Council Directive 93/85/EEC of 4 October 1993 on the control of potato ring rot

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ANNEX I

METHOD FOR THE DETECTION AND DIAGNOSIS OF THE RING ROT BACTERIUM, CLAVIBACTER MICHIGANENSIS (Smith) Davis et al. ssp. SEPEDONICUS (Spieckermann et Kotthof) Davis et al. IN BATCHES OF POTATO TUBERS

1. Removal of heel-end cores
   - 1.1. Wash 200 tubers in running tap water and remove the...
   - 1.2. Carefully remove conical tissue cores from the heel ends with...

2. Visual examination for ring rot symptoms

3. Preparation of samples for Gram staining, immunofluorescence staining (IF) and...
   - 3.1. Homogenize the heel ends until complete maceration has just been...
   - 3.2. Extract bacteria from the homogenate by one of the methods...
   - 3.3. Suspend the pellet in sterile 0,01 M phosphate buffer pH...
   - 3.4. It is imperative that all positive C. sepedonicum controls and...

4. Gram staining
   - 4.1. Prepare Gram stains for all pellet dilutions (5.2.1) and for...
   - 4.2. Prepare Gram stains for known C. sepedonicum cultures and, if...

4.3. Determine which samples contain typical Gram positive coryneform cells. In...

5. Scheme for IF-testing
   - 5.1. Use antiserum to a known strain of C. sepedonicum —...
   - 5.2. Procedure
     - 5.2.1. Prepare three serial ten fold dilutions (101, 102, 103) of...
     - 5.2.2. Pipette a measured standard volume sufficient to cover the window...
     - 5.2.4. Cover appropriate windows which C. sepedonicum antiserum at the recommended...
     - 5.2.5. Incubate in a humid chamber at ambient temperature for 30...
     - 5.2.6. Rinse carefully with 0,01 M PBS pH 7,2. Wash for...
     - 5.2.7. Carefully remove excess moisture.
     - 5.2.8. Cover each window with FITC conjugate at the same dilution...
     - 5.2.9. Rinse and wash as before.
     - 5.2.10. Apply approximately 5 to 10 µl of 0,1 M phosphate...
     - 5.2.11. Examine with a microscope fitted with an epifluorescent light source...

6. Eggplant test
   - 6.1. Distribute the pellet from 3.3 between at least 25 eggplants...
   - 6.2. Slit inoculation I
     - 6.2.1. Support each pot horizontally (a block of expanded polystyrene with...
     - 6.2.2. Using a scalpel, make a longitudinal or slightly diagonal cut...
     - 6.2.3. Hold the slit open with the scalpel blade point and...
     - 6.2.4. Seal the cut with sterile vaseline from a 2 ml...
   - 6.3. Slit inoculation II
     - 6.3.1. Holding the plant between two fingers, pipette a drop (approximately...
     - 6.3.2. Using a sterile scalpel, make a diagonal (at an angle...
     - 6.3.3. Seal the cut with sterile vaseline from a syringe barrel....
   - 6.4. Syringe inoculation
     - 6.4.1. Do not water eggplants for one day prior to inoculation...
     - 6.4.2. Inoculate the eggplant stems just above the cotyledons using a...
   - 6.5. Inoculate 25 plants with a known C. sepedonicum culture and,...
   - 6.6. Inoculate 25 plants with sterile 0,05 M PBS by the...
   - 6.7. Incubate the plants in appropriate conditions (Appendix 5) for 40...
   - 6.8. Prepare a Gram stain (4) for all batches of eggplants...
   - 6.9. Under certain circumstances, in particular where growing conditions are not...

7. Isolation of C. sepedonicum
   - 7.1. Streak suspensions on to one of the following media: (formulae...

Identification

Appendix 1

FORMULATION OF MACERATING FLUID RECOMMENDED BY LELLIOTT AND SELLAR, 1976

Appendix 2

BUFFERS

0,05 M phosphate buffered saline pH 7,0

0,01 M phosphate buffered saline pH 7,2

0,1 M phosphate buffered glycerine pH 7,6

Appendix 3

GRAM STAIN PROCEDURE (HUCKER'S MODIFICATION) (DOETSCH, 1981)

Crystal violet solution

Lugol's iodine

Safranin counterstain solution

Staining procedure

1. Prepare smears, air dry and heat fix.
2. Flood slide with crystal violet solution for one minute.
3. Wash briefly with tap water.
4. Flood with Lugol's iodine for one minute.
5. Wash with tap water and blot dry.
6. Decolourize with 95 % ethanol, added dropwise, until no further...
7. Wash in tap water and blot dry.
8. Flood with safranin solution for 10 seconds.
9. Wash with tap water and blot dry.

Appendix 4

DETERMINATION OF POPULATION OF IF-POSITIVE CELLS

Surface area (S) of window of multispot slide
= πD 2 4 (1)
Where D = diameter of window. Surface area(s) of objective...
= πd 2 4 (2)
where d = diameter of field.
Calculate d either by direct measurement or from the following...
s = πi 2 G 2 K 2 × 4...
from (2) d = 4s π
from (3) d = 4× πi 2 G 2 K...
Count the number of typical fluorescent cells per field (c)....
Calculate the number of typical fluorescent cells per window (C)....
C = c S s
Calculate the number of typical fluorescent cells per ml pellet...
N = C × 1000 y × F

Appendix 5

EGGPLANT CULTURE

Sow seeds of eggplant (Solarium melongena cv. Black Beauty)...
Use eggplants at leaf stage 3 when two, but not...

Eggplants should be grown in a glasshouse with the following...

Appendix 6

MEDIA FOR GROWTH AND ISOLATION OF C. SEPEDONICUM

Nutrient agar (NA)

Nutrient dextrose agar (NDA)

Yeast peptone glucose agar (YPGA)

Yeast extract mineral salts medium (YGM)

Appendix 7

NUTRITIONAL AND PHYSIOLOGICAL TESTS FOR THE IDENTIFICATION OF C. SEPEDONICUM

All media should be incubated at 21 oC and examinated...

— Oxidative and fermentative test (Hugh & Leifson), 1953) - O/F-test....

— Oxidase test (Kovacs, 1956)

— Acid production from lactose, rhamnose, salicin, glycerol

— Catalase test

— Nitrate reductase activity and denitrification (Bradbury, 1970)

— Urease production (Lelliott, 1966)
Utilization of citrate (Christensen) (Skerman, 1967)

— Hydrogen sulphide production (Ramamurthi, 1959)

— Indole production (Ramamurthi, 1959)

— Growth ar 37 oC (Ramamurthi, 1959)

— Growth in 7 % sodium chloride (Ramamurthi, 1959)

— Gelatin hydrolysis (Lelliott, Billing & Hayward, 1966)

— Starch hydrolysis

— Aesculin hydrolase activity (Sneath & Collins, 1974)

REFERENCES

ANNEX II

1. For each suspected occurrence for which a positive immunofluorescence test...

2. In the case of positive confirmation of the organism, there...

ANNEX III

1. The elements to be considered in the determination of the...

2. The elements to be considered in the determination of the...

3. The details of the notification referred to in the first...

ANNEX IV

1. The officially supervised measures referred to in Article 7 (1)...

2. The appropriate use or disposal of tubers or plants determined...

3. The appropriate methods for cleansing and disinfecting of the objects...

4. The series of measures to be implemented by Member States...

(**1**) OJ No C 93, 2. 4. 1993, p. 12.

(**2**) OJ No C 176, 28. 6. 1993, p. 210.

(**3**) OJ No C 161, 14. 6. 1993, p. 18.

(**4**) OJ No L 26, 31. 1. 1977, p. 20. Directive as last amended by Commission Directive 92/103/EEC (OJ No L 363, 11. 12. 1992, p. 1).

(**5**) OJ No L 180, 14. 7. 1980, p. 30.