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

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 epidermis around the heel end of each tuber using a regularly disinfected scalpel or potato peeler; disinfection may be achieved by dipping the peeler in 70 % ethanol and flaming.

1.2. Carefully remove conical tissue cores from the heel ends with a knife or potato peeler. Keep the excess non-vascular tissue to a minimum. Once removed, heel ends should be processed within 24 hours (see paragraph 3) or conserved at -20°C for no longer than two weeks.

2. Visual examination for ring rot symptoms

After removal of heel ends, cut each tuber transversely and observe for the presence of ring rot symptoms.

Squeeze the tubers and look for expression of macerated tissues from the vascular tissue.

The earliest symptoms are a slight glassiness or translucence of the tissue without softening round the vascular system, particularly near the heel end. The vascular ring at the heel end may be slightly darker in colour than normal. The first readily identifiable symptom is one whereby the vascular ring has a yellowish coloration and when the tuber is gently squeezed, pillars of cheese-like material emerge from the vessels. This exudation contains millions of bacteria. Browning of the vascular tissue may develop at this stage. At first, these symptoms may be restricted to one part of the ring, not necessarily close to the heel end and may gradually extend to the whole ring. As the infection progresses, destruction of the vascular tissue occurs; the outer cortex may become separated from the inner cortex. In advanced stages of infection, cracks appear on the surface of the tuber, which are often reddish-brown at their margins. Secondary fungal or bacterial invasion may mask the symptoms and it may be difficult, if not impossible, to distinguish advanced ring rot symptoms from other tuber rots.

3. Preparation of samples for Gram staining, immunofluorescence staining (IF) and eggplant test

3.1. Homogenize the heel ends until complete maceration has just been achieved in a diluent known to be non-toxic to *Corynebacterium sepedonicum* (for example, 0,05 M phosphate buffered saline (PBS) pH 7,0) at a temperature of less than 30°C; the addition of a non-toxic deflocculant is advisable and non-toxic antifoam agent may be needed (Appendices 1 and 2). Excessive maceration should be avoided.

3.2. Extract bacteria from the homogenate by one of the methods as follows[(1)]:

A. (a) Centrifuge at not more than 180 g for 10 minutes.

(b) Centrifuge supernatant at not less than 4 000 g for 10 minutes. Decant and discard the supernatant.

B. (a) Allow the macerate to stand for 30 minutes for the tissue debris to settle. Decant the supernatant.without disturbing the sediment.

(b) Filter the supernatant through filter paper (Whatman No 1) held in a sintered glass filter (No 2 = 40-100|J.m) using a water vacuum pump. Collect the filtrate in a centrifuge tube. Wash the filter with sterile PBS to a maximum filtrate volume of 35 ml.

(c) Centrifuge filtrate at not less than 4 000 g for 20 minutes.

3.3. Suspend the pellet in sterile 0,01 M phosphate buffer pH 7,2 (Appendix 2) to give a total volume of approximately 1 ml. Divide in two equal parts and retain one part for reference purposes by freezing at - 20 °C[(2)] or by lyophilization. Divide the other part into halves using one half for the IF test and Gram stain and the other for the eggplant test.

3.4. It is imperative that all positive *C. sepedonicum* controls and samples are treated separately to avoid contamination. This applies to IF slides and to eggplant tests.

4. *Gram staining*

4.1. Prepare Gram stains for all pellet dilutions (5.2.1) and for any cut tubers (2) which show glassiness, rotting or other suspect symptoms. Samples should be taken from the edge of diseased tissues.

4.2. Prepare Gram stains for known *C. sepedonicum* cultures and, if possible, for naturally infected tissue (5.1).

4.3. Determine which samples contain typical Gram positive coryneform cells. In general, *C. sepedonicum* cells are 0,8 to l,2µm long and 0,4 to 0,60µm wide.

An appropriate staining procedure is given in Appendix 3.

Preparations from natural infections or recently isolated cultures often show a predominance of coccoid rods which are usually slightly smaller than cells from oder agar cultures. On most culture media, *C. sepedonicum* cells are pleomorphic coryneform rods and may give a variable Gram reaction. Cells are single, in pairs with characteristic ‘elbows’ typical of bending division, and occasionally in irregular groups often referred to as palisades and Chinese letters.

5. **Scheme for IF-testing**

5.1. Use antiserum to a known strain of *C. sepedonicum* — ATCC 33113 (NCPPB 2137), or NCPPB 2140. This should have an IF titre of more than 1:600. Include one PBS control on the test slide to determine whether the fluorescein isothiocyanate anti-rabbit immunoglobulin conjugate (FITC) combines non-specifically with bacterial cells. *Corynebacterium sepedonicum* (ATCC 33113 (NCPPB 2137), NCPPB 2140) should be used as homologous antigen controls on a separate slide. Naturally infected tissue (maintained by lyophilization or freezing at - 20°C) should be used where possible as a similar control on the same slide (Figure 2).

5.2. *Procedure*

5.2.1. Prepare three serial ten fold dilutions (10^1, 10^2, 10^3) of the final pellet in distilled water (Figure 1).

5.2.2. Pipette a measured standard volume sufficient to cover the window (approximately 25 µl) of each pellet dilution or *C. sepedonicum* suspension (approximately 10^6 cells/ml) to windows of a multispot slide as shown in Figure 1.

Figure 1

Sample and PBS control slide

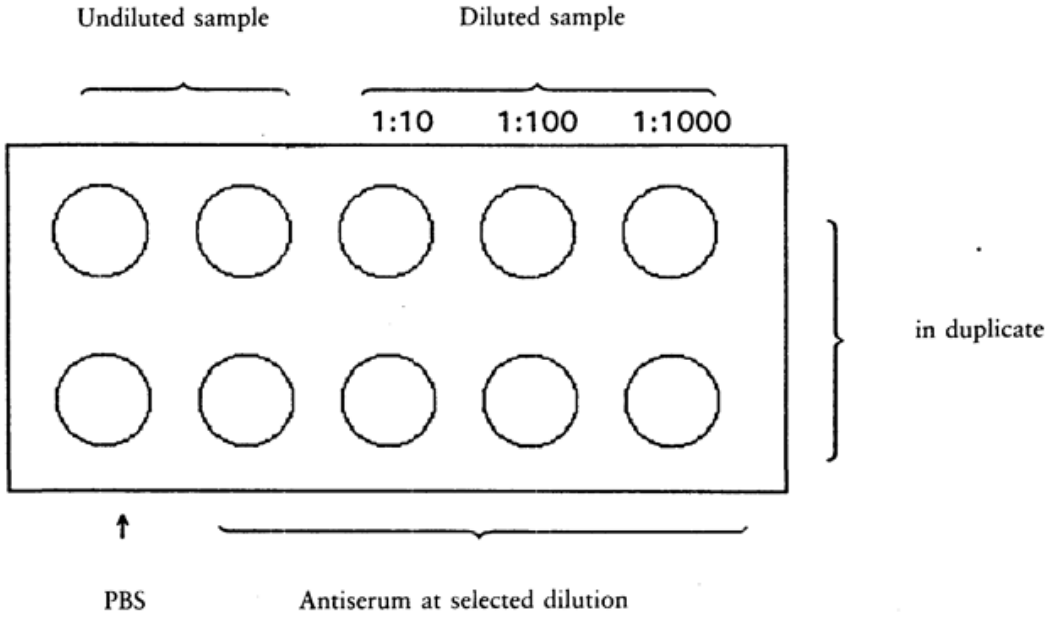
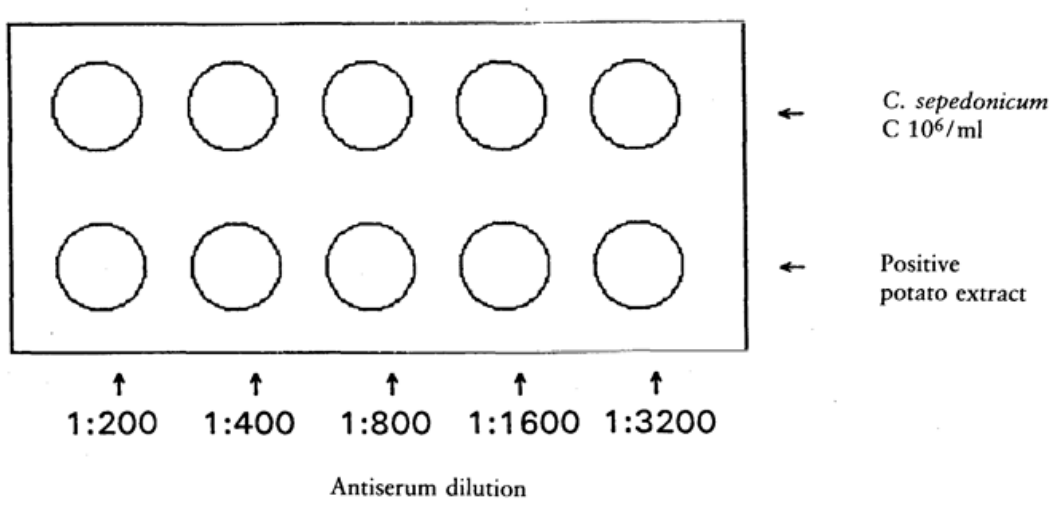


Figure 2

Positive control slide



5.2.4. Cover appropriate windows which *C. sepedonicum* antiserum at the recommended dilutions, 0,01 M PBS pH 7,2 (Appendix 2), as shown in Figure 1. (Use PBS for the FITC control.) The working dilution of the antiserum should be approximately half that of the IF titre. If other antiserum dilutions are to be included, separate slides should be prepared for each dilution to be used.

5.2.5. Incubate in a humid chamber at ambient temperature for 30 minutes.

5.2.6. Rinse carefully with 0,01 M PBS pH 7,2. Wash for five minutes in three changes of 0,01 M PBS pH 7,2.

5.2.7. Carefully remove excess moisture.

5.2.8. Cover each window with FITC conjugate at the same dilution used to determine the titre and incubate in a dark humid chamber at ambient temperature for 30 minutes.

5.2.9. Rinse and wash as before.

5.2.10. Apply approximately 5 to 10 µl of 0,1 M phosphate buffered glycerine pH 7,6 (or a similar mountant with a pH not less than 7,6) to each window and cover with a coverglass (Appendix 2).

5.2.11. Examine with a microscope fitted with an epifluorescent light source and filters suitable for working with FITC. A magnification of 400 to 1 000 is suitable. Scan replicated windows across two diameters at right angles and around the window perimeters.

Observe for fluorescing cells in the positive controls and determine the titre. Observe for fluorescing cells in the FITC/PBS control window and, if absent, proceed to the test windows. Determine in a minimum of 10 microscope fields the mean number of morphologically typical fluorescing cells per field and calculate the number per ml of undiluted pellet (Appendix 4).

There are several problems inherent to the immunofluorescence test.

— Background populations of fluorescing cells with atypical morphology and cross reacting saprophytic bacteria with size and morphology similar to *Clavibacter michiganensis* ssp. *sepedonicus* are likely to occur in potato pellets. Consider only fluorescing cells with typical size and morphology.

  Because of the possibility of cross-reactions, samples with a positive immunoflorescence test should be retested using a different antiserum.

— The technical limit of detection of this method is situated between 10^3 and 10^4 cells per ml of undiluted pellet. Samples with counts of IF typical cells at the detection limit are usually negative for C. *m.* ssp. *sepedonicus* but may be committed to eggplant testing.

A negative immunofluorescence test is identified for any sample where morphologically typical fluorescing cells are not found. The samples shall be considered as *'not contaminated'* with *Clavibacter michiganensis* ssp. *sepedonicus*.

The eggplant test is not required.

A positive immunofluorescence test is identified for any sample where morphologically typical fluorescing cells are found.

Samples for which a positive immunofluorescence test have been identified with both antisera shall be considered as *'potentially contaminated'* with *Clavibacter michiganensis* ssp. *sepedonicus*.

The eggplant test is required for all samples considered as potentially contaminated.

6. **Eggplant test**

For cultural details, see Appendix 5.

6.1. Distribute the pellet from 3.3 between at least 25 eggplants at leaf stage 3 (Appendix 5) by one of the methods given below (6.2, 6.3 or 6.4).

6.2. *Slit inoculation I*

6.2.1. Support each pot horizontally (a block of expanded polystyrene with a piece 5 cm deep x 10 cm wide x 15 cm long, removed from one surface (Figure 3) is adequate

for a 10 cm pot). A strip of sterile aluminium foil should be placed between the stem and the block for each sample tested. The plant may be held in place by a rubber band around the block.

6.2.2. Using a scalpel, make a longitudinal or slightly diagonal cut 0,5 to 1,0 cm long and approximately three quarters of the stem diameter deep, between the cotyledons and the first leaf.

6.2.3. Hold the slit open with the scalpel blade point and paint the inoculum into it using an eyeliner or fine artist's brush charged with the pellet. Distribute the remainder of the pellet between the eggplants.

6.2.4. Seal the cut with sterile vaseline from a 2 ml syringe barrel.

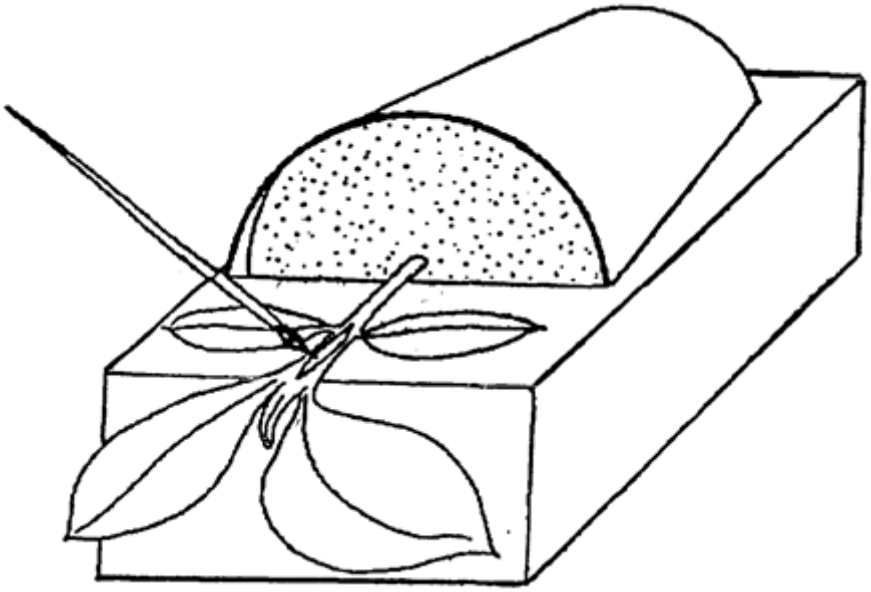


Figure 3

6.3. *Slit inoculation II*

6.3.1. Holding the plant between two fingers, pipette a drop (approximately 5 to 10 µl) of the suspended pellet on the stem between the cotyledons and the first leaf.

6.3.2. Using a sterile scalpel, make a diagonal (at an angle of approximately 5^{o}) slit, 1,0 cm long and approximately 2/3 of the stem thickness deep, starting the cut from the pellet drop.

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 to reduce turgor pressure.

6.4.2. Inoculate the eggplant stems just above the cotyledons using a syringe fitted with a hypodermic needle (not less than 23G). Distribute the pellet between the eggplants.

6.5. Inoculate 25 plants with a known *C. sepedonicum* culture and, where possible, naturally infected tuber tissue (5.1) by the same inoculation method (6.2, 6.3 or 6.4).

6.6. Inoculate 25 plants with sterile 0,05 M PBS by the same inoculation method (6.2, 6.3 or 6.4).

6.7. Incubate the plants in appropriate conditions (Appendix 5) for 40 days. Examine regularly for symptoms after eight days. Count the number of plants showing symptoms. *C. sepedonicum* causes leaf wilting in eggplants which may commence as a marginal or interveinal flaccidity. Wilted tissue may initially appear dark green or mottled but turns paler before becoming necrotic. Interveinal wilts often have a greasy water-soaked appearance. Necrotic tissue often has a bright yellow margin. Plants are not necessarily killed; the longer the period before symptoms develop, the greater the chance of survival. Plants may outgrow the infection. Susceptible young eggplants are much more sensitive to low populations of *C. sepedonicum* than are older plants, hence the necessity to use plants at or just before leaf stage 3.

Wilts may also be induced by populations of other bacteria or fungi present in the tuber tissue pellet. These include *Erwinia carotovora,* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica, Phoma exigua* var. *foveata,* as well as large populations of saprophytic bacteria. Such wilts can be distinguished from those caused by *C. sepedonicum* since whole leaves or whole plants wilt rapidly.

6.8. Prepare a Gram stain (4) for all batches of eggplants showing symptoms, using sections of wilted leaf tissue and stem tissue from plants and isolate on to suitable nutrient media (7). Surface disinfect the eggplant leaves and sterns by wiping with 70 % ethanol.

6.9. Under certain circumstances, in particular where growing conditions are not optimal, it may be possible for *C. sepedonicum* to exist as a latent infection within eggplants even after incubation for 40 days. Such infections may possibly result in stunting and lack of vigour in the inoculated plants. If the IF test is considered positive, it may be considered necessary to test further. It is, therefore, essential to compare the growth rates of all eggplant test plants with the sterile 0,05 M PBS inoculated controls and to monitor the environmental conditions of the glasshouse.

Recommendations for further testing are as follows:

6.9.1. excise the stems above the inoculation site and remove the leaves;

6.9.2. macerate the stems in 0,05 M PBS pH 7,0, as in 3.1 to 3.2;

6.9.3. use half of the pellet to perform a Gram stain (4) and an IF test (5);

6.9.4. use the other half to perform a further eggplant test (6) if the Gram stain and/or IF tests are positive. Use a known *C. sepedonicum* culture and sterile 0,05M PBS controls. If symptoms are not observed in the subsequent test, the sample must be considered negative.

7. **Isolation of***C. sepedonicum*

Diagnosis can only be confirmed if *C. sepedonicum* is isolated and so identified (8). Although *C. sepedonicum* is a fastidious organism, it can be isolated from symptomatic tissue. However, it may be outgrown by rapidly growing saprophytic bacteria and, therefore, isolations directly from the tuber tissue pellet (3.3) are not recommended. Eggplants provide an excellent selective enrichment medium for the growth of *C. sepedonicum* and also provide an excellent confirmatory host test.

Isolations should be made from all symptomatic potato tubers and eggplants (4, 6). Maceration of eggplant stems when necessary should be carried out as in 3. and 6.9.

7.1. Streak suspensions on to one of the following media: (formulae are given in Appendix 6):

nutrient dextrose agar (for subculture only),

yeast peptone glucose agar,

nutrient yeast dextrose agar,

yeast extract mineral salts agar.

Incubate at 21 ^{o}C for up to 20 days.

C. sepedonicum is slow-growing, usually producing pin-point, cream, domed colonies within 10 days.

Re-streak to establish purity.

Growth rates are improved with subculture. Typical colonies are creamy-white or ivory, rounded, smooth, raised, convex-domed, mucoid-fluidal, with entire edges and usually 1 to 3 mm in diameter.
Identification

Many Gram positive coryneform bacteria, with colonial characters similar to those of *C. sepedonicum,* may be isolated from healthy or diseased potatoes and eggplants. In this context *C. sepedonicum* must be identified by the following tests:

IF test (5.1),

eggplant test,

nutrition and physiological tests (Appendix 7),
— oxidation/fermentation test (O/F),
— oxidase test,
— growth at 37^{o}C,
— urease production,
— aesculin hydrolysis,
— starch hydrolysis,
— tolerance of 7 % sodium chloride solution,
— indole test,
— catalase test,
— $H_2$8 production,
— citrate utilization,
— gelatin hydrolysis
— acid from: glycerol, lactose, rhamnose and salicin,
— Gram stain.

All tests should include a known *C. sepedonicum* control. Nutritional and physiological tests should be made using inocula from nutrient agar subcultures. Morphological comparisons should be made from nutrient dextrose agar cultures.

For the IF test, cell populations should be adjusted to 10^6 cells/ml. The IF titre should be similar to that of the known *C. sepedonicum* culture.

For the eggplant test cell populations should be adjusted to c 10^7 cells/ml. Eggplant tests should be made using 10 plants for each of the test organisms, again using known *C. sepedonicum* culture and sterile water controls; with pure cultures typical wilting should be obtained within 20 days but plants not showing symptons after this time should be incubated for a total of 30 days at temperatures conducive to eggplant growth but not exceeding 30°C (Appendix 5). If after 30 days symptoms are not present, the culture cannot be confirmed as being a pathogenic form of *Corynebacterium sepedonicum*.

Test	*C. sepedonicum*
O/F	Inert or weakly oxidative
Oxidase	
Catalase	+
Nitrate reduction	
Urease activity	
H_2S production	
Indole production	
Citrate utilization	
Starch hydrolysis	- or weak
Growth at 37°	
Growth in 7 % NaCl	
Gelatin hydrolysis	
Aesculin hydrolysis	+
Acid from:	
— Glycerol	
— Lactose	- or weak
— Rhamnose	
— Salicin	

Appendix 1

FORMULATION OF MACERATING FLUID RECOMMENDED BY LELLIOTT AND SELLAR, 1976

D C silicone antifoam MS A compound (Hopkins & Williams Ltd, Cat. No 9964-25, Chadwell Heath, Essex, England)	10 ml
Lubrol W flakes (ICI Ltd)	0,5 g
Tetra-sodium pyrophosphate	1 g
0,05 M phosphate buffered saline pH 7,0 (Appendix 2)	1 litre

Appendix 2

BUFFERS

0,05 M phosphate buffered saline pH 7,0

This buffer can be used for tuber tissue maceration (2.1)

Na_2HPO_4	4,26 g
KH_2PO_4	2,72 g
NaCl	8,0 g
Distilled water to	1 litre

0,01 M phosphate buffered saline pH 7,2

This buffer is used for diluting antisera and washing IF slides

Na_2HPO_4 12 H_2O	2,7 g
NaH_2PO_4 2 H_2O	0,4 g
NaCl	8,0 g
Distilled water to	1 litre

0,1 M phosphate buffered glycerine pH 7,6

This buffer is used as a mountant to enhance fluorescence in the IF test

Na_2HPO_4 12 H_2O	3,2 g
NaH_2PO_4 2 H_2O	0,15 g
Glycerol	50 ml
Distilled water	100 ml

Appendix 3

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

Crystal violet solution

Dissolve 2 g crystal violet in 20 ml 95 % ethanol.

Dissolve 0,8 g ammonium oxalate in 80 ml distilled water.

Mix the two solutions.

Lugol's iodine

Iodine	1 g
Potassium iodide	2 g
Distilled water	300 ml.

Grind the solids together in a pestle and mortar. Add to the water and stir to dissolve in a closed container.

Safranin counterstain solution

Stock solution:

Safranin O	2,5 g
95 % ethanol	100 ml.

Mix and store.

Dilute: 1:10 to obtain a working 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 colour is removed or immerse with gentle agitation for 30 seconds.
7. Wash in tap water and blot dry.
8. Flood with safranin solution for 10 seconds.
9. Wash with tap water and blot dry.

Gram positive bacteria stain violet-blue; Gram negative bacteria stain pink-red.

Appendix 4

DETERMINATION OF POPULATION OF IF-POSITIVE CELLS

Surface area (S) of window of multispot slide

$$= \frac{\pi D^2}{4} (1)$$

Where D = diameter of window. Surface area(s) of objective field

$$= \frac{\pi d^2}{4} (2)$$

where d = diameter of field.

Calculate d either by direct measurement or from the following formule:

$$s = \frac{\pi i^2}{G^2 K^2 \times 4} (3)$$

where	i	= field coefficient (depends upon ocular type and varies from 8 to 24),
	K	= tube coefficient (1 or 1,25),
	G	= magnification of 100 ×, 40 × etc) objective,

from (2)

$$d = \sqrt{\frac{4s}{\pi}}$$

from (3)

$$d = \sqrt{\frac{4 \times \frac{\pi i^2}{G^2 K^2 \times 4}}{\pi}} = \frac{i}{GK}$$

(4)

Count the number of typical fluorescent cells per field (c).

Calculate the number of typical fluorescent cells per window (C).

$$C = \frac{c\,S}{s}$$

Calculate the number of typical fluorescent cells per ml pellet (N)

$$N = C \times \frac{1000}{y} \times F$$

where	y	= volume of pellet on window,
where	F	= pellet dilution factor.

Appendix 5

EGGPLANT CULTURE

Sow seeds of eggplant (*Solarium melongena* cv. Black Beauty) in pasteurized seed compost. Transplant seedlings with fully expanded cotyledons (10 to 14 days) into pasteurized potting compost.

Use eggplants at leaf stage 3 when two, but not more than three, leaves are fully uncurled.

Eggplants should be grown in a glasshouse with the following environmental conditions:

day length:	14 hours or natural day length if greater;
temperature: day:	21 to 24°C,
night:	15°C.

NB: C. sepedonicum will not grow at temperatures >30°C. If night temperatures do not fall to 15°C, chromophore damage (silvery necrosis) may occur.

Root damage caused by sciarid larvae can be overcome by the application of an appropriate insecticide.

Eggplant cv. Black Beauty may be obtained from:

1. AB Hammenhögs Frö,
   270 50 Hammenhög,
   Sweden;
2. HURST Seeds Ltd,
   Avenue Road,
   Witham,
   Essex CM8 2DX,
   England;
3. ASGRO Italia Sp A,
   Corso Lodi, 23,
   Milan;
4. KÜPPER
   Mitteldeutsche Samen GmbH,
   Hessenring 22,
   D-37269 Eschwege

Appendix 6

MEDIA FOR GROWTH AND ISOLATION OF *C. SEPEDONICUM*

Nutrient agar (NA)

Difco bacto nutrient agar in distilled water at manufacturer's rate. Sterilize by autoclaving at 121 ^{o}C for 15 minutes.

Nutrient dextrose agar (NDA)

Difco bacto nutrient agar containing 1 % D(+) glucose (monohydrate). Sterilize by autoclaving at 115 ^{o}C for 20 minutes.

Yeast peptone glucose agar (YPGA)

Difco bacto yeast extract (No 0127)	5 g
Difco bacto peptone (No 0118)	5 g
D(+)-glucose (monohydrate)	10 g
Difco bacto purified agar (No 0560)	15 g
Distilled water	1 litre

Sterilize 0,5 litre volumes of medium by autoclaving at 115 ^{o}C for 20 minutes.

Yeast extract mineral salts medium (YGM)

Difco bacto yeast extract	2,0 g
D(+)-glucose (monohydrate)	2,5 g
K_2HPO_4	0,25 g
KH_2PO_4	0,25 g
$MgSO_4$. $7H_2O$	0,1 g
$MnSO_4$. H_2O	0,015 g
NaCl	0,05 g
$FeSO_4$. $7H_2O$	0,005 g
Difco bacto purified agar	18 g
Distilled water	1 litre

Sterilize 0,5 litre volumes of medium by autoclaving at 115 ^{o}C for 20 minutes.

Appendix 7

NUTRITIONAL AND PHYSIOLOGICAL TESTS
FOR THE IDENTIFICATION OF *C. SEPEDONICUM*

All media should be incubated at 21 °C and examined after six days. If no growth has occurred, incubate for up to 20 days.

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

Basal medium:

KCl	0,2 g
$MgSO_4$. $7H_2O$	0,2 g
$NH_4H_2PO_4$	1,0 g
Difco bacto peptone	1,0 g
Difco bacto purified agar	3,0 g
D(+)-glucose (monohydrate)	10,0 g
Bromothymol blue	0,03 g
Distilled water	1 litre

Mix and adjust to pH 7,0 to 7,2 with 1N KOH.

Dispense in Pyrex culture tubes 16 mm x 100 mm (12 ml capacity) in 5 ml and 10 ml volumes.

Sterilize by autoclaving at 115 °C for 10 minutes.

Stab inoculate 5 ml and 10 ml tubes for each culture. Aseptically add 1 to 2 ml sterile liquid paraffin to the 10 ml tube. Incubate.

POSITIVE REACTION:

Tube	**Colour**		**Interpretation**
Open	Yellow		Fermentative
Closed	Yellow		
Open	Yellow		Oxidative
Closed	Blue-green		
Open	Greenish		Oxidative or inert
Closed	Blue-green		

— **Oxidase test** (Kovacs, 1956)

Kovacs'oxidase reagent:

1 % aqueous solution of tetramethyl paraphenylenediamine dihydrochloride (BDH No 30386) in distilled water.

This reagent should be freshly made up in 1 ml volumes or can be stored in a brown glass bottle at 5 ^{o}C for 1 to 4 weeks.

Place a drop of reagent on filter paper in a clean Petri dish. Immediately rub some of the test culture from nutrient agar using a platinum loop.

Positive reaction: development of purple coloration within 10 seconds. Cultures with times of 10 to 30 seconds are weakly positive.

NB: It is essential to use a platinum loop and NA cultures, since traces of iron or high sugar content in the growth medium may give false positive results.

— **Acid production from lactose, rhamnose, salicin, glycerol**

Prepare Hugh & Leifson's O/F medium without the glucose. Distribute into 5 ml volumes in tubes. Sterilize by autoclaving at 115 ^{o}C for 10 minutes. To the molten base at 45 ^{o}C, aseptically add 0,5 ml of filter sterilized 10 % aqueous solutions of either glycerol, lactose, rhamnose or salicin. Mix carefully.

Positive reaction: colour change from blue-green to yellow indicates production of acid.

— **Catalase test**

Place a drop of hydrogen peroxide (30 volume) onto a clean slide, and emulsify with a loopful of culture using a platinum loop.

Positive reaction: production of oxygen bubbles in the drop indicates the presence of catalase.

— **Nitrate reductase activity and denitrification** (Bradbury, 1970)

Culture medium:

KNO_3 (nitrite free)	1 g
Difco bacto yeast extract	1 g
K_2HPO_4	5 g
Distilled water	1 litre

Dispense into 10 ml volumes in 20 ml bottles. Sterilize by autoclaving at 121 ^{o}C for 15 minutes.

Reagent A:

H_2SO_4	8g
5N acetic acid	1 litre

Reagent B:

naphthylamine	5 g
5N acetic acid	1 litre

Inoculate the nitrate medium in duplicate. Test after 10 and 20 days, by adding one drop of Lugol's iodine, 0,5 ml reagent A and 0,5 ml reagent B. If medium does not turn reddish, add approximately 50 mg zinc powder. Observe the colour reaction.

POSITIVE REACTION:

	Colour reaction	
	Stage 1	**Stage 2**
No reduction of nitrate	colourless	red
Reduction of nitrate as far as nitrite (nitrate reductase only)	red	—
Reduction of nitrate beyond nitrite (denitrification — nitrate and nitrite reductase)	colourless	colourless

— **Urease production** (Lelliott, 1966)

Basal medium:

Oxoid urea agar base (CM53)	2,4 g
Distilled water	95 ml

Sterilize by autoclaving at 115 ^{o}C for 20 minutes. Cool the molten base to 50 ^{o}C and aseptically add 5 ml of filter-sterilized 40 % aqueous urea solution (Oxoid SR20). Mix well.

Distribute into 6 ml volumes in sterile tubes (16. x 100 mm) and allow to set as slopes with a good butt.

Positive reaction: the yellow-orange medium develops a cherry red or magenta pink coloration if urease activity has occurred.
Utilization of citrate (Christensen) (Skerman, 1967)

Citrate agar base (Merck 2503)	23 g
Distilled water	1 litre

Mix and dissolve by heating. Dispense into 6 ml volumes as for urea medium. Sterilize by autoclaving at 121 ^{o}C for 15 minutes and allow to set as slopes.

Positive reaction: citrate utilization is indicated by a change in the colour of the medium from orange to red.

— **Hydrogen sulphide production (Ramamurthi, 1959)**

Medium:

Difco bacto tryptone (No 0123)	10 g
K_2HPO_4	1 g
NaCl	5 g
Distilled water	1 litre.

Dissolve and dispense into 6 ml volumes in 16 x 100 mm tubes. Sterilize by autoclaving at 115 ^{o}C for 10 minutes.

Inoculate and aseptically suspend a lead acetate paper (Merck 9511) from the lip if the tube. Hold in place with the cap. Incubate for up to 20 days.

Positive reaction: H_2S production from tryptone is indicated by the development of a black-brown coloration of the test paper.

— **Indole production** (Ramamurthi, 1959)

Medium:

As for H_2S test.

Remove the lead acetate paper and add 1 to 2 ml of diethyl ether and shake gently. Allow the layers to separate (five minutes). Add 0,5 ml Kovacs' reagent (Merck 9293) carefully down the inside of the sloped tube.

Positive reaction: the presence of indole is indicated by the development of a red colour in the yellow layer between the ether and aqueous fractions.

— **Growth** ar 37 ^{o}C (Ramamurthi, 1959)

Medium:

Difco bacto nutrient broth (No 0003)	8 g
Distilled water	1 litre

Mix, dissolve and distribute into 6 ml volumes in tubes.

Sterilize by autoclaving at 121 ^{o}C for 15 minutes.

Inoculate and incubate at 37 ^{o}C.

Positive reaction: look for growth.

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

Medium:

Difco bacto nutrient broth	8g
NaCl	70 g
Distilled water	1 litre

Mix, dissolve and distribute into 6 ml volumes in tubes.

Sterilize by autoclaving at 121 ^{o}C for 15 minutes.

Positive reaction: look for growth.

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

Medium:

Difco bacto gelatine (No 0143)	120 g
Distilled water	1 litre

Mix, dissolve by heating and distribute into 6 ml volumes in tubes.

Sterilize by autoclaving at 121 ^{o}C for 15 minutes.

Positive reaction: liquefaction of gelating even when held at 5 ^{o}C for 30 minutes.

— Starch hydrolysis

Medium:

Difco bacto nutrient agar (molten)	1 litre
Difco bacto soluble starch (No 0178)	2 g

Mix, sterilize by autoclaving at 115 ^{o}C for 10 minutes.

Pour plates. Spot inoculate the plates.

After good growth has occurred (10 to 20 days), remove part of the growth and flood with Lugol's iodine.

Positive reaction: starch hydrolysis is indicated by clear zones under or around the bacterial growth; the remainder of the medium stains purple.

— **Aesculin hydrolase activity** (Sneath & Collins, 1974)

Medium:

Difco bacto peptone	10 g
Aesculin	1 g
Ferric citrate (Merck 3862)	0,05 g
Sodium citrate	1 g
Distilled water	1 litre

Mix to dissolve and distribute into 6 ml volumes in tubes.

Sterilize by autoclaving at 115 ^{o}C for 10 minutes.

The medium is clear but has a bluish fluorescence.

Positive reaction: aesculin hydrolysis is indicated by the development of a brown colour together with a disappearance of fluorescence. This can be checked using an ultraviolet lamp.

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(**1**) An alternative method for extraction is given by Dinesen, 1984.

(**2**) There is evidence (Janse and Van Vaerenberg, 1987) that freezing may reduce viability of Corynebacterium sepedonicum. Suspension of the pellet in 10 % glycerol may overcome this problem.