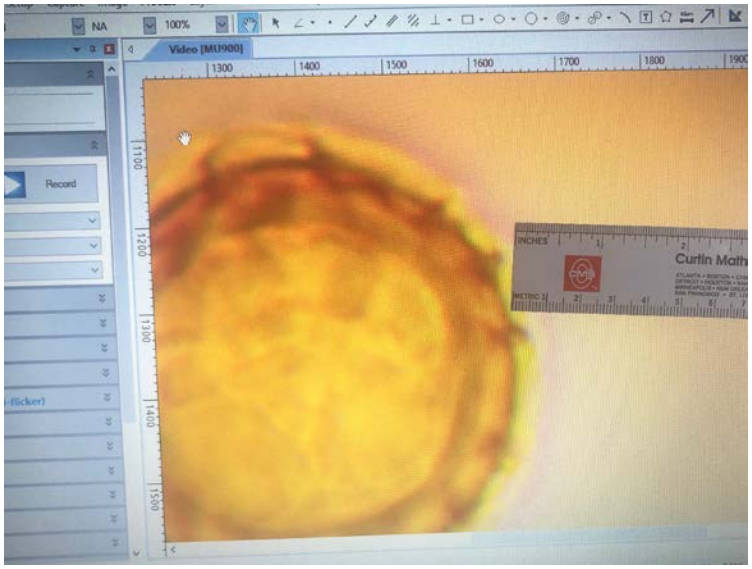
Note: *At 1000X very slight movements of the microscope stage are greatly magnified so it will take some practice to focus and move the teliospore to a position on the monitor where it can be measured. With a digital camera there is also slight delay in what you see when you move the slide on the microscope stage and what appears on the monitor.*

Step 6

Using a plastic millimeter ruler, measure the depth of the reticulum from the center of this dark line to the top of the reticulum "spike". Take four measurements from each spore, choosing points that closely correspond to north/south/east/west points on a compass. You may have to refocus as you change to different points on the teliospore. Measure to the nearest 0.5 mm. Record all measurements on the data sheet. Do not measure more than 30 teliospores.



Measuring *T. controversa*



Measuring *T. tritici*

Note: *If you have very few teliospores in the sample, such as one teliospore per microscope field or less, you should stop and measure each teliospore as you encounter them. If the teliospore count is greater than one teliospore per microscope field, it is faster to first count all the teliospores on the slide, then go back to the beginning and measure 30 teliospores.*

c. Sample Analysis

Step 1

Multiply the number of teliospores counted on the slide (**C**) by the number of drops in the tube (**D**) to obtain the number of teliospores in the 50g sample (**T**)

C*D=T

Step 2

For each measured teliospore, determine the average of the four reticulum measurements and multiply by the conversion factor for your microscope/monitor system to determine the average reticulum depth in micrometers (Appendix IV).

Step 3

The mean reticulum depth for each teliospore is converted to a Logistic Index. This can be done by using the Logistic Indices Table in Appendix V, choosing the closest Indices value corresponding to the average micrometer value for each teliospore or by using the formula below:

Logistics Index

$$\text{Index} = (e^{a+bR})/(1+e^{a+bR})$$

a= -10.6914 b= 11.6214 R= reticulum depth e= 2.71828

Excel functions for calculating LI is presented in Appendix V

Step 4

The Logistics Index for each teliospore can be interpreted as the probability that the teliospore is *Tilletia controversa* rather than *Tilletia tritici.* A Logistic Index of greater than 0.50 is presumed to be *T. controversa*.

Summing the logistic Indices for all of the teliospores yields the **Absolute Index** which is interpreted as the expected number of Dwarf bunt teliospores in the sample.

Averaging the logistic Indices for all of the teliospores yields the **Relative Index** which is interpreted as the proportion of Dwarf bunt teliospores in the 50g sample

Appendix I: Equipment

1. Microscope:

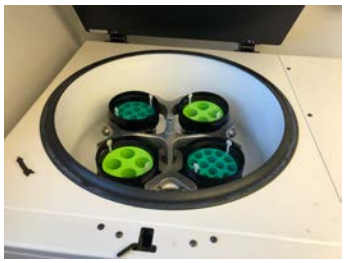
The minimum requirement is a trinocular compound brightfield microscope with 10X, 20X, 40X, and 100X objectives and 10X oculars equipped with a 5 to 10 mega pixel camera coupled with a monitor or laptop.

ARS-FDWSRU tested and found a satisfactory system sold online by AmScope (https://www.amscope.com), Model T940B-9MP, which meets the above microscope specifications (except the 20X achromatic objective, which can be purchased separately; AmScope part number is A20X-V300, $24)
including a 9MP camera and software to connect it to a PC laptop (not included). It also includes a spare halogen light bulb, stage micrometer, and immersion oil for 1000X magnification. At the time of this writing the list price was $570.99. Transport cases are also available.

2. Centrifuge

a. Horizonal centrifuge that takes 50 ml and 15 ml conical centrifuge tubes and reaches at least 600 X *g*

Horizonal Bucket



Fixed Angle

b. Fixed angle centrifuge that accommodates both 50 ml and 15 ml conical centrifuge tubes and reaches at least 800 X *g*. ARS-FDWSRU tested a small tabletop unit made by Southwest Science, Model SCL456 Clinical Centrifuge that

handles both 50 ml and 15 ml conical centrifuge tubes and runs up to 1,800 X *g*. At the time of this writing the list price was $568.00.

3. Orbital Shaker

If you are processing multiple samples at the same time you will want to use an orbital shaker table that reaches at least 200 revolutions per minute. If you are only processing a single sample, you can forgo the shaker and swirl the flask by hand for 5-minutes.

Southwest Science makes an orbital shaker that has had good reviews (not tested by ARS-FDWSRU). The model SBT300 costs $629, however the tabletop (SBT300-UNV top $63) and flask clamps for 500-ml flasks are sold separately (Model F-CLAMP-500 $25 ea.) for a total of $792 at the time of this writing.

4. Digital Balance

A digital balance for weighing grain samples with a minimum accuracy of 0.1g. This can be obtained from a scientific supply company such as Fisher Scientific or Lab Depot.

Appendix II: Materials and supplies

Item	Notes
Beakers 600 ml	Kimble No. 14000
Beakers 250 ml	
Glass Pasteur pipettes	5 ¾ inch
Bulbs for Pasteur pipettes	
10 ml Plastic disposable pipettes	
Pipette pump	
500 ml Erlenmeyer flasks	
Standard microscope slides, frosted end	
Coverslips 22 X 22 mm	
Lens cleaning solution (Non-alcohol)	
Lens cleaner tissues	
Microscope immersion oil	
Tween-20	100 ml bottle
50 ml plastic capped conical centrifuge tubes	
15 ml plastic capped conical centrifuge tubes	
95% Ethanol (ACS reagent grade)	
Glycerin	
Potassium acetate	
500 ml crew cap bottles or a 6 to10L carboy with spigot for Tween-20 water	
Clear nail polish	
Weighing dishes or 3 oz paper cups	
Stage micrometer 1.0mm with 0.01mm divisions	
52 μm nylon mesh screen	See Appendix VII
3” PVC drainpipe	See Appendix VII
Plastic storage box with lid for bleach solution to decontaminate sieves	Approximately 16 - quart size
Tally Counter for teliospore counting	Free version for smart phones on-line

Appendix III: Solutions

a. Shears Mounting Medium

Distilled water	300 ml
Glycerin	120 ml
Ethanol 95%	180 ml
Potassium acetate	6.0 g

Dividing this final solution among several smaller bottles is recommended due to the risk of accidental contamination with teliospores.

b. Bleach Solution

Add 660 ml of standard Clorox bleach (5.25% sodium hypochlorite) to 1,340 ml of water. Pour into a plastic box with lid for decontaminating sieves. This 33% bleach solution should be changed weekly.

c. Tween-20 Solution

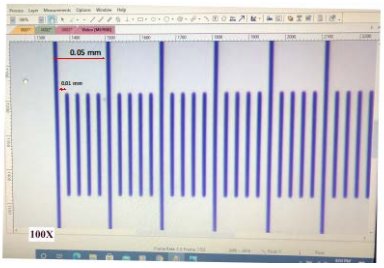
Tween-20 solution is mixed at a rate of 2 drops in 100 ml of water. For 10 L of this solution add 4 ml of Tween-20.

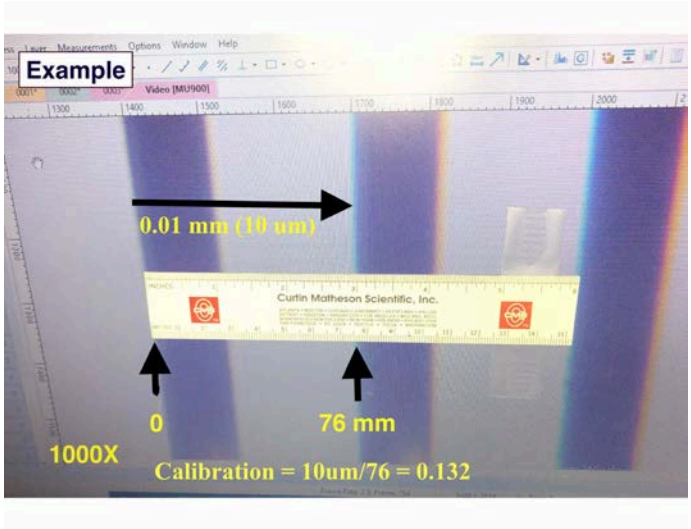
Appendix IV: Microscope Calibration

Using a 1.0 mm stage micrometer, which is divided into one hundred 0.01 mm marks, observe the stage micrometer at 400X then switch to the 1000X oil objective and bring a portion of the stage micrometer into focus on the viewing monitor. At that magnification you may only see three wide vertical lines on the screen. Use your ruler to measure in millimeters from the left edge of the first vertical line to the left edge of the next vertical line. There is 0.01 mm or 10 mm between lines on the micrometer. Take the distance in millimeters you measured between the lines and divide 10 mm by that measurement to obtain the conversion factor. As long as the equipment remains the same, write the conversion factor on a piece of tape and attached it to the base of the microscope.

For example, on one scope that distance between the two lines is 76.0 mm. Divide the 10 mm by 76 mm gives you 0.132. This is the conversion factor. If you measure the height of a reticulation on the monitor and it is 14 mm, then the reticulation depth is 14 mm * 0.132 = 1.8 mm.

1.0 mm Stage Micrometer





Appendix V: *Tilletia controversa* Logistic Indices

Reticulum (μm)	Logistics index	Reticulum (μm)	Logistics index
0.05	0.000	1.00	0.717

0.10	0.000	1.05	0.819
0.15	0.000	1.10	0.890
0.20	0.000	1.15	0.935
0.25	0.000	1.20	0.963
0.30	0.001	1.25	0.979
0.35	0.001	1.30	0.988
0.40	0.002	1.35	0.993
0.45	0.004	1.40	0.996
0.50	0.008	1.45	0.998
0.55	0.013	1.50	0.999
0.60	0.024	1.55	0.999
0.65	0.042	1.60	1.000
0.70	0.072	1.65	1.000
0.75	0.122	1.70	1.000
0.80	0.199	1.75	1.000
0.85	0.307	1.80	1.000
0.90	0.442	1.85	1.000
0.95	0.586	1.90	1.000

LI >0.50 is presumed to be *T. controversa*

Logistics Index

$LI = (e^{a+bR})/(1+e^{a+bR})$

a= -10.6914 b= 11.6214 R= reticulum depth e= 2.71828

The LI can be programed into Excel using the formula syntax below:

	A	B	C
1	Avg. Ret. µm	(e^{a+bR})	Logistic Index
2	R	=EXP(((-10.6914+(11.6214*A2))))	=(B2)/(1+B2)

Example: Excel Output

	Avg. Ret. Depth	(e^{a+bR})	Logistic Index
1	0.95	1.417549958	0.58635808
2	1.1	8.102235409	0.89013688
3	0.5	0.007591698	0.0075345

Appendix VI: Example of Data Sheet

Identification: SS Minnow Date Loaded: 2/31/2021

Calibration: 0.22 Date Tested: 3/1/2021

No. drops (**D**) in sample: 24 Teliospores (**C**) in drop: 63

Reticulum (R) Measurements (no more than 30 teliospores)

Teliospore	R1	R2	R3	R4	Avg R	R*.22=µm	L I*
1	6	4	5	5	5	1.1	0.89
2	5	5	4	4	4.5	0.99	0.586
3	5	5	5	5	5	1.1	0.89
4	7	6	7	7	6.75	1.485	0.996
5	6	5	4	4	4.75	1.045	0.819
6	5	5	5	5	5	1.1	0.89
7	6	5	5	5	5.25	1.155	0.935
8	4	4	4	4	4	0.88	0.442
9	5	4	5	5	4.75	1.045	0.819
10	4	6	4	4	4.5	0.99	0.586
11	5	4	4	4	4.25	0.935	0.586
12	5	4	5	5	4.75	1.045	0.819
13	6	5	6	6	5.75	1.265	0.979
14	5	7	5	5	5.5	1.21	0.963
15	5	4	4	4	4.25	0.935	0.586
16	5	4	4	5	4.5	0.99	0.586
17	6	4	5	6	5.25	1.155	0.953

*See Appendix IV

Total teliospores in 50g (C*D=T): **1,512**
Relative Index (AVG LI): **0.78** (78% of the sample teliospores are *T. controversa*)
Absolute Index (SUM LI): **13.3** (13 of the 17 teliospores measured were T. *controversa*)

Technician: A. Wadyko

Note: *Example datasheet is suitable as an official record for an FGIS or certified laboratory.*

Appendix VII: 52µm Nylon Mesh Sieves

The frames for the 52-µm nylon mesh sieves are fabricated from 3-inch white PVC, Schedule 40 drainpipe. The pipe is cut into equal numbers of ¼ inch and 3-inch-long pieces. A power radial miter saw with a 60 tooth/inch or greater blade works well. Remember to wear safely goggles and dust mask when cutting PVC pipe. Cut slowly. It is best to clamp the pipe to the rear fence of the saw when cutting rather than holding in place with your hand.

You may need to slide the cut pieces across some sandpaper or a belt sander to remove any burrs from the surface.

There are several sources for the 52-µm nylon mesh screen material. One source is Component Supply Company (https://componentsupplycompany.com/product-pages/nylon-screening-mesh.php) which sells the screen material by the ½ yard. One yard will make over 50 sieves.

Components are assembled using J-B Clear Weld. Pre-cut the 52-µm nylon mesh screen material into 4 X 4-inch squares. The glue cures rapidly so do one sieve at a time. In a ventilated area apply the cleaner to one surface of the ¼ inch pipe and the 3-inch pipe with a cotton swab or acid brush. Center the piece of screen onto the 3-inch pipe and press down and out to prevent wrinkles. Place the glue side of the ¼ inch pipe over the 3-inch to sandwich the screen between. Press down for 30 seconds, set a flat weight (brick, book etc.) on top and leave for an hour. Trim the excess screen from the outside with a razor blade.

Note*: Don't substitute the grey CPVC or black ABS pipe for the white PVC. We experimented with stainless steel and polyester screens and found that over time the decontamination with Clorox solution roughed up the surface of the screen threads which resulted in teliospores hanging up in the screens.*

Appendix VIII: Warning about Shipping Grain Samples

In 2000, the Wheat Marketing Center (WMC) in Portland, OR was established as the official *T. controversa* laboratory for testing PNW wheat exports to China. ARS provided initial training at WMC and FGIS. WMC is certified by FGIS annually after an inspection of the facility to affirm the testing protocols, record keeping, and personnel training requirements were being met. As part of that review, quality control (QC) testing of 10% of the China export file samples retained at WMC were retested by the National Grain Center AMS, FGIS in Kansas City, MO and results compared to those obtained by WMC. During the first quality control testing in Kansas City, the number of teliospores reported by FGIS average less than 20% of those reported by WMC.

In an investigative study conducted by ARS, *T. controversa* grain samples were analyzed at ARS-FDWSRU, Fort Detrick, MD, then shipped by commercial carrier to and back from ARS, Raleigh, NC, and retested again. Results show a reduction of 70 to 80 % of teliospores after shipping. Similar results were obtained when dry grain was placed in an Erlenmeyer flask and shaken vigorously for a few minutes then retested. The conclusion of the study was that the brittle teliospores were being ground-up between the seeds during shipping. The problem with the QC evaluation was remedied by conducting the annual QC testing on site at the WMC facility in Portland.

In general, this means we cannot expect comparable results, if for example, a grain sample is tested in League City, TX then sent by courier to College Station, TX or Kansas City, MO for confirmation testing. Likewise, if you sample grain in a rail car in Tulsa, OK and mail it to League City, TX for testing, the teliospore numbers may not accurately reflect the contamination level in the rail car. However, the extracted sample suspended in Shears medium can be shipped with no damage to the possible teliospores in the sample.

Appendix IX: Quality Control Verification

Quality control verification testing is conducted as part of the annual laboratory inspection for certification by USDA FGIS. The inspection requires FGIS to test 10% of the samples evaluated by the testing facility. Samples are selected randomly from the file samples the facility is required to retain in cold storge. The facility will provide FGIS with copies of their test results for the selected samples.

FGIS personnel will perform the testing analysis at the facility being certified.

The analysis is based on the equation developed by Dr. Thomas Whitaker of ARS which estimates the variability associated with TCK export sampling (Whitaker et al, 2001). The equation says the standard deviation is approximately 1/2 (0.551) the TCK concentration. Ninety-five percent of the sample results will vary from a low of T-1.96*s, to a high of T+1.96*s. Our best estimate of the lot TCK concentration, T, is the average, A, of the 2 sample test results, *X1* and *X*2. The average is: A = (*X* 1+ *X* 2)/2 = T, and the expected random variation among samples from a lot at T is 0 to 2T. So it is acceptable for *X* 1 and *X* 2 to differ by 2T (Hi - Lo or 2T - 0). Take the difference (D) between *X* 1 and *X* 2 and compare D to 2A or 2T. If D <= 2T, then results are acceptable. If D > 2T, then results are unacceptable, and some action is required.

Below is an example of a QC analysis conducted by ARS for FGIS from samples tested at WMC:

WMC Number	WMC TCK	ARS TCK	D	Average (T)	2T	Accept if D ≤ 2T
120061264	5	6	1	5.5	11	Yes
120071040	1534	552	982	1043	2086	Yes
120070127	2651	558	2093	1605	3209	Yes
120071182	1536	384	1152	960	1920	Yes
120060054	54	10	44	32	64	Yes
120060141	0	0	0	0	0	Yes
120061078	1	0	0	0.5	1	Yes
120061249	1	0	0	0.5	1	Yes

Analysis shows that the numbers of TCK spores found in the ARS over-sight sample extractions are not statistically different from those obtained by WMC.

* Whitaker, T.B., Wu, J., Peterson, G.L., Giesbrecht, F.G., and Johansson, A.S. 2001. Variability associated with the official USDA sampling plan used to inspect export wheat shipments for *Tilletia controversa* spores. Plant Pathology 50:755-760

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