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Export Program Manual

Special Procedures

Commodity • Tobacco (Blue Mold)

Contents

Phytosanitary Protocol for Exporting Tobacco Leaves to the People's Republic of China

In order to prevent the introduction of the tobacco blue mold (*Peronospora tabacina* (hyoscyamt)) pathogen and other regulated pests into China and to ensure the safety of China's tobacco production, the State Administration for Entry-Exit Inspection and Quarantine of the People's Republic of China (hereafter referred to as CIQ SA) and the U.S. Department of Agriculture (USDA), represented by the Animal and Plant Health Inspection Service (APHIS) have conducted discussions, considered CIQ SA's pest risk analysis, and agree to the following plant quarantine requirements for exporting tobacco leaves from the U.S. to China:

Article 1

Both sides agree that U.S. tobacco leaves exported to China shall **not** pose any threat to the production of China's tobacco.

Article 2

Tobacco leaves referred to in this protocol are flue-cured and burley tobacco produced, cured, and processed (threshed and redried) in the U.S.

Article 3

Tobacco buying teams from China will include CIQ SA inspectors who will preinspect tobacco leaves being considered for purchase and export to China. Tobacco leaves that have passed preinspection shall be allowed to be exported to China.

Every year, APHIS will provide CIQ SA a report on Tobacco Blue Mold (TBM) investigations in the U.S., including the methodology, oospore survey, and results. Based on this information and TBM occurrence in the U.S., CIQ SA will consult with APHIS, and if agreed, CIQ SA will send two experts for investigation if the weather is particularly suitable for oospore formation. Expenses will be paid by the U.S. industry.

APHIS should cooperate with the Chinese experts conducting preinspection and investigation, and provide them with technical support.

Article 4

APHIS should conduct strict quarantine inspection of tobacco leaves designated for export to China and ensure these tobacco leaves are free from TBM oospores, live sporangiospores, live mycelium of *Peronospora tabacina*, and other regulated pests of concern to China. An official export certificate should be issued by APHIS for the tobacco leaves that meet the quarantine requirements. If APHIS detects an oospore during inspection of a lot being exported to China, APHIS will reject the lot and immediately transmit the interception data to CIQ SA. The export certificate should indicate the lot number (contract number) along with the counties and States where the tobacco was grown.

Article 5

The processing of the tobacco leaves exported to China should be conducted under APHIS' supervision. The tobacco leaves for export to China should be sealed to avoid contamination by *Peronospora tabacina* and other regulated pests. Each carton of tobacco leaves should be labeled to include information such as: type, lot number (contract number), harvest year, grade, and processing plant.

Article 6

When the tobacco leaves arrive at the Chinese port of entry (POE). CIQ SA will examine the relevant certificates and the information on the carton label to check whether the commodity conforms to the certificate and whether the tobacco leaves have been preinspected, as well as conduct a quarantine inspection. If it is found that the tobacco leaves have **not** been preinspected or are **not** accompanied by an export certificate issued by APHIS, the tobacco leaves will be denied entry.

If TBM oospores, live sporangiospores, or live mycelium of *Peronospora tabacina* are found on the lot of tobacco leaves at the POE, the lot of tobacco leaves will be denied entry and the interception data will be immediately transmitted to APHIS. If any other regulated pest of concern to China are found, the shipment will be dealt with according to the "Law of the People's Republic of China on Entry and Exit Animal and Plant Quarantine" and the relevant implementing regulation.

Article 7

A. If oospores of *Peronospora tabacina* are found in U.S. tobacco fields, APHIS will immediately invite CIQ SA to participate in the relevant investigation. CIQ SA and APHIS will determine if the situation will allow the resumption of the previously agreed

coexperiments. Based on the results of the research, CIQ SA will alter its regulations accordingly: if it is determined that the TBM oospores in dried tobacco leaves are noninfectious, CIQ SA will eliminate all restrictions on the import of U.S. tobacco leaves related to TBM oospores. If it is determined that the TBM oospores in dried tobacco leaves are infectious, CIQ SA will prohibit the exportation of U.S. tobacco leaves unti1 both sides can find measures to kill the oospores.

Only imports of tobacco leaves from that year and from that county in which oospores of *Peronospora tabacina* were found shall **not** be eligible for export to China. The relevant county will be surveyed the following growing season by CIQ SA and APHIS. If **no** oospores are found, tobacco leaves from that county can once again be exported to China.

Above expenses will be paid by the U.S. industry.

B. If a TBM oospore is detected in a lot of dried tobacco leaves being inspected for export to China, that lot will be rejected from entry and all tobacco leaves from that production area from that year will **no** longer be eligible for export to China.

If a TBM oospore is found in a lot, survey will be conducted by APHIS, with CIQ SA participation, the following growing season in the county(s) affected, and if **no** oospores are found, tobacco leaves from that production area will again be eligible for export. CIQ SA participation will be determined by the following criteria. If timing is suitable, CIQ SA officials, with the buying team, will be invited to participate in the APHIS survey. If timing is **not** suitable, scientists from both sides will conduct the survey in the affected production area. Expenses associated with CIQ SA's participation in the survey will be paid by the U.S. industry.

Article 8

The protocol is valid for 1 year. If neither side requests revision or termination of the protocol within 2 months before the expiration date, the period of validity of the protocol wi1l be automatically extended for another year.

The protocol was signed on February 5, 2001 in Beijing with duplicate in Chinese and English languages, both texts being equally authentic.

The Quarantine Inspection Sampling Procedure of Tobacco from Abroad

This procedure is made relative to China and international standards, and is suitable for quarantine inspection sampling of tobacco from abroad. The particular procedure is as follows.

1. A quarantine lot will consist of the same variety, same grade, same production area, same year, and same producer of tobacco.
2. The quantity of the sampling is based on the following:
   - **A.** If the total amount of every lot is less than 10 pieces, take whole pieces to do the sampling.
   - **B.** If the total amount of every lot is between 10 and 100 pieces, take a random sample of every 10 pieces.
   - **C.** If the total amount of every lot is over 100 pieces, use the square root of the number of lots to ca1culate the number of pieces to randomly sample.
   - **D.** For single lots of tobacco (carton, box) that are randomly sampled, take the random sample at same amount in different levels, such as top, middle, and down 3 levels. If some suspect leaves are found, the sampling amount will be increased.
   - **E.** Every lot of quarantine sampling consists of single sample and the sampling amount should **not** be less than 8.5 kg.

Tobacco Blue Mold Field Survey for Oospores Field Sampling Protocol

Objective

A field survey and sampling of blue mold infected flue-cured and burley tobacco is required to determine if oospores of the pathogen have formed in the leaves. The samples **must** be examined by a trained and certified microscopist to determine the presence of oospores.

Survey Personnel

PPQ will cooperate with State plant pest regulatory personnel to provide guidance in conducting the survey. Each State involved will identify a coordinator to conduct and/or oversee the survey/sampling. State coordinators will have samples forwarded to a certified laboratory for evaluation and the laboratory will forward the sampling information to the PPQ coordinator. Draft reports produced by PPQ will be submitted to the appropriate State coordinator for review.

Scope

When the occurrence of blue mold disease has been reported on flue-cured or burley tobacco in a State by an agent or other reliable source, the State regulatory coordinator will make arrangements for sampling one field with blue mold per county.

Representative Areas

When blue mold disease is widespread in a tobacco marketing area in a State, e.g., several adjacent counties have been reported to have blue mold, the State regulatory coordinator may elect to sample in a few of the counties as representative of all the counties in the infected area.

Minimum Field Sample

A minimum sample consists of sampling 5 leaves of 5 different plants in a field identified for sampling. Samples will be taken from leaves with clearly identified tobacco blue mold lesions.

Sample Records

A Field Sample Record form is provided to record the required survey information and to guide the process of field sampling.

Sample Identification

A unique ID number will be assigned by each State to samples collected in that State. The ID number **must** consist of the last 2 digits of the current year and the 2 letters of the State postal abbreviation. You may also include a county code or abbreviation along with a chronological numbering system identifying samples submitted for analysis. (Note: the NAPIS 3-digit county code may be used as part of the sample identification in place of a 2-digit number. It will **not** work if more than 1 sample per county is taken.)

EXAMPLE The first sample collected in Georgia in 2002 may have the ID number 02GA0I.

Field Location

The location where a field sample is collected should be identified by written description and a GPS coordinate description. Names, addresses, and other appropriate information listed on the form should be as complete as possible. In some instances it may be necessary to return to a particular location the following year. This will **not** be possible unless the information on the form is complete and accurate.

Selecting Leaves

Leaves selected for sampling **must** have clearly visible mold lesions. The leaves for samples **must** have been infected for at least 3 weeks prior to sampling. This is because oospores usually form later in the disease development. Light green or yellow blue mold lesions form first and the lesions become brown as necrotic tissue forms. Oospores, when they form, are usually found in the necrotic tissue. This should **not** be a problem because most blue mold is usually reported after the disease has progressed for 2 weeks. Sampling of lower or mid-stalk leaves with older brown lesions is preferred. Avoid leaves that are in a state of severe decay or rot as they may decompose in transit.

Removing Lesions

Cut 2 X 2 inch squares of leaf tissue containing the 3-week-old blue mold lesions from the selected leaves. Avoid the mid-rib. The purpose is to get the lesions with a minimum of leaf tissue that could result in rot during transit. Leaf tissue will rot if it stays moist. Allow the samples to dry during transit.

Preparing Samples

Leaf tissue will rot if it stays moist. Allow the sample to continue to dry before and during transit. Once dried, the squares may be kept indefinitely for laboratory testing. **Do not** dry samples to the point of brittleness prior to mailing, because they can be damaged in transit. Given this, we suggest the following preparation and mailing procedure for all samples:

- Spread out the 2 X 2 inch squares on a paper towel in a cool dry place (like your office) and air dry for an hour or so to remove surface moisture and field heat.
- Package samples by assembling a "sandwich" as follows: a layer of stiff cardboard cut to underfit the mailing envelope, several layers of paper towels, the sample squares laid flat and **NOT** overlapping each other, several layers of paper towels, and a layer of stiff cardboard. The edges of the two cardboard pieces can then be taped in a few places to hold it together.
- Place assembled sample in appropriate envelope for forwarding to the certifying laboratory.

Submitting Samples

Sampling personnel will forward samples as directed by the State coordinator. The State coordinator will ensure the samples are forwarded to the certified laboratory, indicated below, for microscopic evaluation along with the Field Sample Records. Send all samples to the laboratory by September 1st of the present survey year. If oospores are found in any sample, the Field Sample Record, immediately forward to the PPQ coordinator via the certified laboratory. Include an email address, because the Plant Disease and Insect Clinic will report back **only** by email.

ATTN: Tom Creswell, Blue Mold Project Manager
Plant Disease and Insect Clinic
North Carolina State University, 100 Derieux Place
Campus Box 7211, Room 1227 Gardner Hall
Raleigh, NC 27695-7211
Lab: 919-515-3619
Fax: 919-882-1842
http://www.ces.ncsu.edu/depts/ent/clinic/

Tobacco Blue Mold Field Survey for Oospores Field Sampling Record 2005

Date Sampled: _____

Sample ID Number Assigned by State Coordinator: _____

Nearest Town: _____ County: _____ State:_____

Field Location (Describe): _____

GPS Location: _____

Grower/Farm Name: _____

Address: _____

Phone: _____ Email: _____

County Agent (optional): _____

Address: _____

Phone: _____ Email: _____

Name of Sampler: _____

Address: _____

Phone: _____ Email: _____

State of Agency: _____

Address: _____

Phone: _____ Email: _____

Tobacco Type: Flue-Cured Burley

Please indicate who should be billed for this sample (give name and contact information)

Send completed form to appropriate state coordinator and laboratory at the address below:

ATTN: Tom Creswell, Blue Mold Project Manager
Plant Disease and Insect Clinic
North Carolina State University, 100 Derieux Place
Campus Box 7211, Room 1227 Gardner Hall
Raleigh, NC 27695-7211
Lab: 919-515-3619; Fax: 919-882-1842
http://www.ces.ncsu.edu/depts/ent/clinic/

Tobacco Blue Mold Export Sampling Record

Company Requesting Sampling: _____

Contact Person: _____

Address: _____

Phone: _____ Fax: _____ Email: _____

Date Sampled: _____ Crop Year: _____

Contract of Sale Number: _____

Sample ID Number Assigned by State Coordinator: _____

Sampling/Warehouse Site (Describe): _____

_____

_____

_____

Phytosanitary Certification Issuer: _____

Name: _____

Agency: _____

Address: _____

Phone: _____ Fax: _____ Email: _____

Tobacco Type: Flue-Cured Burley

Please remit $500.00 for each 50 disc sample. Make check payable to North Carolina State University.

Send completed form with check attached to the address below:

ATTN: Tom Creswell, Blue Mold Project Manager
Plant Disease and Insect Clinic
North Carolina State University, 100 Derieux Place
Campus Box 7211, Room 1227 Gardner Hall
Raleigh, NC 27695-7211
Lab: 919-515-3619
Fax: 919-882-1842
http://www.ces.ncsu.edu/depts/ent/clinic/

China: Tobacco Blue Mold Protocol Standard Certification Procedures

1. Sampling of Tobacco Leaves For Export

1.1: APHIS or its agent shall accompany tobacco buying team from China.

1.2: APHIS/Agent will split the tobacco sample selected by the State Administration of China Entry-Exit Inspection and Quarantine of the People's Republic of China (CIQ-SA) inspector for analysis of tobacco blue mold (*Peronospora tabacina*) oospores. These samples will be submitted to an APHIS-certified laboratory for analysis. This will be used for export certification of a given lot.

1.3: If APHIS/Agent is unable to accompany CIQ-SA inspector, samples will be drawn from boxes in storage.

1.3.1: Samples will be drawn at random.

1.3.2: Sample size will be determined by using the hypergeometric table for random sampling.

1.3.3: Samples will consist of aggregate cores of leaves, taken from top, middle, and bottom of box.

1.3.4: A subsample will be taken from an aggregate core sampling of at least 8.5kg.

1.3.5: The subsample will be randomly reduced to a final sample of 50 discs.

1.3.6: APHIS will provide sampling training to certifying officials.

1.4: APHIS/Agent, at requesting industry's expense, will submit tobacco leaf samples to an APHIS-certified laboratory for determination of live tobacco blue mold oospores, sporangiospores, and/or mycelium.

1.4.1: Each sample lot will indicate tobacco type (flue-cured, burley), crop year, location of tobacco sampling site, and applicable identification number (lot number, contract of sale number).

1.4.2: Samples shall be packaged to minimize damage to leaf sample.

1.5: The laboratory will submit analysis results to the U.S. Industry representative and APHIS/Agent certifying officials for issuance of an export certificate.

1.6: APHIS/Agent will issue an export certificate as indicated in EXCERPT.

2. Tobacco Blue Mold Field Survey for Oospores

2.1: Participating States will survey tobacco areas for tobacco blue mold oospores.

2.1.1: Survey will be a representative sampling of fields within a tobacco marketing area for flue-cured and burley tobacco. (Appendix A & B)

2.1.2: Samples will include representative samples from each State reporting tobacco blue mold within that marketing area by county.

2.1.3: APHIS will provide training to participating States relevant to blue mold recognition, sampling procedures, and shipping instruction.

2.1.4: The States will designate what agency within their State will conduct the tobacco blue mold oospore survey and provide APHIS with the designated agency officials.

2.2: Sampling procedure

2.2.1: Samples will be taken from tobacco blue mold infected tobacco leaves.

2:2.2: Samples will be identified by its representative area in State or county.

2.2.3: Dried leaves with tobacco blue mold will be submitted to an APHIS-certified laboratory for oospores presence analysis.

2.2.4: The laboratory will provide analysis results to APHIS and to U.S. Industry representative.

2.2.5: APHIS will compile results of tobacco blue mold oospores survey and submit report to CIQ-SA.

3. Detection of Oospores in Survey

3.1: APHIS will conduct a survey of a suspect positive tobacco blue mold county.

3.1.1: If positive find is close to an adjacent county(s), a representative survey for oospores will be conducted in the adjacent county(s).

3.1.2: Tobacco from the affected county(s) will be ineligible for export to China during the production year discovered.

3.2: The following growing season the affected county(s) will be surveyed.

3.2.1: APHIS, with CIQ-SA participation, will survey the affected county(s).

3.2.2: If **no** oospores are found, tobacco will be allowed for export to China that production year.

4. Tobacco Blue Mold Report

4.1: APHIS will provide CIQ-SA with a report on tobacco blue mold in the U.S.

4.1.1: Report will be based on the information provided by the North American Blue Mold Forecasting Center in Raleigh, NC.

4.1.2: The report will include information on areas affected by tobacco blue mold, methodology for tobacco blue mold oospore survey, and survey results.

4.1.3: CIQ-SA will be notified of discovery of tobacco blue mold oospores.

Field Sampling Blue Mold Lesions Laboratory Analysis

Field Sampling

1. Examine field of tobacco and identify tobacco plants with blue mold disease or examine processed tobacco prepared for sale.
2. Select leaves or pieces of tobacco with blue mold lesions.

3. Visually inspect blue mold lesions with hand lens to find oospores. **Note:** Oospores are observed in lesions as red to black specks or small dots. When present, oospores are concentrated in large numbers in lesions.

Field Sampling for Detailed Microscopic Observation of Pathological Structures

1. Select 5 leaves from 5 plants with blue mold lesions in 1 field.
2. Cut 5 circular pieces of tissue in vial for processing.
3. Place 5 pieces of tissues in vial for processing.
4. Label vial with date, farm, county, State.

Clearing Tissue

1. Add 10cc of 1 M KOH to vial with 5 tissue pieces.
2. Drop vial in boiling water for 10 to 30 minutes to clear tissue.
3. If tissues **do not** clear:
   - **A.** Drain KOH from vial, rinse tissues with water 3 times.
   - **B.** Add 5cc GAA ALC (1 part glacial acetic acid to 1 part 100% ethanol).
   - **C.** Rinse 3 times with water.
   - **D.** Add 10 cc 1M KOH and drop vial in boiling water for 10 to 30 minutes.

Staining Tissue

1. Drain KOH from vial, rinse 3 times with water.
2. Add 5cc of 0.05% aniline blue stain in 0.067 K2PH04, pH 9.0.
3. Wait 30 minutes before making slides.

Preparing and Viewing Slides

1. Pour stain and tissue onto a watch glass.
2. Lift tissue pieces carefully with a spatula and place 5 tissue specimens on a slide and cover with glass cover slip.
3. Examine tissues with microscope using incandescent light and a mercury vapor light with filters (HBO 100- W/2 mercury burner and G365nm exciter: LP 42nm barrier fluorescence filter). **Note:** blue mold pathogen structures fluoresce.

August 25, 2000
H.W. Spurr, Jr.

Sampling Plan to Estimate Presence/Amount of Viable Blue Mold Spores in Stored Tobacco

(North Carolina State University, November 1995)

The population to be sampled is tobacco of the year 1990, 1991, and 1992 stored in 440 1b. boxes. Each numbered box will be listed by year and by the 4 type-locations. In accordance with the suggested protocol, 5 boxes will be drawn as simple random samples from each of the l2 lists (3 years by 4 types). Each list is a stratum.

Each sampled box will be located and brought to the warehouse floor and subsampled using a probe sampler inserted down into a randomly chosen quadrant to provide about a 2.0 lb. (900 gm) core increment.

Each of the 12 sets of 5 increments will be handled as follows. A random 2 of the 5 increments will be sieved and the pieces retained by 1 inch mesh will be examined by pathology specialists trained in diagnosing blue mold symptoms. The total weight and number of those pieces will be recorded along with the number of pieces that contain “any evidence of blue-mold-like lesions.”

All 5 increments will be ground in a Whiley mill to yield the gross sample. This will be put into a container and blended. Two 100 gm laboratory samples will be drawn from the 4.5 kg gross sample. One will be used in a soil assay and the other in a foliar assay.

For soil assay, the 100 gm of ground tobacco will be mixed with potting soil and put into a tray to grow 200 cells of tobacco seedlings. For foliar assay two trays of seedlings will be grown, then each sprinkled at two times with 25 gm of ground tobacco, each time.

These procedures wil1 be repeated for each of the 12 Year-Type strata.

In the soil assay any number from 0 to 200 cells will be found to have blue mold. The average number of cells with blue mold over the 12 trays will represent the estimated cell root viability found in the survey. An estimate of the variance in this estimate can be obtained from a 2-way (years by types) analysis of variance of the 12 separate proportions cells with blue mold. A similar analysis will be made on the data from the 400 sprinkled plants. The data on pieces-with-lesions will provide 2 observations (proportions of pieces with lesions) in each of the 12 strata and can be ana1yzed using a 2-way analysis of variance with 2 observations per year-type combination.

Statistical Considerations

(North Carolina State University, November 1995)

The population is a solid bulk in air. That is, the boxes contain about 75% air by volume and the rest is the largely fibrous leaf. Since the grinding operation will reduce particle size to, say, 1 millimeter or less, we will take 1.0 cubic mm (the sphere) as the elementary cluster (EC) size. In fact, this is an "effective" size since by far the greatest number of particles will be smaller. [See Proctor, C. H. (1990), "Statistical considerations in bulk sampling," Institute of Statistics Mimeograph Series No. 1988, North Carolina State University, Ra1eigh, NC.]

B1ue mold spores are around 30 microns in diameter so there can be from none to as many as 30,000 in an elementary cluster. A tobacco leaf contains about 17cc of fibrous volume, so there are about 17,000 e1ememary clusters in each leaf. By rough calculations, each 440 lb. box contains the equivalent of around 30,000 leaves. If, for example, a particular stratum were to be composed of 2500 boxes, the population size would be 2500 x 30.000 x 17,000 = 1.3 x 102 elementary c1usters. In sampling theory notation N = 1.3 x 102. If all the tobacco in the 2500 boxes were ground there would be 1.3 x 102 grains of l mm. diameter.

Each of the 200 bioassay cells will receive 1/200th of the 100 gm from the laboratory sample or about 0.5 gm in each cell. Converting the 19 lbs. per cubic foot density of tobacco leaf to 3.3cc/gm allows us to see that each cell contains about 1600 elementary clusters. The total sample size is thus 3.3 x 105 from N = 1.3 x 102 ECs in the population.

Suppose there were exact1y one EC containing a (laboratory) viable spore on every 10th tobacco leaf in the population. With 17,000 ECs in each leaf, we are supposing that 1 in 170,000 ECs has a viable spore. This level of viability is essentially 6 parts per million (ppm). The chance of missing a viable spore in 1600 trials is roughly what happens when a bioassay cell shows **no** infection and this probability is: 9904 = (1 - 6 x 106)1600. The chance of missing in all 200 cells is: 15 = .9904200, which is becoming small. The sampling method will pick up the condition of 1 EC having viable spores in every 170,000 with 85% probability.

The above suppositions are unrealistic in oversimplifying a complex of events. Viable spores usually occur in bunches (in clumps), while the sampling is **not** simply random. That is, even if viable spores occurred on 1ess than 1 in 10 leaves, they would occur, multiply, and thus, increase the chances of detection. Even though the bu1k is ground to elementary cluster size and well mixed, this occurs **only** at the last stage of subsampling. Any box contains portions of leaves from a wide range of fields, but still the pieces are fairly large clusters of ECs. This clustering reduces the chances of detection. What we have tried to do is describe a simplified setting to illustrate the accuracy of such a sampling survey.

Basically, 2 levels of viability incidence characterize a bulk sampling scheme. We will write them as p_1 and p_2. (They have been called the producer's quality level and the consumer's quality level in acceptance sampling terminology. See Schillling, E. G. Acceptance Sampling.) The value of p_1 is such that the sampling plan will, 95% of the time, find nothing viable, and p_2 is such that 90% of the time it will be detected as viable.

In the previous calculations we have taken the assay sample size of 320,000 ECs to be 320,000 independent trials with a chance of (1-*p*) **no** disease arising at each trial, where *p* is the underlying rate of viable ECs to all ECs. Under this naive viewpoint, the values for p_l and p_2 are found as:

$(1-p_1)^{320000} = .95$ and $(1-p_2)^{320000} = .10$, so that

$p_1 = 1 - \exp(\ln(.95)/320000)$ and $p_2 = 1- \exp(\ln(.1)/320000)$

or

p_1 = 1 in 6.2 million and p_2 = 1 in 139,000

(0.2 ppm)(7 ppm)

Two somewhat questionable assumptions were used previously; they were: (1) the independence of the trials; and (2) the size uniformity of all the ECs. That is, the particle sizes actually vary from 1 particle to the next and the independence assumption will be correct **only** if the entire lot is ground and thoroughly mixed and then sampled.

On balance we can expect, in line with experiences with other natural materials, that the lack of independence caused by viability being clumped in the lot will cause the effective sample size to drop by ½, 1/3, or ¼. Values, such as 2, 3, or 4 are called design effects. Supposing a design effect of 4 would bring effective sample size to around 80,000 ECs, say, and now we find:

p1 = 1 in 1.6×10^6 with *p2* = 1 in 35,000

(0.6 ppm) (29 ppm)

If sample size were increased to 50 boxes from a stratum, ½ would drop to 3 ppm. That is, a 10-fold (1 order of magnitude) increase in sample size would decrease p_1 and p_2 by an order of magnitude. All calculations have thus far considered **only** the soil assay. If the efficacy of the foliar method is found acceptable, sample size will be approximately doubled and the values of p_1 and p_2 can be halved.

Sampling Methods for Boxes, for Scanning, and for Assays

Sample Selection of Boxes

1. Sort the computer listing of boxes by year and type into 12 strata or lots.
2. Number boxes of any 1 lot from 1 to say, N.
3. Use approved random generator to get selection digit in range 1 to N. Repeat 15 times. Remove any duplicate selection digits. Use digits in the order they were produced.
4. Locate in the warehouse the boxes corresponding to the first 5 selection digits. If any have been sold, use the 6th, 7th, etc. selection digits to locate, in all, 5 boxes.
5. Repeat steps 2.to 4. for all 12 lots, and bring the 5 x 12 = 60 boxes to the warehouse floor to be sampled.

Box Sampling

1. Open top of box and looking down on top surface of tobacco, label centers of 4 quadrants as: HH, HT, TH, and TT.
2. Flip a coin twice to get: head-head, head-tail, tail-head, or tail-tail, and thereby decide where to insert probe sampler.
3. Insert sampler perpendicular into top surface. Remove when it binds, extract core into plastic bag, and reinsert until reaching 27 in. depth. Seal and label bag.
4. Repeat steps 1. to 3. for all 5 boxes in a lot.
5. Identify 2 bags from first 2 selected boxes with ribbon.
6. Put 5 bags in container.
7. Repeat steps 1. to 6. for all 12 lots.
8. Send 12 gross samples to Oxford lab.

Scanning and Subsampling Gross Sample

1. Put the contents of the first bag with a ribbon into the hopper of the mechanical siever.
2. In the hopper, break up large pads to about ¼" thickness.
3. Run through sieve and return the plus 1-inch portion to hopper.
4. Break up large pads to less than ¼" thickness and run through siever.
5. Remove plus 1-inch to table.
6. Lay pieces out in lattice arrangement. Count them and examine each for evidence of blue mold lesions.

7. Record counts and repeat steps 1. to 6. for second bag.
8. Combine all 5 bags of a lot into 1 container and feed into a Whiley mill.
9. Grind to less than 1 mm grain size.
10. Cone and quarter. Pour (4500 gm) powder onto paper making a cone. Flatten it somewhat and divide into quarters labeled H, T, H, T. Flip a coin and if H appears use the H quarters for soil assay subsample or if T appears use the T quarters for soil assay and use the other quarters for foliar assay.
11. Further cone and quarter each subsample 4 times removing separate halves to separate papers and then flipping a coin to decide which half to save and which half to return.
12. Spread final selected portion on paper and slide off into 100 gm container used as a cutter moving through the falling stream, thus, obtaining 100 gm for soil assay and repeat to get 100 gm for foliar essay.

Field Sampling Blue Mold Lesions

Field Sampling

1. Examine field of tobacco and identify tobacco plants with blue mold disease or examine processed tobacco prepared for sale.
2. Select leaves or pieces of tobacco with blue mold lesions.
3. Visually inspect blue mold lesions with hand lens to find oospores. **Note:** Oospores are observed in lesions as red to black specks or small dots. When present, oospores are concentrated in large numbers in lesions.

Field Sampling for Detailed Microscopic Observation of Pathological Structures

1. Select 5 leaves from 5 plants with blue mold lesions in 1 field.
2. Cut 5 circular pieces of tissue in vial for processing.
3. Place 5 pieces of tissues in vial for processing.
4. Label vial with date, farm, county, State.

Clearing Tissue

1. Add 10cc of 1M KOH to vial with 5 tissue pieces.
2. Drop vial in boiling water for 10 to 30 minutes to clear tissue.
3. If tissues **do not** clear:
   - **A.** Drain KOH from vial, rinse tissues with water 3 times.
   - **B.** Add 5cc GAA ALC (1 part glacial acetic acid to 1 part 100% ethanol).
   - **C.** Rinse 3 times with water.
   - **D.** Add 10cc 1 M KOH and drop vial in boiling water for 10 to 30 minutes.

Staining Tissue

1. Drain KOH from vial, rinse 3 times with water.
2. Add 5cc of 0.05% aniline blue stain in 0.067 K2PH04, pH 9.0.
3. Wait 30 minutes before making slides; tightly capped vials can be held in the dark a year or more.

Preparing and Viewing Slides

1. Pour stain and tissue onto a watch glass.
2. Lift tissue pieces carefully with a spatula and place 5 tissues specimens on a slide and cover with glass cover slip.

3. Examine tissues with microscope using incandescent light and a mercury vapor light with filters (HBO 100- W /2 mercury burner and G365nm exciter: LP 42nm barrier fluorescence filter). **Note:** blue mold pathogen structures fluoresce.

August 25, 2000
H.W. Spurr, Jr.

Time/Cost Estimate Blue Mold Oospore Microscopy

1. Start with leaves with blue mold lesions.
2. Bring leaves in order (2 hours)
3. Punch out 5 disks from blue mold lesions (10 minutes)
4. Place disks in 15cc screw cap vial w/Teflon seal caps, add 10 mL KOH and cap (10 minutes)
5. Drop vials in boiling water to clear (10 to 30 minutes)
6. Check for clearing, if **not** clear, change KOH, repeat step 5. (30 minutes)
7. Cool, triple rinse disks with water (10 minutes)
8. Add 5 mL aniline blue stain solution (10 minutes)
9. Incubate (minimum of 12 hours)
10. Pour disks into watch glass and fish out disks with spatula, place disks on slide (10 minutes)
11. Examine disks with microscope, record data (20 minutes for 5 disk samples, up to 3 hours for 50 disk samples)

The total time required for processing a 5 disk sample has been estimated at roughly 4.5 hours. A trained technician can prepare the samples, but a skilled diagnostician is required for examination of the disks. Examination time is markedly higher for the 50-disk samples taken from cured tobacco leaves, due to the fragile nature of the samples.

Blue Mold Microscopy Key

Slide 10 int: Sample Date, 10 Number, Method ID, Slide Prep. Date

T/B:Top/Bottom Leaf Surface based on sporangiophore density
Sp:Sporangiophores
Ss:Sporangiospores
Hy:Hyphae
Os:Oospores
Og:Oogonia
A:Antheridia

R:Reworked samples from GA-EOH Fixative
OF:Other Fungi
BM:Blue Mold
SG:Starch Grains
S:Sand
G:Gritty appearance in tissue, **not** completely cleared
BD:Blue Dots or spheres or vesicles of unknown origin visible in AB stain
RB:Round Bodies, unknown origin, often with Altemaria
E:Edge

P:Pollen
HM:Hyphal Mat
Y:Yeast
B:Bacteria
F:Fungi

Aa:*Alternaria altemata*
Asp:*Aspergillus*
Tc:*Thanatephorus cucumerus*
Phy:*Phyllosticta*
Cn:*Cercospora nicotianae*

L:Light
M:Moderate
H:Heavy
-:No Structure Observed

ADDENDUM A

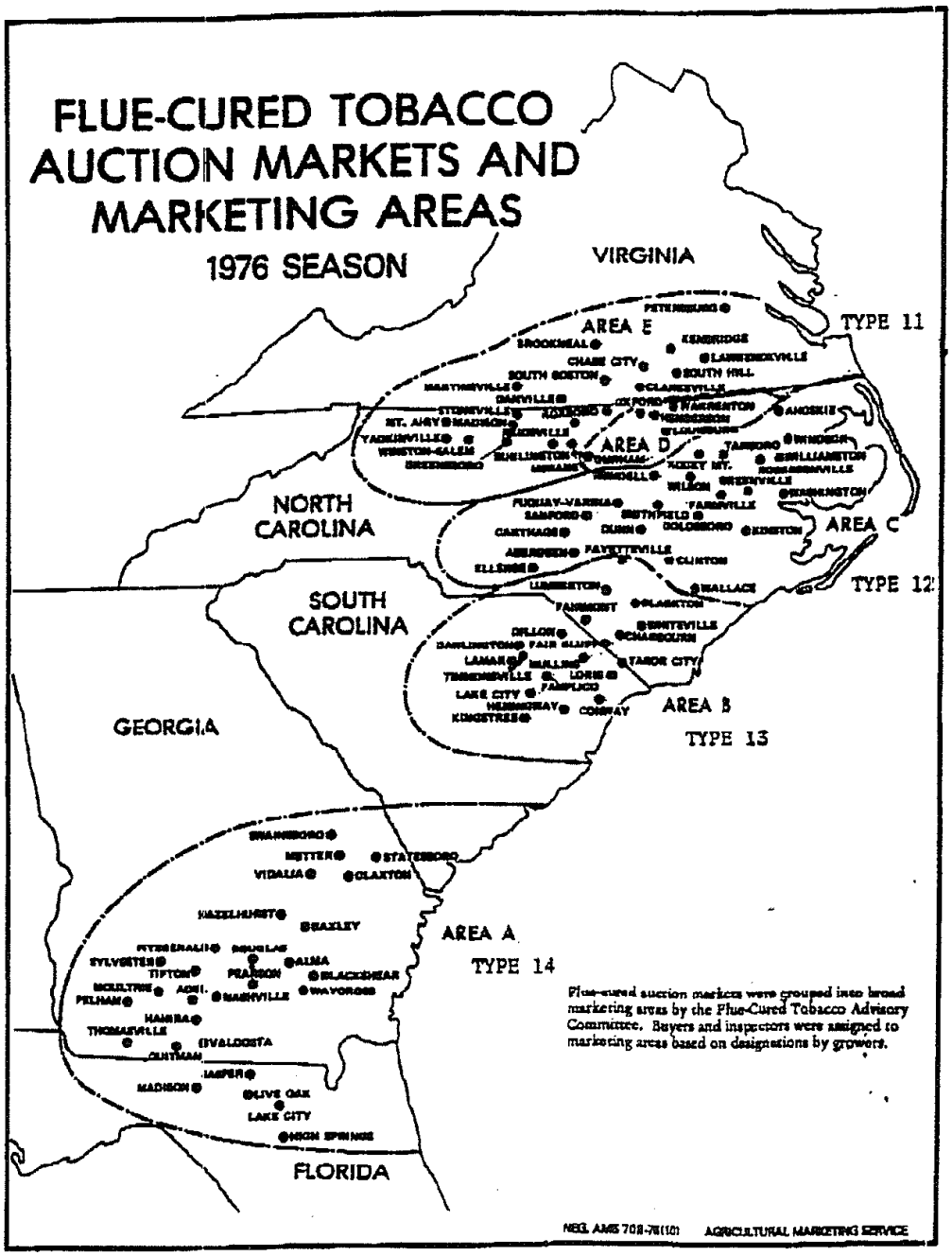

FIGURE 4-8-1: Addendum A — Flue-Cured Tobacco Auction Markets and Marketing Areas