OREGON SEED CERTIFICATION SERVICE

PROTOCOL

FOR

BACTERIAL RING ROT

INDEXING AND CONFIRMATION OF DIAGNOSIS

JANUARY 27, 1998

(modified 3-15-12 with input from Sherry Laug, ICIA)

Bacterial Ring Rot of Potatoes
Oregon Seed Certification Service
Protocol for Indexing and Confirmation of Diagnosis [a]

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Bacterial ring rot has been managed quite successfully by the employment of the disease management principle of exclusion. The pathogen, *Clavibacter michiganensis* subsp. *sepedonicus,* is excluded from certified seed lots by use of the 'zero tolerance' regulation. For exclusion to be successful, seed must first be free of the pathogen. This has been done using a variety of seed production techniques. Most recently, pathogen-free seed stock has been developed using *in vitro* tissue culture techniques. Extensive testing for the pathogen (indexing) is performed on "mother plants" at entry level and annually after meeting entry level testing. Greenhouse plants and mini-tubers are randomly tested to ensure that the material is free of the pathogen. A field inspection is conducted to visually detect bacterial ring rot symptoms. A plant visually suspected of being infected with *C.m. sepedonicus* is **confirmed** in the laboratory using a variety of tests.

Testing for the presence of *C.m. sepedonicus* can involve any or all of the following tests: chemical/colorimetric test (Gram stain), inoculation to an indicator plant (eggplant), serological tests (IFAS, ELISA, LAT), or standard culturing techniques (Richardsons's medium). These assays (Table 1) can be used for detecting the pathogen in symptomless plants (*in vitro* mother plants, greenhouse-grown plants and mini-tubers), and in field grown plants with typical symptoms or with latent infections.

An element of any diagnostic test is the criterion established by the clinician for determining a positive or a negative test result. The purpose of this report is to describe discrete positive/negative thresholds for *C.m sepedonicus* in 1) *in vitro* mother plants, 2) greenhouse plants and/or mini-tubers, and 3) field grown plants and/or tubers.

ENTRY LEVEL (Plant materials(s) from sources outside the production lab)

I. ***In vitro* mother plants:**

1. Symptoms of bacterial ring rot have not been reported in tissue culture plants. If *C.m. sepedonicus* is present in this material, population size of the pathogen would be very small.

2. **Protocol**

   a. Rationale: *C.m. sepedonicus* is a systemic pathogen; it is not known to survive meristem tip procedures because the vascular tissue is not developed in this region. Furthermore, the tissues of the meristem divide faster than the pathogen. These two factors account for, in part, the success of producing pathogen-free stock.
   b. All plantlets will be generated from meristem tip culture.

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[a] Accepted by Oregon Potato Certification and Foundation Seed and Material Advisory Committee January 28, 1997/Amended January 27, 1998 by same committee.

[1] Minor technical modifications 3-15-12 based on input from Sherry Laug, ICIA Lab Manager – Jeff McMorran)

c. Basal portion of plantlets will be indexed annually for cells of *C.m. sepedonicus* by both IFAS and ELISA.
d. Gram stain will no longer be required.

e. Positive threshold: IFAS: no fluorescent cells are allowable in 20 fields of 1000x. ELISA: DeBoer's threshold calculation from EPPO Bull. 26:391-398, (note: this *replaces the previously used "4x the standard deviation of the healthy control", see pg. 5*). Healthy tissue of the same cultivar will serve as the control. If either test is positive, the material is discarded.

II. **Established* *in vitro* mother plants and/or Pre-Nuclear material used for establishing a new crop of PN plants/minitubers.**

a. All plants will be generated from meristem tip culture, or nodal tip cuttings.
b. Basal portions of plantlets will be indexed annually for cells of *C.m. sepedonicus* by ELISA.
c. Positive threshold: ELISA threshold values 4 times the standard deviation of the healthy control. Healthy tissue of the same cultivar will serve as a control. All seed stocks testing positive will be discarded.

* "Established" – is defined as those mother plants that have completed at least two cycles of testing by the Entry Level testing protocol.

III. **Greenhouse production (plants and/or mini-tubers)**

1. Symptoms of bacterial ring rot in early generation seed potatoes increased under greenhouse conditions have not been reported; thus, population size of the pathogen in this material will be small.

2. **Protocol**

a. A total of 1% of mini-tubers and/or plants indexed for *C.m. sepedonicus* by **either** IFAS **and/or** ELISA.
b. Gram stain will no longer be required.
c. Positive threshold: IFAS: average of $\geq$1 fluorescent cell of correct size and morphology per field in 20 fields at 1000x. ELISA: threshold values 4 times the standard deviation of the healthy control. Healthy tissue of the same cultivar will serve as the control. If either test is confirmed positive, the material is discarded.

IV. **Field grown seed potatoes**

1. Development of classic disease symptoms is associated with an increase in the population of the ring rot pathogen in the host. A single bacterial cell can infect a plant, but for disease symptoms to develop, the bacterium must multiply in the host. When typical symptoms of bacterial ring rot are expressed, the number of *C.m. sepedonicus* cells in the vascular tissue is very large.

2. **Protocol**

   a. Cultivar with classic symptoms of bacterial ring rot (See attachment) will be tested for presence of the pathogen by Gram stain, IFAS, and Latex agglutination or similar test using "Express" (Neogen) that uses Staph aureus cells, rather than latex beads (see pg. 5).

   b. Positive threshold; Gram stain: ≥50 gram positive bacteria of correct size and morphology per field in 20 fields at 1000x. IFAS: average of ≥50 fluorescent cells of correct size and morphology per field in 20 fields at 1000x. LAT: flocculation in 3 sub-samples from a single stem. For serological tests, healthy tissue of the same cultivar will serve as the control. If two of the three tests are positive, the sample will be scored as positive for bacterial ring rot.

TABLE 1. Description of Diagnostic Tests and Their Sensitivity

Gram stain: nonspecific test; cell morphology (size and shape) and bending pattern of dividing cells. With symptomatic plants, there is a high number of uniform Gram positive cells.

Immunological tests:

Latex Agglutination Test (LAT)[4]
latex beads - polyclonal antibody
15 minutes
flocculation
sensitivity: 10^6 - 10^7 cells/g tissue

Indirect Immunofluorescence Antibody Staining (IFAS) [2, 3]
Fluorescent dye – monoclonal antibody (9A1)*
4 hour
fluorescence
sensitivity: 10^4 - 10^5 cells/g of tissue

Enzyme Linked Immunosorbant Assay (ELISA)[2]
Alkaline phosphatase – monoclonal antibody (1H3)*
24 hour
yellow color
sensitivity: 10^5 - 10^6 cells/g of tissue

*The monoclonal antibody 9A1 in IFAS detects both mucoid and nonmucoid strains. The monoclonal antibody in ELISA detects mucoid strains only.

References

See also: NAPPO-RSPM 3 Annex 7 - Detection and Identification of Clavibacter michiganensis subsp. sepedonicus (attached)

1. DeBoer, S.H. Wieczorek, A., and Kummer, A. 1988. An ELISA Test For Bacterial Ring Rot of Potato With a New Monoclonal Antibody. Plant Disease 72:874-878.

2. DeBoer, S.H. 1990. Immunofluorescence for Bacteria. Pages 295-298 in: Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens. R. Hampton, E. Ball, and S.H. DeBoer, eds. APS Press, St. Paul, MN.

3. Gudmestad, N.C., Baer, D., and Kurowski, C.J. 1991. Validating Immunoassay Test Performance in the Detection of *Corynebacterium sepedonicum* During the Growing Season. Phytopathology 81:475-480.

4. Slack, S.A., Sanford, H.A., and Manzer, F.E. 1979. The Latex Agglutination Test as a Rapid Serological Assay for *Corynebacterium sepedonicum.* American Potato Journal 56:441-446.

5. Slack, S.A., Westra, A.A.G. Foliar Symptoms of Bacterial Ring Rot of Potatoes. Special Publication 1996.

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Note from Sherry Laug, Idaho Crop Improvement Association Lab, 3-15-12

Hi Jeff,

Always good to do the 'housekeeping'! I looked over the protocol. There are a couple of changes, but most things are the same. One change is that we no longer use Latex agglutination. We ran out of the antiserum that we used to coat the beads. Instead I am using a product called "Express" that I purchase from Neogen. It is still an serological agglutination test, but it uses Staph aureus cells, rather than latex beads. Its sensitivity is similar to the latex test (10^6 to 10^7 cells/ml of sample.) The positive threshold for this test would be the same as the Latex test. Of course this test is only applicable to field grown seed potatoes with classic symptoms.

The only other change that I found was the ELISA threshold calculation. I use DeBoer's threshold calculation from EPPO Bull. 26:391-398, instead of 4x the standard deviation of the healthy control. (I have the reference if you need it.)

Let me know if you have any questions. Hope all is well in Oregon. We are finishing up our winter test and enjoying some spring like weather!

Sherry
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This annex was adopted by the NAPPO Executive Committee on October 17, 2011.
The annex is a prescriptive part of the standard.

Annex 7: Detection and Identification of *Clavibacter michiganensis* subsp. *sepedonicus*

1. Introduction

To mitigate dissemination of *Clavibacter michiganensis* subsp. *sepedonicus* (Cms), the casual agent of bacterial ring rot of potato through latently infected certified seed potato tubers, laboratory tests are available to detect and confirm identity of the bacterium (De Boer et al., 2005). This annex outlines methods agreed upon by NAPPO member countries for the testing of seed potato lots and individual tubers. This annex does not address field inspection or testing of plants from the field, which are fundamental components of seed potato certification and bacterial ring rot control. In the context of this annex, indexing refers to the process of screening a seed potato lot for Cms, confirmation refers to the test requirements to substantiate a positive result in an index test, and verification refers to additional testing to further corroborate a positive test.

2. Sample Collection and Sample Size

For post harvest testing, the sample should be a minimum of 400 tubers randomly collected from a seed lot at harvest or from storage. This sample size, however, only provides a 0.9975 probability of detecting a 1.5% incidence of Cms infected tubers in a given population.

- The probability of detecting Cms in a seed lot is limited by sample size, pathogen incidence, and diagnostic methodology.
- Only persons officially designated by the exporting country's national plant protection organization (NPPO) may collect samples.
- Samples must be identified in a manner that enables trace-back to the specific seed lot from which they were collected.
- Samples must be protected during collection, transport, and storage from conditions that might interfere with the detection of Cms or sample integrity. **3. DiagnosticMethodologies** The methodologies used for indexing, confirmation and verification must be agreed upon in principle by the importing and exporting country's NPPO and should adhere to the following guidelines:
- Tests must be conducted according to standard protocols agreed upon by the NPPO.
- Tests must be done under the auspices of a qualified plant pathologist or within a quality assurance system approved by the importing and exporting country's NPPO.
- A positive diagnosis for Cms must be based on positive results from at least two diagnostic methodologies.
- The recommended scheme for indexing seed potato lots for the presence of Cms is shown in Figure 7.1.
- Positive and negative control samples must be run along with all test samples.

3.1 Enzyme-linked immunoassay (ELISA) ELISA should be the initial index test methodology. ELISA has a high degree of sensitivity for Cms, is rapid and well suited to testing large numbers of samples because it can be applied directly to the sample extract.

- A triple antibody ELISA procedure with commercially available antibodies should be used. The specific monoclonal antibody 1H3, or equivalent, is recommended and commercially available.

- The positive and negative threshold values should be based on absorbance of positive and negative samples included on each plate (De Boer et al., 1996). 3.2 Indirectimmunofluorescence(IMF) IMF is recommended as a test methodology for confirmation of a positive ELISA index test.

- Monoclonal antibody 9A1, or equivalent, is recommended for this methodology and commercially available.

- Consistent detection of five or more typical fluorescing coryneform cells per microscope field at 1000X is considered positive for Cms.

. 3.3 Polymerase chain reaction (PCR) PCR offers the highest degree of sensitivity and specificity for Cms and should, therefore, be retained as a confirmation methodology for a positive ELISA index test.

- Specific primers and probe that are useful for conventional and real time PCR are given in De Boer et al. (2005); efficacy data must be available for other primers and probes used for detecting Cms.

- Negative controls must be clearly negative to ensure that no cross- contamination occured, a particular risk with PCR technologies.

- Conventional PCR amplicons from positive samples must be characterized by hybridization, restriction analysis, or DNA sequencing.

- Melt temperature of real time PCR amplicons from positive samples must agree with the melt temperature of amplicons from positive control samples.

. 3.4 Bioassay A biological assay for Cms to verify a positive confirmation test is considered optional or necessary only if conflicting previous test results have occurred. Eggplant (Solanum *melongena)* cv. Black Beauty is the recommended host for bioassay but the assay may require up to 40 days to complete, making the test too protracted for most certification and trade-related applications.

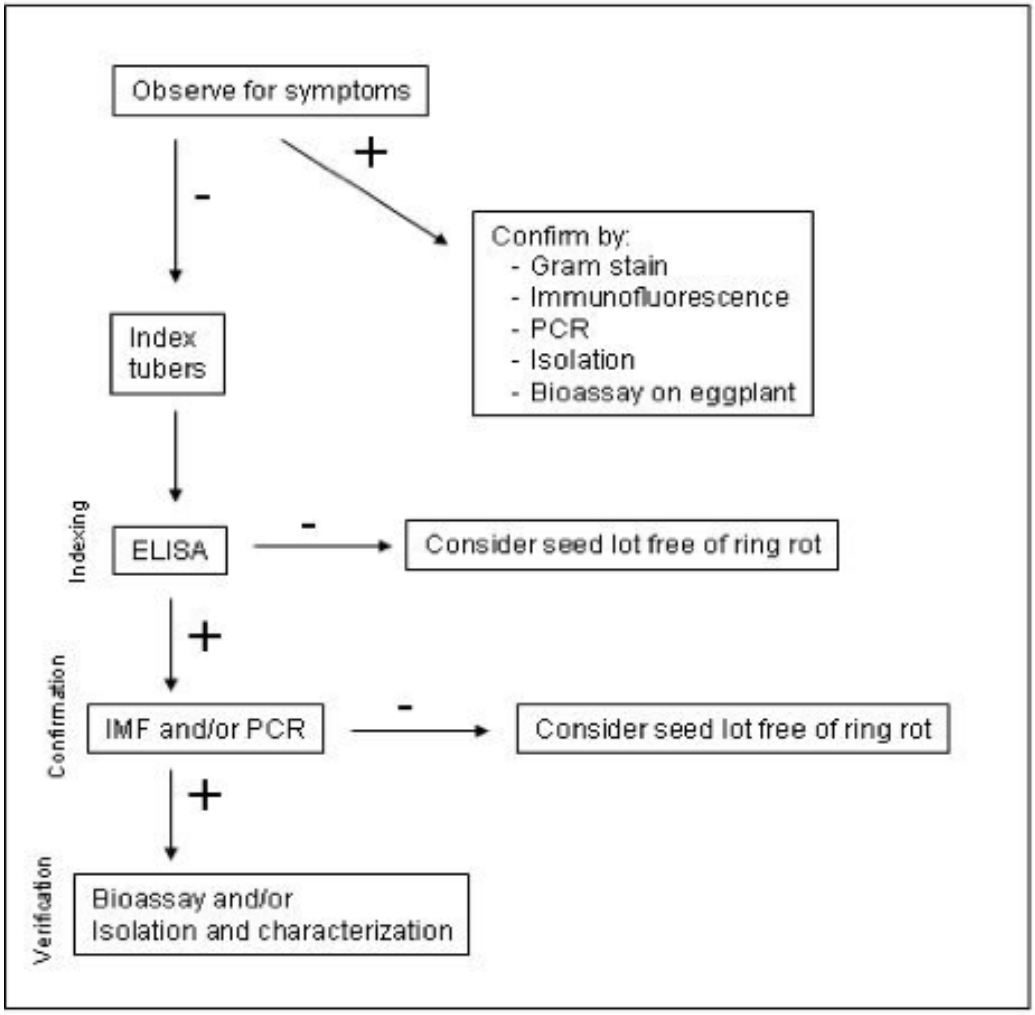
3.5 Isolation and characterization Ultimate verification of Cms, subsequent to other positive diagnostic methodologies, can be achieved by isolation and characterization of the bacterium. Obtaining a pure culture of Cms for characterization is, however, problematic, time consuming and typically requires inoculation to eggplant to selectively increase the Cms population prior to isolation on a nutrient medium. Verification of Cms to this degree is not routine for certification or trade-related applications, but rather utilized for research and/or archiving of isolates.

References

De Boer, S.H., A. Boucher and T.L. DeHaan.1996. Validation of thresholds for serological tests that detect *Clavibacter michiganensis* subsp. *sepedonicus* in potato tuber tissue. Bull. OEPP/EPPO Bull. 26:391-398.

De Boer, S.H., A. O. Charkowski, R. T. Zink, J. P. Martinez-Soriano, and A. Flores-Olivas. 2005. Procedure for detection and identification of *Clavibacter michiganensis* subsp. sepedonicus (Spiekermann and Kotthoff) Davis, Gillespie, Vidaver and Harris in potato (Solanum *tuberosum* L.) tubers. Revista Mexicana de Fitopatologia 23:329-334.

Figure 7.1: Scheme for indexing seed potato lots for the presence of *Clavibacter michiganensis* subsp. *sepedonicus,* causal agent of the bacterial ring rot disease



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