

Reference Manual B **(RM-B)**

NATIONAL SEED HEALTH SYSTEM

***SEED HEALTH TESTING AND
PHYTOSANITARY FIELD INSPECTION
METHODS MANUAL***

Version dated
02/27/01

"Standard" or "Temporary Standard" test results are considered valid for determining phytosanitary eligibility or seed health when conducted by a NSHS accredited entity or other PPQ officially recognized regulatory laboratory.

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SECTION 1

LABORATORY SEED HEALTH TESTING METHODS

Criteria for approving Laboratory Seed Health Testing Methods for the National Seed Health System (NSHS)

Introduction

The National Seed Health System (USA) has developed a peer review system to establish standardized seed health test methods for the NSHS. Technical Panels of 3-4 experts evaluate seed health test methods for groups of seed-borne pathogens. Technical Panel members are drawn from academia and the seed industry throughout the world on the basis of their expertise in pathogens of the assigned crop.

The following criteria are used by the Technical Panels to evaluate seed health testing methods for each host/pathogen combination and recommend their acceptance as standardized methods to be published in Reference Manual B. Final approval of methods is given, on behalf of USDA-APHIS, by the Seed Technical Working Group, a standing committee of NSHS that defines policies and procedures of the system.

Technical Package Development

1. The NSHS Administration Unit (AU) develops technical packages of scientific information for each host/pathogen combination. This is accomplished by conducting a computerized literature review of the pathogen and by direct contact with experts and organizations with special knowledge and/or information of the seed-borne aspect of the pathogen.

Information is assembled on:

- Laboratory seed health tests that are published
- Laboratory seed health tests in use but not published
- Proprietary methodologies in use * (if any)
- Data on test standardization by ISHI, ISTA, or other organizations
- Relevant data on seed transmission or detection of the pathogen

* The evaluation of proprietary methods will be governed by a confidentiality agreement.

2. The AU forwards to each panel member a description of all known laboratory seed health tests for the particular pathogen. Included in this description will be all relevant scientific documentation on the test development and any records of use as routine tests.

3. The AU will eliminate those laboratory methods judged to be trivial, out of date etc.

Technical Panel Responsibilities

Technical Panel members evaluate seed health test methods included in the technical package based upon the following criteria:

Criterion 1. Empirical Test Data

New techniques should have the following parameters included in the development process.

Sensitivity. Determine how sensitive the assay is in terms of either percent-infected seed or target pathogen quantification, such as CFU, number of conidia, etc.

Panel members may need to make an arbitrary determination whether the methods in question are sensitive enough, or they may make a recommendation that the test requires further work. The panel members should also make recommendations on sample size if this is not clearly spelled out in the existing procedures for that test.

Specificity. Determine whether the assay is capable of detecting a range of isolates of the pathogen from different geographical regions, races, etc. Determine whether other closely related organisms are separated with the technique.

Repeatability and Reliability. Replicate experiments of 1 and 2 will help determine these parameters. Additional experimentation including varying types of seed (varieties, production areas, etc.) samples will further refine these parameters.

Criterion 2. Comparative Test Data

Comparisons with already established techniques will yield useful information regarding new techniques. If a new technique performs as well or better than an established technique then it should be accepted. This information is generated internally through the developmental process or through group comparative testing. (These criteria may be of limited applicability to panel members evaluating existing test methodologies.)

Criterion 3. Historical Data

If a technique has been used in industry or academia there may be some indication or record of the number of uses of that technique. There may also be a record of the number of complaints associated with a particular assay. These records may be a good indicator of the effectiveness of the assay.

Other Criteria

Panel members should consider any other criteria that might have significant impact on the recommendation for use of a test. These include the cost of the test, facilities required to perform it, time to perform test, etc.

Technical Panel Report

1. Each Technical Panel (TP) reviewer prepares a report on the form titled “NSHS TECHNICAL PANEL REVIEW - Individual Report Form”, rating each of the methods evaluated by these criteria. He or she then should state their reasons for their rating, and recommend any further research that may be needed.

The panel members should use the following rating system for each method:

Class A – Acceptable as a Standard Test Method.

Class B –Acceptable as a Temporary Standard Test Method. This should include recommendation for further research that would make the method acceptable as a Standard Method Test.

Class C –Test should not be approved as a Standard Test Method.

2. Panel members then return their individual report to the Technical Panel Chair.

3. The Technical Panel Chair evaluates the individual reports from all TP members and prepares a Technical Report that includes a recommendation for disposition of tests and the scientific basis for the recommendations. The recommendation will be made on the form titled “NSHS TECHNICAL PANEL REVIEW - Summary Report Form”. This indicates classification of each test as A (Standard), B (Temporary Standard), or C (Not Approved).

4. The Technical Panel Chair submits the Technical Report and recommendation to the Seed Technical Working Group of NSHS for final approval.

5. The Seed Technical Working Group examines the report of the Technical Panel and makes the final decision on test classification and publication in Reference Manual B.

6. Approved methods are then entered into Reference Manual B as NSHS Standard or Temporary Standard seed health testing methods.

VEGETABLE CROPS

PATHOGEN: *Pseudomonas syringae* pv.
phaseolicola

HOSTS: Bean (*Phaseolus* spp.)

METHOD 1 Plate Test (STA Laboratories, Longmont, CO)

METHOD 1 Plate Test (STA Laboratories, Longmont, CO)

METHOD CLASS: STANDARD

PATHOGEN: *Pseudomonas syringae* pv. *phaseolicola*

SAMPLE: 5000 seeds

PROCEDURE:

1. 5 subsamples of 1,000 seeds are placed in 0.85% NaCl buffer in a ratio of 1:3.
2. Samples are shaken vigorously for 3 to 4 hours, or refrigerated overnight.
3. Part of the extract is centrifuged at 10,000 rpm for 10 minutes. Most of the supernatant liquid is discarded, and pellet is resuspended.
4. Two more dilutions are made, one directly from the bean extract, and a 1:10 dilution from this original solution.
5. 0.1ml of each of the three dilutions is plated in duplicate onto KBBC and MSP media. Liquid is spread evenly over each plate, and plates are incubated at 27-30°C for 5 to 7 days.
6. Plates are evaluated for suspected *P.s. pv. phaseolicola* colonies. Suspect colonies are confirmed by fluorescence, oxidase, reaction on BBD media, and pathogenicity testing.
7. Pathogenicity is shown by mixing a slightly turbid solution of the bacterium in sterile water, and inoculating the undersides of susceptible bean leaves with a cotton swab. Plants are incubated at high humidity for 7 to 10 days, then checked for lesions surrounded by chlorotic halos.

MEDIA PREPARATION:

KBBC:

King's B media 900ml

After autoclaving filter sterilize the following adding to media just prior to pouring:

Boric acid 100ml of 1.5% aqueous solution

Cephalexin 80mg

Cycloheximide 20mg

MSP:

Sucrose 20g

Peptone 5g

K₂HPO₄ 0.5g

MgSO₄ * 7H₂O 0.25g

Agar 20g

H₂O 1 liter

After autoclaving filter sterilize the following adding to media just prior to pouring:

Cycloheximide 200mg

Cephalexin	80mg
Vancomycin	10mg
Bromthymol blue	15mg

BBD:

Yeast extract	1g
Glycerol	5g
Bacto peptone	5g
Bacto agar	15g
H ₂ O	750ml

Autoclave separately for no more than 15 minutes:

Powdered milk	15g
H ₂ O	250ml

REFERENCES:

Saettler, A.W. 1991. Halo blight. Page 30 in: Compendium of Bean Disease. Robert Hall (edit.) American Phytopathological Society, St. Paul, MN.

Van Vuurde, J.W.L. and Van den Bovenkamp, G.W. 1989. Detection of *Pseudomonas syringae* pv. *phaseolicola* in bean. Pages 30-40 in: Detection of bacteria in seed and other planting material. A.W. Saettler, N. W. Schaad, and D.A. Roth (Eds.) American Phytopathological Society. St. Paul, MN. 122 pp.

Mohan, S.K. and Schaad, N.W. 1987. An improved agar plating assay for detecting *Pseudomonas syringae* pv. *syringae* and *Pseudomonas syringae* pv. *phaseolicola* in contaminated bean seed. *Phytopathology* 77: 1390-1395.

PATHOGEN: *Xanthomonas campestris* pv. *carotae*

HOSTS: Carrot (*Daucus carota*)

METHOD 1 Seed wash (Kuan et al., 1985)

METHOD 1 Seed wash (Kuan et al., 1985)

METHOD CLASS: STANDARD

PATHOGEN: *Xanthomonas campestris* pv. *carotae*

SAMPLE: 20g of seed

PROCEDURE:

1. Twenty grams of seed is added to 100 ml of 0.85% sterile cold aqueous NaCl (saline) in a 250ml flask.
2. The flask is swirled to submerge the seeds and incubated in the dark for 16-18 h at 5°C (a 2 hour incubation was found to be as effective as a 16-18 hour incubation).
3. Following incubation, two drops of sterile Tween 20 are added to the flask, and the flask is vigorously shaken for 1 minute.
4. The resulting suspension is filtered through two layers of sterile cheesecloth, and the seed is rinsed with an additional 25 ml of saline.
5. The pooled washings are centrifuged at ca 8000 g for 15 minutes.
6. Pellets are resuspended in 10ml sterile cold saline and serially diluted to 0.000001.
7. Aliquots of 100 µl of each dilution are then plated in triplicate onto MDS and/or XCS media.

MEDIA PREPARATION:

XCS:

Lactose		10g
D+ Trealose	4g	
Thiobarbituric acid		0.2g
K ₂ HPO ₄		0.8g
KH ₂ PO ₄		0.8g
NH ₄ Cl		1g
Yeast Extract		0.5g
Agar		15g
H ₂ O		1 liter

After autoclaving filter sterilize the following adding to media just prior to pouring:

Ampicillin		1mg
Vancomycin	0.5mg	
Cycloheximide		100mg

MDS:

K ₂ HPO ₄	10g
NaH ₂ PO ₄	1g
NH ₄ Cl	1g
MgSO ₄ * 7H ₂ O	0.3g
Agar	15g
H ₂ O	1 liter

After autoclaving filter sterilize the following adding to media just prior to pouring:

Cellobiose	10g
Cycloheximide	100mg

REFERENCES:

Kuan TL, Minsavage GV, Gabrielson RL. 1985. Detection of *Xanthomonas campestris* pv. *carotae* in carrot seed. Plant Disease. 69(9):758-760.

PATHOGEN: *Septoria apiicola*

HOSTS: Celery (*Apium graveolens*)

METHOD 1 Seed Wash (STA Laboratories,
Longmont, CO)

METHOD 1 Seed Wash (STA Laboratories, Longmont, CO)

METHOD CLASS: STANDARD

PATHOGEN: Celery (*Apium graveolens*)

SAMPLE: 10,000 seeds

PROCEDURE:

1. Two reps of 5,000 seeds are added to water and shaken for two hours.
2. 1ml of liquid is pipetted into microcentrifuge tubes and centrifuged at 8000rpm for 10 minutes.
3. Discard supernatant and resuspend pellet in DI water.
4. Examine for spores of *Septoria* on a hemacytometer at 100 to 400x magnification.
5. If spores are found, run a pathogenicity test using the spore suspension from Step 3.
6. For pathogenicity testing, excise leaves from a healthy celery plant, disinfect with 1% NaOCl solution and rinse. Place leaves in a 45°C water bath for 15 seconds, then blot leaves dry and place in a humid box. Dip a cotton swab in the spore suspension and wipe onto leaves. Incubate samples at 20-25°C under light and examine for pycnidia in 7 to 19 days.

REFERENCES:

Hewitt, P.D. 1968. Viable *Septoria* ssp. in celery seed samples. Ann. Appl. Biol. 61:89-98.

Updates: Cubeta M.A. North Carolina State Research Station.

Disease and pests of vegetable crops in Canada: an illustrated compendium. 1994.

PATHOGEN: Lettuce Mosaic Virus

HOSTS: Lettuce (*Lactuca sativa*)

METHOD 1 Chenopodium (Kimble et al. 1975)

METHOD 2 ISHI Seed (van Ettekoven, 2000)

METHOD 1 Chenopodium (Kimble et al. 1975)

METHOD CLASS: STANDARD

PATHOGEN: Lettuce Mosaic Virus

SAMPLE: 30,000 seeds

PROCEDURE:

1. Grind sub-samples of 500 lettuce seeds (total sample 30,000 seeds) in 4ml 0.05M phosphate buffer pH 7.7, containing 0.1% thioglycolic acid.
2. Mechanically inoculate five *Chenopodium quinoa* seedlings with each extract (within 16 minutes of making the extract).
3. Observe indicator plants for symptoms 18-20 days after inoculation (chlorotic local lesions followed by systemic yellow vein-netting and leaf distortion).

REFERENCES:

Kimble KA, Grogan RG, Greathead AS, Paulus AO, House JK, 1975. Development, application, and comparison of methods for indexing lettuce seed for mosaic virus in California. *Plant Disease Reporter*: 59(6):461-464.

METHOD 2 ISHI Seed (van Ettekoven, 2000)

METHOD CLASS: STANDARD

PATHOGEN: Lettuce Mosaic Virus

SAMPLE: 10,000 seeds

PROCEDURE:

Samples and buffers:

1. Count 20 x 500 seeds for each seed lot, at least 8 x 500 seeds of negative seed lot as negative control, and 2 x 500 seeds of a positive seed lot (contaminated leaf sample can be used as additional positive control).
2. Prepare the buffer solutions as follows:

PBS-Tween:

Dissolve 8.0g NaCl, 1.0g KH₂PO₄, 14.5 g Na₂HPO₄.* 12H₂O, and 0.5ml Tween 20, in 950ml dionized water, adjust pH to 7.2, and adjust volume to 1 liter.

Coating buffer:

1.59g Na₂CO₃, 2.93g NaHCO₃ in 1 liter dionized water, pH 9.6.

Extraction buffer:

Dissolve 20g of polyvinyl pyrrolidone PVP-10, in 950 ml PBS-Tween, adjust to 1 liter.

Conjugate buffer:

Dissolve 5.0 g of bovine serumalbumine (or ovalbumine) in 950 ml PBS-Tween, adjust to 1 liter.

Coating of ELISA microplates:

1. Dilute LMV-antisera in coating buffer to appropriate working dilution.
2. Transfer 200µl of diluted antisera to each well.
3. Incubate microplate 3 hours at 37⁰C, or overnight at 4⁰C.

Extraction from seeds:

1. Grind seeds of each subsample in ca. 5ml extraction buffer using a power driven crusher (Erich Pollahne type). Alternative extraction devices can be used.
2. Collect the complete seed extract in tube. Process extracts within 4 hours after crushing. Store extracts at 4⁰C if not processed immediately.
3. Rinse rollers of crusher between each subsample with water to prevent cross-contamination between subsamples.
4. Wash thoroughly rollers between seed lots.

Seed extract incubation:

1. Remove coating buffer with diluted antisera from the plates.
Rinse microplate three times for 30 seconds with PBS-Tween, to reduce background.
2. Transfer 200µl of seed subsample extract in two wells for each seed lot, each positive and negative subsamples as well. Several templates are in use.
3. Incubate microplates overnight at 4°C.

Incubation of conjugated LMV antiserum:

1. Remove seed extracts.
2. Rinse microplate three times 30 seconds with PBS-Tween, to reduce background.
3. Dilute conjugated LMV-antiserum in conjugate buffer to appropriate working dilution.
4. Transfer 200µl of diluted conjugated antiserum to each well.
5. Incubate microplate 3 hours at 37°C, or overnight at 4°C.

Incubation of substrate:

1. Dissolve 20mg of para-nitrophenylphosphate per 20ml of substrate buffer shortly before use.
2. Rinse microplate three times 30 seconds with PBS-Tween, to reduce background
3. Transfer 20µl of substrate buffer to each well of the microplate.
4. Incubate at room temperature in the dark. Measure optical density at 405nm after 30 minutes, 1 hour, and longer if necessary. Verify always first whether positive and negative controls show appropriate reaction, otherwise the test should be repeated.
5. Use negative threshold to determine how many subsamples were infected with LMV.
6. Use Table 1 of ISHI-Veg Manual (2000) to determine the estimated LMV infection rate of the seed lot.

REFERENCES:

van Ettehoven, K. (editor). 2000. Seed Health Testing Methods Reference Manual. ISHI-Veg.

Falk BW, Purcifull DE. 1983. Development and application of an enzyme-linked immunosorbent assay (ELISA) test to index lettuce seeds for lettuce mosaic virus in Florida. *Plant Disease*: 67(4):413-416.

PATHOGEN: *Xanthomonas campestris* pv.
vesicatoria

HOSTS: **Pepper** (*Capsicum annuum*), **Tomato**
(*Lycopersicon esculentum*)

METHOD 1 **ISHI Seed wash for pepper and tomato** (van
Ettekoven, 2000)

METHOD 1 ISHI Seed wash for pepper and tomato (van Ettehoven, 2000)

METHOD CLASS: TEMPORARY STANDARD

PATHOGEN: *Xanthomonas campestris* pv. *vesicatoria*

SAMPLE: Minimum number of seeds to be tested is 2000 (protected cultivation in temperate conditions) or 30,000 (outdoor cropping in humid and warm conditions), which will detect with 95 % reliability seed contamination of 0.15 % and 0.01 % respectively.

PROCEDURE:

Sample preparation:

1. Put every subsample into a sterilized flask or other liquid-proof container.
2. Add 0.05M PBT to each flask at a ratio of 3:1 (v:w).
3. Incubate overnight (minimum 14 hrs) at 4°C.

Serial dilution:

1. Directly from the settled seed wash, serial dilute the supernatant to 10^{-1} by aseptically pipeting 1.0 ml of the seed wash supernatant into 9ml of sterile 0.85% NaCl in a sterile culture tube. Label the dilution with the appropriate sample number.
2. From the 10^{-1} dilution, dilute the supernatant to 10^{-2} by aseptically pipeting 1.0ml of 10^{-1} dilution into 9ml of sterile 0.85% NaCl in a sterile culture tube. Label the dilution with the appropriate sample number.
3. Vortex each tube to assure thorough mixing.

Liquid plating:

1. Pipette direct from each sample flask and from the 10^{-1} and 10^{-2} dilution, 100µl on two plates (duplicate plating) of two semi-selective media.
2. With a sterile glass rod spread the 100µl aliquot evenly over the semi-selective media.
3. Label the plates with the appropriate sample number and dilution factor (direct, 10^{-1} , and 10^{-2}).
4. Inoculate plates from both media with a known culture of Xcv.

Incubation:

1. Incubate the plates in a dark incubator at 26-28°C.

Examination:

1. Evaluate the plates for suspect Xcv colonies at day 3, 5, and 7 days.
2. Compare them to the known descriptions of Xcv on the used selective media given below.

3. Record the number of suspect Xcv colonies and background.
4. Purify the suspect colonies by transferring suspect colonies to a general rich medium plates.

Additional confirmation tests as ELISA, PCR, IF, or biochemical tests are optional.

Xcv descriptions on semi-selective media:

Xcv on TMB:

1. Xcv colonies typically appear and can be identified in 4-7 days.
2. Xcv colonies are yellow, slightly mucoid, mounded and round (circular).
3. Xcv utilizes Tween and in 3-7 days a white crystalline halo usually forms around the yellow colony.
4. Some strains of Xcv form only a weak halo, clear halo, or only clear on the media under the colony (not visible). (NOTE: The white crystalline halo is due to calcium deposits in the clear halo caused by Tween utilization.)

Xcv on CKTM:

1. Xcv colonies typically appear and can be identified in 4-7 days.
2. Xcv colonies are similar to the colonies on TMB; yellow, mucoid, mounded and round (circular). In 3-7 days a white halo of fine calcium crystals usually forms around the yellow colony. Some strains of Xcv form only a weak halo, clear halo, or only clear the media under the colony (not visible).

Xcv on NA-Starch:

1. No information provided. Check appearance with known Xcv cultures.

Pathogenicity testing:

1. For best results pepper/tomato seedlings should be at 2-3 true leaves (approx. 3-4 week old seedlings).
2. Purify Xcv colonies (suspects) on a general medium suitable for rapid colony growth such as YDC. Purify a known Xcv colony for a positive control. Incubate the plates for 24 to 48 hours at 26 to 28°C.
3. Transfer a mass of the colony aseptically to a culture tube with sterile distilled water.
4. Adjust the inoculum to 10^8 CFU per ml by visual means or with optical density methods (0.1 to 0.2 o.d. at 600nm).
5. Infiltrate a leaf of a susceptible pepper/tomato cultivar with the suspension by gently forcing the liquid into the underside of the leaf using a sterile syringe without a needle and label the leaf with the appropriate sample identification number.
6. Infiltrate a leaf with the positive check and a blank water check (negative control)
7. Incubate the inoculated plants at 90-100% relative humidity and at 27 -32°C with approx. 12 hour light.
8. Observe the plants daily and record the day that the water-soaked lesion appears. Generally, pathogenic Xcv will develop a water-soaked lesion within 48-96 hours. Non-pathogenic bacteria will produce a hypersensitive reaction in about 24 hours, or will not develop a lesion.

MEDIA PREPARATION:

PBT:

0.05M Phosphate buffer
0.5% Tween 20
pH 7.2

mTMB:

H ₂ O	990 ml
H ₃ BO ₃	0.1 g
Bacto peptone	10.0 g
KBr	10.0 g
CaCl ₂	0.25 g
Agar	15.0 g

After autoclaving filter sterilize the following adding to media just prior to pouring:

Tween 80	10.0 ml
Cephalexin	65 mg
5-fluorouracil	12 mg
Tobramycin	0.2 mg
Cycloheximide	200 mg

YDC:

H ₂ O	1000 ml
Yeast extract	10.0 g
CaCO ₃	20.0 g
Bacto agar	15.0 g
Glucose	20.0 g

NA-Starch:

H ₂ O	1000 ml
Difco nutrient agar	23.0 g
Soluble potato starch	12.0 g

After autoclaving filter sterilize the following adding to media just prior to pouring:

Penicillin G	50 mg
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CKTM:

H ₂ O	990 ml
Soy peptone	2.0 g
Bacto typtone	2.0 g
Dextrose	1.0 g
L-glutamine	6.0 g
L-histidine	1.0 g
(NH ₄) ₂ HPO ₄	0.8 g
MgSO ₄ . * 7H ₂ O	0.4 g
CaCl ₂	0.25 g
Pourite (optional)	1 drop
Difco bacto agar	15.0 g

After autoclaving sterilize the following adding to media just prior to pouring:

Tween 80	10 ml
Cephalexin	65 mg
5-fluorouracil	12 mg
Tobramycin	0.4 mg
Cycloheximide	100 mg
Bacitracin	100 mg
Neomycin sulfate	10 mg

REFERENCES:

van Ettehoven, K. (editor). 2000. Seed Health Testing Methods Reference Manual. ISHI-Veg.

FIELD CROPS

PATHOGEN: *Clavibacter michiganensis subsp. nebraskensis*

HOSTS: Maize (*Zea mays*)

METHOD 1 sCNS Culture Plate Method (Shepherd, 1999)

METHOD 1 sCNS Culture Plate Method (Shepherd, 1999)

METHOD CLASS: STANDARD

PATHOGEN: *Clavibacter michiganensis* subsp. *nebraskensis*

SAMPLE: 400 seeds

PROCEDURE:

1. Divide a representative sample of 400 corn seeds into 4 sub-lots of 100.
2. Surface sterilize seeds with 0.5% NaOCl for 3 minutes. Rinse thoroughly with sterile water.
3. Grind each sub-sample until finely pulverized and add to 100ml phosphate buffer saline solution (seeds may be ground dry or soaked overnight at 6°C before grinding).
4. Shake sub-samples at room temperature for 1 hour.
5. Prepare a dilution series comprising the original suspension and two 10-fold dilutions.
6. Plate each of the three dilutions onto sCNS in triplicate.
7. Incubate at 28°C for 5 to 7 days.
8. Any orange colonies that appear in 1 to 3 days should be disregarded.
9. Round and peach colored colonies (Figure 1) that appear after 3 days and by 9 days are putative colonies of *C. michiganensis* subsp. *nebraskensis* (Cmn).
10. Cmn can be confirmation by plant injections of culture suspensions into seedlings of Goss' wilt susceptible corn varieties (such as A632). Seedlings are then examined for typical Goss' wilt symptoms on leaves.
11. Alternatively, Cmn can be confirmed by CORYNE strip tests, performed according to manufacturer's (bioMeriux-Vitek Inc., Hazelwood, MO) directions on colonies plated on TSA agar. The positive code for Cmn is 6550004 or 6150004.

MEDIA PREPARATION:

sCNS:

Distilled water	1 liter
Nutrient broth	8.0g
Yeast extract	2.0g
K ₂ HPO ₄	2.0g
KH ₂ PO ₄	0.5g
Dextrose	5.0g
MgSO ₄ * 7H ₂ O	0.25
Agar	15g
Bravo 720 (Chlorothalonil)	0.2ml diluted 1:50 in water
Mertect 340F (Thiabendazole)	50µl (1 drop)
Potassium dichromate	0.02g

After autoclaving filter sterilize the following adding to media just prior to pouring:

Naladixic acid	40mg
Cycloheximide	100mg

REFERENCES:

Shepherd, LM. 1999. Detection and transmission of *Clavibacter michiganensis* subsp. *nebraskensis* of corn. Ms Thesis, Iowa State University, Ames, IA.

PATHOGEN: *Pantoea stewartii* (syn. *Erwinia stewartii*)

HOSTS: Maize (*Zea mays*)

METHOD 1 **ELISA (Enzyme-linked immunosorbent assay)**
(Lamka et al, 1991)

METHOD 1 ELISA (Enzyme-linked immunosorbent assay) **(Lamka et al, 1991)**

METHOD CLASS: STANDARD

PATHOGEN: *Pantoea stewartii* (syn. *Erwinia stewartii*)

SAMPLE: 400 seeds

PROCEDURE:

Diagnostic kit:

This method uses an ELISA kit developed by Agdia, Elkhart, Indiana. The monoclonal antiserum used in the kit was made at Iowa State University as described (Lamka et al, 1991).

Extraction of the bacterium:

1. Place 4 replicates of 100 corn kernels, per sample, into 100ml of General Extraction Buffer and soak overnight at room temperature.
2. Grind seeds to fine particles.
3. Disinfect the grinder between replicates and samples by rinsing with distilled water, followed by a rinse with a laboratory or hospital standard detergent, then a final rinse with distilled water.

Preparation of Buffers:

PBS-Tween Buffer/Wash

1. Empty contents of the PBS-Tween satchel into 1 liter container. Add distilled water to make one liter PBS-Tween (buffer/wash).
2. To make from scratch, dissolve the following in 1 liter distilled water: 8.0g sodium chloride, 1.15g sodium phosphate (dibasic), 0.2g potassium phosphate (monobasic), 0.2g potassium chloride, and 0.5g Tween 20.

General Extraction Buffer

1. Add dry Extraction Buffer provided to 1 liter distilled water provided.
2. Mix the buffer powder with a small amount of distilled water to make a slurry, then gradually add more distilled water to the final volume.
3. Add 20ml Tween 20 per liter of Extraction Buffer and mix well.
4. Store at 4°C.
5. To make from scratch, dissolve the following in 900ml PBS-Tween buffer: 1.3g sodium sulfite, 2.0g powdered chicken albumin, 20g polyvinylpyrrolidone, 20g Tween 20.

Preparation of *Pantoea stewartii* controls:

Add 2ml of the General Extraction Buffer to each of the provided control vials, positive (+) ES and negative (-) ES, and mix well.

Loading ELISA plates:

1. Add 100µl of the sample filtrate, of each replicate, to one well on a pre-coated ELISA plate for *P. stewartii*. Change micropipette tips between each replicate.
2. Add 100µl of positive (+) ES and negative (-) ES to control wells.
3. Incubate for 2 hours at room temperature or overnight at 4°C in a humid container.
4. Wash the plates approximately 6 times with PBS-Tween. Tap plate gently after final rinse to remove remaining wash buffer.

Addition of enzyme conjugate:

1. Dispense 100µl of prepared enzyme conjugate (as prepared below) into each well and into two blank control wells.
2. Add enzyme conjugates A and B at the dilution of 1:100.
3. Incubate for 2 hours at room temperature or overnight at 4°C in a humid container.
4. Wash 6 times with PBS-Tween.

Preparation of enzyme conjugate

To make enzyme conjugate diluent, mix 4 parts of PBS-Tween to 1 part MRS component.

Note: The volume of enzyme conjugate prepared is dependent on the number of test wells that are being used. On the basis of 100µl needed for each test well, a helpful way of estimating the amount of diluent needed is to prepare 1ml for each 8-well column of test wells.

Addition of substrate:

1. Dispense 100µl of substrate per well.
2. Incubate for 20 minutes at room temperature in the dark, or until the positive control is showing significant color.
3. Add 50µl (one drop) 3M sulfuric acid to stop the color reaction.

Substrate preparation

1. Add 1 OPD stick to 10ml of OPD buffer solution. The paper end of the stick is submerged into room temperature OPD buffer solution for 2 min.
2. Swirl the stick slightly before removing from the solution. Always prepare OPD buffer solution immediately before use.

Evaluating ELISA plates:

Evaluate visually, or measure on an ELISA plate reader at 490nm. The threshold for a positive reaction on the plate reader should be >2X that of the negative control.

REFERENCES:

Lamka GL, Hill JH, McGee DC and Braun EJ. 1991. Development of an immunosorbent assay for seed-borne *Erwinia stewartii*. *Phytopathology* 81:839-846.

PATHOGEN: *Cercospora kikuchii*

HOSTS: Soybean (*Glycine max*)

METHOD 1 Culture Plate (Jordan et al., 1986)

METHOD 2 Culture Plate (McGee and Nyvall, 1984)

METHOD 1 Culture Plate (Jordan et al., 1986)

METHOD CLASS: STANDARD

PATHOGEN: *Cercospora kikuchii*

SAMPLE: 400 seeds

PROCEDURE:

1. Surface sterilize seeds in 0.5% NaOCl for 4 minutes; wash in distilled water.
2. Plate on potato dextrose agar.
3. Incubate at 25°C in the dark for 10 days.

REFERENCES:

Jordan EG, Manandhar JB, Thapliyal PN, Sinclair JB, 1986. Factors affecting soybean seed quality in Illinois. *Plant Disease*. 70(3):246-248.

METHOD 2 Culture Plate (McGee and Nyvall, 1984)

METHOD CLASS: STANDARD

PATHOGEN: *Cercospora kikuchii*

SAMPLE: 400 seeds

PROCEDURE:

1. Seeds are surface sterilized in 1% NaOCl for 30 seconds and then rinsed in sterile water.
2. Incubate on a moistened blotter at 25°C for 10 days under continuous light.
3. Seeds are evaluated for the presence of *Cercospora kikuchii* as indicated by purple staining on the seed coat under fungal growth.

REFERENCES:

McGee DC, Brandt CL, Burris JS, 1980. Seed mycoflora of soybeans relative to fungal interactions, seedling emergence, and carry over of pathogens to subsequent crops. *Phytopathology*. 70(7):615-617.

McGee DC, Nyvall RF, 1984. Soybean seed health. Coop. Ext. Serv. Iowa State University, Pm-990.

PATHOGEN: *Phomopsis/Diaporthe* complex

HOSTS: Soybean (*Glycine max*)

METHOD 1 **Culture plate** (McGee, 1986)

METHOD 2 **Blotter** (McGee and Nyvall, 1984)

METHOD 1 Culture plate (McGee, 1986)

METHOD CLASS: STANDARD

PATHOGEN: *Phomopsis/Diaporthe* complex

SAMPLE: 400 seeds

PROCEDURE:

1. Seed are surface sterilized in 0.5% NaOCl for 1 minute and then rinsed in sterile water.
2. Plate seeds on potato dextrose agar (PDA) adjusted to pH 4.5.
3. Seeds incubation at 25°C for 7 days under continuous light.
4. Seeds are evaluated for presence of the three fungi associated with the *Phomopsis/Diaporthe* complex in soybeans according to the following descriptions.

Note: There is considerable controversy regarding the taxonomy of this group. These descriptions should, therefore only be used to identify the *Diaporthe/Phomopsis* complex and not the individual pathogens.

Phomopsis longicolla, previously known as *Phomopsis* sp. are floccose, dense and white, with occasional greenish yellow areas on PDA. In reverse, colonies are colorless, with black, spreading stromata. Pycnidia are black, stromatic, solitary or aggregated, unilocular or multilocular, with prominent necks more than 200µm long. Alpha conidia are hyaline, ellipsoid to fusiform, guttulate, and measure 5-9 x 1.5-3.5µm. Beta conidia, which are rarely formed, are hyaline, filiform, and hamate. No perithecia are formed on PDA.

Diaporthe phaseolorum var. *caulivora* cultures on PDA are white, closely appressed, with tufted aerial mycelium. In reverse, colonies are colorless, with black circular stromata, less than 2mm in diameter. Perithecia are black and globose, measuring 165-340 x 282-412µm, and have a long protruding beak. Asci measure 30-40 x 4-7µm. Ascospores are hyaline, elongate-ellipsoidal, two-celled, slightly constricted at the septum, biguttulate in each cell, and measure 8-12 x 3-4µm. Pycnidia rarely occur on PDA.

Diaporthe phaseolorum var. *meridionales* cultures on PDA are white even colonies with brown chlamydospores. Stromata are irregular in shape (2-10mm long). Perithecia are similar in to those of *D. phaseolorum* var. *caulivora*, but they have wider neck width, i.e. 100 compared to 55µm.

Diaporthe phaseolorum var. *sojae* cultures on PDA are floccose and rope-like, turning tan to brown with age. In reverse, the colonies are tan to dark brown with black, pulvinate stromata. Pycnidia are black, stromatic, solitary or aggregated, and usually unilocular, with no beak, or a beak less than 200µm long. Alpha conidia are hyaline, usually fusiform, guttulate, and measure 5.5-10.5 x 1.3-3.5µm. Beta conidia, which are more commonly produced, are hyaline, filiform, and hamate. Perithecia are nearly spherical and measure 148-282 x 185-346µm. They have long, tapered beaks, measuring 60-100 x

60-150µm, and usually are solitary, not clustered. Asci are elongate and clavate, measuring 35-51 x 3.3-10µm. Ascospores are similar in shape to alpha conidia, but are larger, 9-13 x 2-6µm, and bicellular. They are biguttulate in both cells.

REFERENCES:

McGee DC, 1986. Prediction of *Phomopsis* seed decay by measuring soybean pod infection. Plant Disease 70:329-333.

METHOD 2 Blotter (McGee and Nyvall, 1984)

METHOD CLASS: STANDARD

PATHOGEN: *Phomopsis/Diaporthe* complex

SAMPLE: 400 seeds

PROCEDURE:

1. Two sterilized blotters are placed in plastic boxes.
2. Moisten blotters with 80ml of sterile water, containing 40mg of 2,6 dichloro-6-nitroaniline (Botran 75W).
3. Four sets of 100 seeds are surface sterilized in 1% NaOCl for 30 seconds and then rinsed in sterile water.
4. Seeds are incubated at 25°C for 10 days under continuous light.
5. Seeds are evaluated for the presence of fungi in the *Phomopsis/Diaporthe* complex characteristic dense, white fungal growth.

REFERENCES:

- McGee DC, Brandt CL, Burris JS, 1980. Seed mycoflora of soybeans relative to fungal interactions, seedling emergence, and carry over of pathogens to subsequent crops. *Phytopathology*. 70(7):615-617.
- McGee DC, Nyvall RF, 1984. Soybean seed health. *Coop. Ext. Serv. Iowa State Univ*, Pm-990.

PATHOGEN: *Sclerotinia sclerotiorum*

HOSTS: Soybeans (*Glycine max*)

METHOD 1 Culture plate (Totir, 2000)

METHOD 1 Culture plate (Totir, 2000)

METHOD CLASS: STANDARD

PATHOGEN: *Sclerotinia sclerotiorum*

SAMPLE: 400 seeds

PROCEDURE:

1. Four sub-samples of 100 seeds are surface sterilized in 1.75% NaOCl for 30 seconds.
2. Seeds are rinsed three times in sterile water.
3. Incubate seeds on potato dextrose agar for 10 days at 25°C (5 seeds/plate)
4. Seeds with characteristic white mycelium of *Sclerotinia sclerotiorum* are marked after 3, 5, and 7 days to account for overgrowth of colonies compromising the final count.
5. A final count is made of seeds with characteristic white mycelium and/or large black sclerotia, at 10 days.

REFERENCES:

Totir, C. 2000. Seed transmission and control of *Sclerotinia sclerotiorum* in soybean seeds. Ms. Thesis, Iowa State University, Ames, IA.

SECTION 2

PHYTOSANITARY FIELD INSPECTION METHODS

Criteria for Phytosanitary Field Inspection Methods Approved for the National Seed Health System (NSHS)

Phytosanitary field inspection procedures described in this manual were developed by a sub-committee of the NSHS-STWG, representing USDA-APHIS, the National Plant Board, and the Association of Official Seed Certification Agencies. The procedures are designed to meet fundamental standards of quality but to be flexible enough to accommodate commonly-used practices.

Field diagnostic aids are developed by plant pathologists with expertise in symptomatology of diseases in each crop.

PHYTOSANITARY INSPECTION PROCEDURES

EQUIPMENT REQUIREMENTS

1. Field maps
2. 10x hand lens
3. Pocket knife/scissors
4. Tape measure
5. Sampling bags or envelopes (paper)
6. Labels
7. Ice chest
8. Hand counter
9. Diagnostic illustrations
10. Report forms

FIELD INSPECTION STRATEGY

Field overview

Find a point near the field that allows the best opportunity to look the field over. This overview enables:

- a. Verification that the field is correct by comparison between the map or information provided and the observed field.
- b. Identification of special areas or micro-climates in the field that appear different enough to warrant special attention when inspected. These would include:

Locations in which high moisture levels may be retained such as proximity to:

- Rivers and streams
- Drainage areas
- Low spots
- Weedy areas

Areas of the field affected by borders, such as:

- Field edges
- Tree lines in the field
- Adjacent fields of a similar crop
- Presence of buildings or bins

Drought stress areas, such as:

- High spots
- Light textured soils
- Margins or overhead irrigation areas

Establish the seed field inspection pattern

1. The seed field inspection pattern should ensure that all parts of the field are adequately and proportionately represented in the plants inspected within the various usual micro-climates of the field.
2. As long as these requirements are met, the pattern of field inspection can vary.
3. Examples of established inspection patterns are as follows. Other formats may, however, be acceptable.

Stagger “X” pattern. (CDFA Phytosanitary Certification Manual, 1985)

This is used for cereal crops and requires examination of plants along one side of the field, then diagonally in a stagger pattern across rows to the far corner, across the far side, and diagonally back to starting corner (Figure 1). Additional examinations may be necessary for field environments not covered by the inspection pattern.

Equidistant passes pattern. (CDFA Phytosanitary Certification Manual, 1985)

This system is used for crops other than cereals. Table 1 lists the minimum number of field passes (Figure 2) in relation to field size to give a minimum of 95% confidence level in detecting an infection level of 0.1%.

Table 1. Minimum field passes per acre.

Field size (acres)	Minimum # passes
0 - 1	6
1 - 5	9
5 - 10	11
10 - 20	13
20 - 50	17
50 - 100	20
100 - 200	24
200 - 500	30
500 - 1000	36
1000 +	42

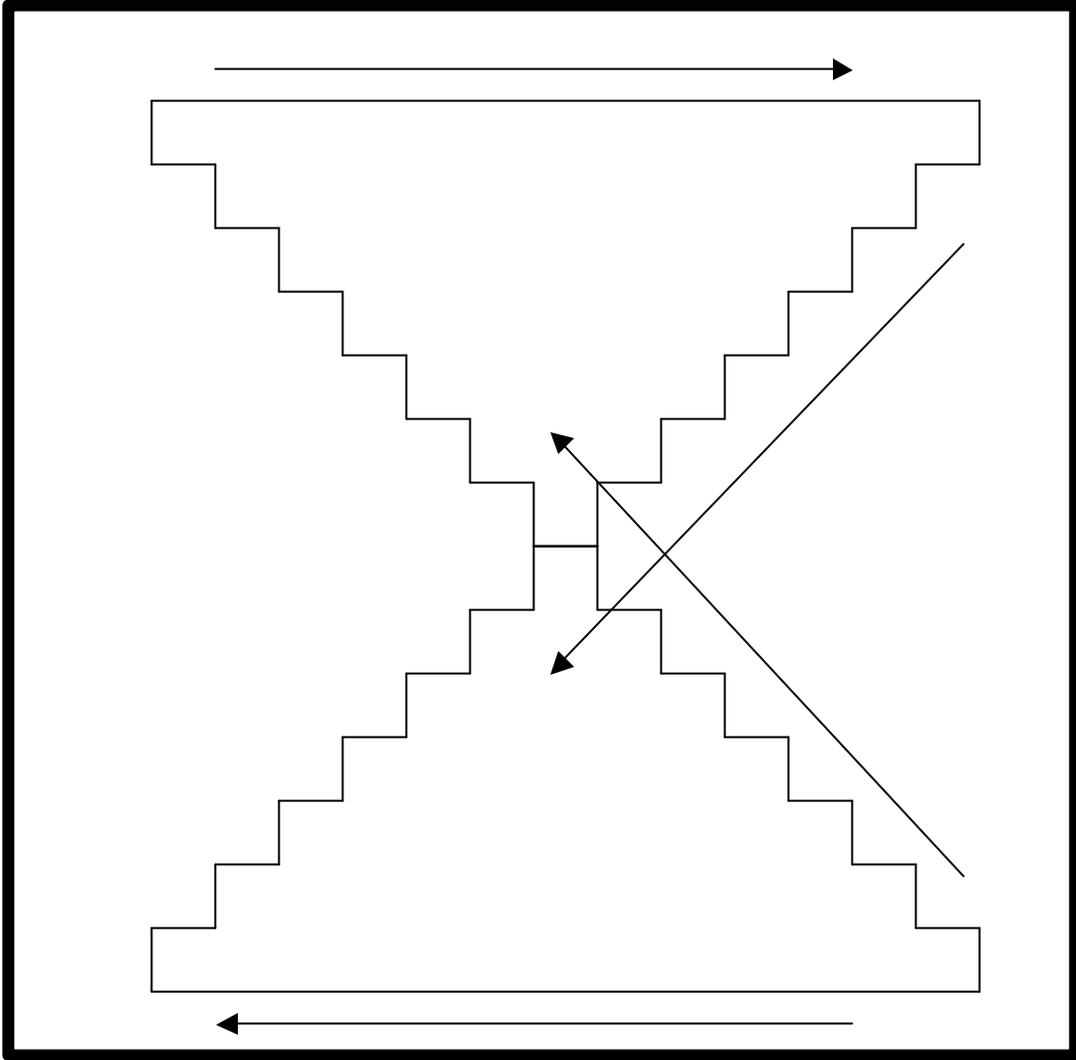


Figure 1 "X" Field Inspection Pattern

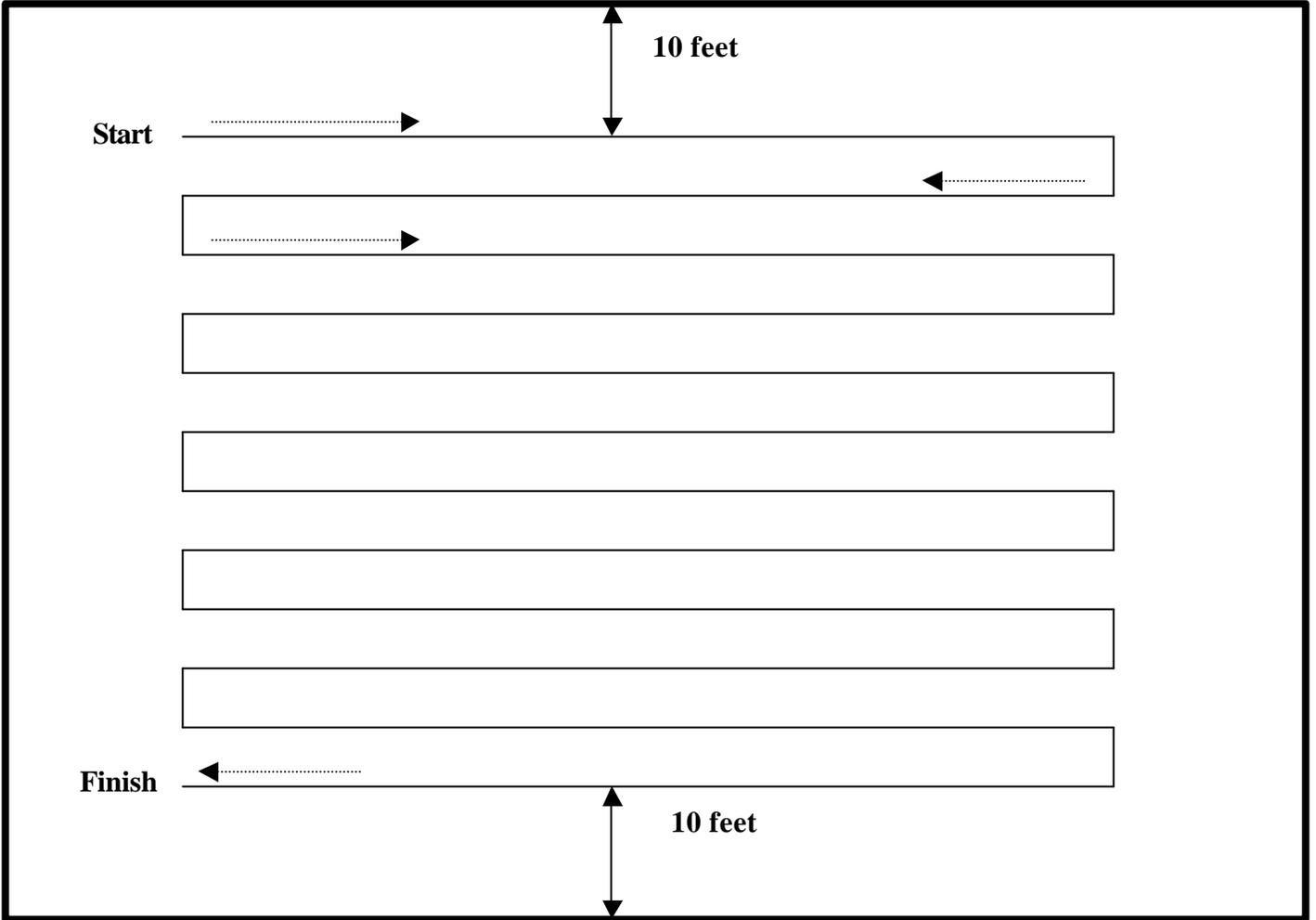


Figure 2 - Equidistant Pass Pattern

Customized field inspection pattern.

This system allocates appropriate numbers of plants to be inspected in the various environments in a field. An example is shown in Figure 3.

Disease Diagnosis in the Field

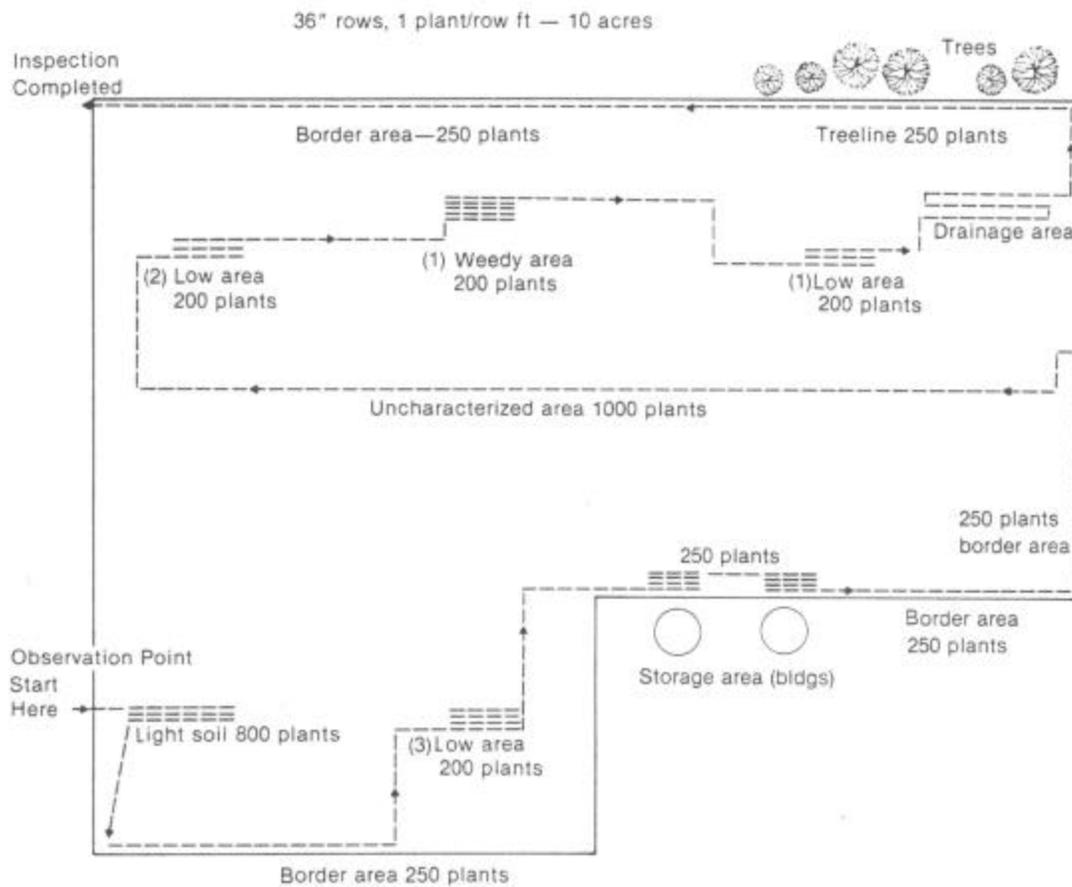
1. The presence or absence of diseases relevant to the inspection requirements is first determined by visual examination of plants in the field. Descriptions of signs and symptoms are provided in this manual for the individual diseases of the major seed crops. Other established aids to identification may also be used.
2. Inspections have to be made at crop growth stage when signs or symptoms of a disease are evident. Appropriate inspection times for particular pests or diseases are indicated in this manual.
3. An appropriate number of plant samples, representative of diseases in the field, should be taken for laboratory confirmation of the visual diagnosis. More extensive sampling should be carried out when visual symptoms are insufficient to ensure accurate diagnosis. Samples of suspected disease tissue should be kept flat in paper envelopes or towels in a plastic bag in ice chest. All samples should be correctly labeled to indicate date, time, locations, crop, and plant part.

Disease Diagnosis in the Laboratory

1. Samples should be processed systematically in a laboratory facility with demonstrated proficiency in diagnosing plant diseases.
2. Information on diagnostic tests for particular diseases is provided in this manual.

Reports

Inspection reports should be made on a standard form that is indicated below.



This is an example of how a field inspection map might look. Notice that the inspector has shown the field shape, areas inspected, number of plants inspected in each area, code for each special area (example, low areas 1, 2, 3) and inspection pattern.

Figure 3 - Example of field inspection by customized pattern

PHYTOSANITARY GROWING SEASON INSPECTION REPORT

Crop _____

Accredited Entity

PLEASE PRINT

Company Name _____

Variety _____

Company Contact Official _____

Field # _____ Acres _____

Address _____

TYPE OF FIELD:
 Increase/Production

Telephone Number _____

Contract Grower _____ Phone _____

County _____

Growth Stage & Date: _____

1st Insp.

2nd Insp.

3rd Insp.

INSPECTION DATA

(Refer to list of plant diseases/pests on separate pages)

Code	Severity (Optional) Low, Moderate, High L, M, H	Lab Sample Submitted		Lab Confirmation Field Diagnosis		Additional Sample Number	Lab
		Yes	No	Confirmed Yes/No	Pathogens Identified Code		
_____	_____	[]	[]	[]	[]	_____	
_____	_____	[]	[]	[]	[]	_____	
_____	_____	[]	[]	[]	[]	_____	
_____	_____	[]	[]	[]	[]	_____	
_____	_____	[]	[]	[]	[]	_____	
_____	_____	[]	[]	[]	[]	_____	
_____	_____	[]	[]	[]	[]	_____	

No Other Diseases Noted

I inspected these fields during active growth and determined the above diseases/pests were found as indicated.

Remarks

INSPECTOR _____ ID _____
 NUMBER: _____ DATE: _____

LAB MANAGER_(If sample submitted) _____ ID _____
 NUMBER: _____ DATE: _____

DIAGNOSTIC AIDS

Pantoea stewartii (syn. *Erwinia stewartii*)

Time of inspection:

Corn plants in early flowering stage

Field symptoms:

Leaf: Leaves show linear, pale-green to yellow streaks with irregular or wavy margins that run parallel to veins and may extend the length of the leaf (Figure 1). These streaks soon become dry and brown. Masses of bacteria may also stream from the cut edges of infected leaf tissue. When diseased tissue was placed near the edge of a water drop, the drop quickly became cloudy.

Stalk: Conducting vessels become plugged with bright-yellow slime. If infected stems are cut in cross section, the yellow slime will often exude. Cavities form in the stalk near the soil line in severely infected plants.

Whole plant: Plants infected early in the season may show wilting and stunting (Figure 2).

Indicators of disease presence:

The corn flea beetle, *Chaetocnema pulicaria*, is generally recognized as the most important carrier of inoculum for *E. stewartii* in the USA (Pepper, 1967). The pathogen overwinters in the alimentary tract of this insect, which emerges from hibernation and feeds on young maize. The occurrence of substantial numbers of the corn flea beetle at the beginning of the growing season (Figure 3) and subsequent feeding scars of this insect on leaves of the growing corn plant are strong indicators of infection by *E. stewartii*.

In the US Corn Belt, survival of the corn flea beetle is greatly reduced by low winter temperatures. Severity of Stewart's wilt can be forecast on the basis of the average temperatures during December, January, and February.

Laboratory diagnosis:

Leaf sections with suspect Stewart's wilt lesions may be cut across veins and observed under the microscope for bacterial streaming from the vascular tissue.

Suspect colonies of *E. stewartii* on culture medium can be tested for pathogenicity on susceptible seedlings.

The presence of the *E. stewartii* in plant tissue can also be detected with an ELISA test kit (Agdia, Elkhart, Indiana). This kit is adapted from the method of Lamka et al, 1991). The method, described in Method 2.1 in Section 1 of this manual for detection of seedborne *E. stewartii*, can also be used to detect the pathogen in leaf or stalk tissue and in insects.

References:

Lamka GL, Hill JH, McGee DC, and Braun, EJ. 1991. Development of an immunosorbent assay for seed-borne *Erwinia stewartii*. *Phytopathology* 81:839-846.

Pepper EH, 1967. Stewart's Bacterial Wilt of Corn. Monogr. 4. St. Paul, Minnesota, USA: American Phytopathological Society.

Alternaria dauci

Time of inspection:

Buds on carrot plants are just beginning to flower and tops still green

Field symptoms:

Leaf: Lesions produced on leaf and petiole tissues are generally dark-brown to black, and chlorosis of surrounding tissues is observed. Gradually, the spots increase in size and become confluent. Finally, the whole leaf becomes grayish-black, while the leaflets become curly and convolute. The older leaves are more heavily infected than the young ones (Figure 2).

Stalk: The stem bark is discolored.

Root: Root lesions are irregular in shape, dark-brown to black. The decay is dark-brown to black, firm and shallow.

Floral structures: *Alternaria dauci* causes dark, longitudinal spots on flower-stalks and umbels, and attacks flowers and immature seeds, causing them to be discolored (Figures 3 & 4).

Whole plant:

When infection is severe, the top part of the may be killed (Figure 1).

Indicators of disease presence:

The pathogen requires the presence of moisture for infection. Heavy dews and rains are favorable for this process. The optimum temperature for infection is 82°F.

Laboratory diagnosis:

For lesions on plant parts, incubation in a moist chamber on blotters at 20-25°C or on agar media will lead to the production of conidiophores and conidia for final identification (Figures 5 & 6). The methods of isolation of *A. dauci* from diseased carrot leaves, seedlings and seeds and the ways to achieve abundant sporulation are described in detail by Strandberg (1987).

References:

Strandberg JO. 1987. Isolation, storage, and, inoculum production methods for *Alternaria dauci*. *Phytopathology*. 77(7):1008-1012.