National Diagnostic Protocol

Clavibacter michiganensis subsp. *sepedonicus*

the cause of potato ring rot

NDP 8 V2

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Purpose

National Diagnostic Protocols (NDPs) are diagnostic protocols for the unambiguous taxonomic identification of plant pests. NDPs:

- are a verified information resource for plant health diagnosticians
- are consistent with ISPM No. 27 – Diagnostic Protocols for Regulated Pests
- provide a nationally consistent approach to the identification of plant pests enabling transparency when comparing diagnostic results between laboratories; and,
- are endorsed by regulatory jurisdictions for use (either within their own facilities or when commissioning from others) in a pest incursion.

Where an International Plant Protection Convention (IPPC) diagnostic protocol exists it should be used in preference to NDPs although NDPs may contain additional information to aid diagnosis. IPPC protocols are available on the IPPC website:

https://www.ippc.int/core-activities/standards-setting/ispms

Process

NDPs are facilitated and endorsed by the Subcommittee on Plant Health Diagnostics (SPHD). SPHD reports to Plant Health Committee and is Australia's peak technical and policy forum for plant health diagnostics.

NDPs are developed and endorsed according to Reference Standards developed and maintained by SPHD. Current Reference Standards are available at http://plantbiosecuritydiagnostics.net.au/sphd/sphd-reference-standards/

NDPs are living documents. They are updated every 5 years or before this time if required (i.e. when new techniques become available).

Document status

This version of the National Diagnostic Protocol (NDP) for *Clavibacter michiganensis* subsp. *sepedonicus* is current as at the date contained in the version control box below.

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Further information

Inquiries regarding technical matters relating to this project should be sent to: sphds@agriculture.gov.au

Contents

1 INTRODUCTION

Clavibacter michiganensis subsp. *sepedonicus* (Spieckermann and Kotthoff 1914) Davis *et al*. 1984 (Cms) is the causal agent of the potato disease, bacterial ring rot (Davis *et al*. 1997; Franc 1999). The disease derives its name from the characteristic internal breakdown in the vascular ring of an infected tuber (Manzer and Genereux 1981). This can be seen as a brown, cheesy decay of the vascular tissue. Above ground the disease is usually seen as a progressive wilt (Lelliott and Stead 1987).

Infected seed potatoes produce infected plants. After planting, bacteria multiply and spread to the vascular tissue of stems, petioles, roots and developing tubers. Symptoms rarely develop quickly and infections usually remain latent for long periods. Some cultivars tolerate infection so that symptoms may not develop for several plant generations, even though the bacteria can multiply in both plants and tubers. The latent period may encompass almost the entire period of host growth from vegetative propagule to mature plant (Bishop and Slack 1987).

1.1 Host range

Potato (*Solanum tuberosum*) is the main host.

The bacterium has been isolated from sugarbeet seed and roots (Bugbee and Gudmestad, 1988) and is described as a natural symptomless host. In inoculation tests many members of the Solanaceae, including tomatoes and aubergines, were found to be susceptible (EPPO 2006).

1.2 Transmission

Important means of spread are the planting of infected seed potatoes and contamination of containers, equipment and premises. When an infected seed piece is planted, bacteria move from the seed through the vascular tissue into the stem and lower leaves of the growing plant (Babadoost 1990). The plant will start to show foliar and stem symptoms mid-season or later (Davis *et al*. 1997). Late in the season, bacteria migrate from the stem down into the stolons, infecting the new tubers (Babadoost 1990). Internal symptoms may be present within tubers at harvest but are more commonly observed toward the end of the storage period (Lelliott and Stead 1987).

Planters, graders and knives which have been contaminated by bacteria from diseased potatoes are also a potent infection source. Disease spread in the field from plant to plant is usually poor, but there is experimental evidence that some insects, including the potato flea beetle, *Epitrix cucumeris* (Harris), the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), the green peach aphid, *Myzus persicae* (Sulzer) and the fruit fly are possible vectors of Cms (Christie *et al*. 1991; Christie *et al*. 1993; De Boer *et al*. 1988).

Bacteria can also survive and remain infectious for several years on potato bags, bulk bins, store walls and other surfaces that have been contaminated by rotting ooze. The bacterium is able to overwinter in the soil, usually in association with “groundkeepers” (unharvested potatoes from the previous crop) and debris from infected crops. Infected groundkeepers lifted with an otherwise clean seed or ware crop can infect that crop (DEFRA, 2002).

The pathogen can survive in water for more than a month but there is no known aquatic weed host to build up inoculum levels. Contaminated wash water from infected tuber lots can transmit the pathogen to subsequent lots washed in the same water (DEFRA 2002).

2 TAXONOMIC INFORMATION

Phylum:	Actinobacteria
Class:	Actinobacteria
Subclass	Actinobacteridae
Order:	Actinomycetales
Suborder:	Micrococcineae
Family:	Microbacteriaceae
Genus:	*Clavibacter*
Species:	*Clavibacter michiganensis*
Subspecies:	*Clavibacter michiganensis* subsp. *sepedonicus*

Name: *Clavibacter michiganensis subsp. sepedonicus* (Spieckermann and Kotthoff) Davis *et al*.

Synonyms:

- *Aplanobacter sepedonicum* (sic) (Spieckermann and Kotthoff 1914) Smith 1920
- *Bacterium sepedonicum* Spieckermann and Kotthoff 1914
- *Clavibacter michiganensis* subsp. *sepedonicus* corrig.(Spieckermann and Kotthoff 1914) *Davis et al*. 1984
- *Corynebacterium michiganense* subsp. *sepedonicum* (Spieckermann and Kotthoff 1914) Carlson and Vidaver 1982
- *Corynebacterium sepedonicum* (Spieckermann and Kotthoff 1914) Skaptason and Burkholder 1942 (AL1980)
- *Corynebacterium michiganense* subsp. *Sepedonicum*
- *Mycobacterium sepedonicum* (Spieckermann and Kotthoff 1914) Krasil'nikov 1949
- *Phytomonas sepedonica* (Spieckermann and Kotthoff 1914) Magrou 1937
- *Pseudobacterium sepedonicum* (Spieckermann and Kotthoff 1914) Krasil'nikov 1949

Equivalent name: *Clavibacter michiganense sepedonicum*

Common names: Bacterial ring rot (English), Bactériose annulaire, flétrissement bactérien (French), Bakterienringfäule (German), Podredumbre anular (Spanish)

3 DETECTION

3.1 Symptoms

3.1.1 Foliar and stem symptoms

If foliar symptoms appear, they usually occur mid-season or later and are first seen on nearly full-grown plants (Davis *et al*. 1997). Lower leaves usually wilt first, becoming slightly rolled upward at the margins, and are paler green than healthy leaves (Rowe *et al*. 1995). As wilting progresses, the leaf margins and interveinal regions become chlorotic, then necrotic (Figs 1, 2), turning the leaves brown as if burnt (Rowe *et al*. 1995; ACP, 2003). Whole stems can wilt and die, but it is unusual for all the stems on a plant to be killed (DEFRA, 2002). Frequently, only one or two stems per plant develop symptoms and, in some cases, there are no aboveground symptoms at all (Glick *et al*. 1944).

In advanced stages of the disease, the vascular tissue near the base of an infected stem turns brown and exudes a milky bacterial ooze when squeezed (Babadoost 1990; Lelliott and Stead 1987).

Figure 1 Potato leaf showing symptoms of infection by Cms in USA (© Regents, University of California).

Figure 2 Potato plant exhibiting infection by Cms in USA (© Regents, University of California).

3.1.2 Tuber symptoms

Late in the growing season when bacterial populations become established in new tubers, internal symptoms may begin to develop. The first symptom appears as a glassiness of the vascular tissue. This is best seen immediately below the point of tuber attachment (heal end) (Lelliott and Stead 1987). Early symptoms can be confused with tuber infections caused by *Ralstonia*.

After harvest the disease continues to develop within stored tubers. The breakdown of the vascular tissue becomes more evident as a broken, sporadic dark line or as a continuous, yellow discoloration (Fig. 3). As the disease advances, the vascular ring separates and a creamy or cheesy exudate can be forced from the tissue when gentle pressure is applied to the outer skin (Fig. 4) (Rowe *et al*. 1995). Externally, tubers may appear normal. In severely diseased tubers pressure created by the breakdown can cause external swelling, ragged cracks and reddish brown discoloration, especially around the eyes (Fig. 5) (Manzer and Genereux 1981). At this stage, secondary infections by common soft rot

bacteria can result in the complete breakdown of the tuber (Fig. 6**Error! Reference source not found.**) (ACP 2003; Rich 1983; Rowe *et al*. 1995).

Figure 3 Bacterial ring rot symptoms of the vascular ring often appear only as sporadically broken dark line or continuous yellow discoloration (© Ohio State University).

Figure 4 As bacterial ring rot progresses the breakdown of vascular tissue becomes more severe. A brown, cheesy decay can be seen seeping from the vascular ring (© Cornell University).

Figure 5 Severely infected tubers may show swelling, ragged cracks and reddish brown discoloration, especially around the eyes (© Regents, University of California).

Figure 6 Varying degrees of vascular tissue breakdown in a number of Cms infected tubers (© Regents, University of California).

3.1.3 Symptom variability

Bacterial ring rot symptom expression can vary markedly depending on the potato cultivar, environmental conditions and infection by other organisms. Norchip and Red Pontiac usually have easily recognised symptoms, Russet Burbank has moderate symptom expression and Desiree and Belrus have less apparent disease symptoms. Cultivars such as Teton and Urgenta rarely exhibit any symptoms when infected, but the bacterium can readily be recovered from infected symptomless tubers of both varieties (De Boer and McCann 1990; ACP 2003). Some varieties develop atypical symptoms such as dwarf rosette foliage (Nelson *et al*. 1992). Symptom expression is generally favoured by warm growing conditions, while under cool conditions; few or no symptoms may develop (ACP 2003). Symptoms can also be confusing when other disorders such as early blight, late blight, blackleg, brown rot, freezing injury or water damage are present. Viruses, such as Potato leaf roll virus, can mask the effects of bacterial ring rot (Babadoost 1990; Nelson and Torfason 1974). Disease symptoms can be further complicated by the latent phase of the bacterium, and may be absent altogether, although bacterial population in the tubers is high (Lelliott and Stead 1987). Bacterial ring rot can be confused in the early stages with brown rot caused by *Ralstonia solanacearum*.

3.2 Sampling

Formal identification is based on tuber symptoms and tests on the bacterial exudates from the affected tubers. While the bacteria can be detected in other plant parts (Appendix 1) these are usually sampled only during surveillance.

4 IDENTIFICATION

Many of the identification procedures in this protocol have been sourced from the EPPO protocol PM 7/59 (EPPO 2006), drafted by D. Stead, Central Science Laboratory, York (GB) and revised by P. Müller, Biologische Bundesanstalt für Land- und Forstwirtschaft, Kleinmachnow (DE).

Note: *While these procedures are still suitable for identification, new procedures are available and will be incorporated into the protocol at a future date. These include real time PCR, sequencing and serological methods.*

Suspect Gram positive cultures of *Clavibacter* should be identified using at least two tests based on different biological principles (e.g, biochemical, serology, or PCR). Final confirmation should be by a pathogenicity test using the eggplant bioassay.

4.1 Morphological methods

The bacterium is Gram positive, non-motile, non spore-forming and approximately 0.4-0.6 µm x 0.8-1.2 µm. Cells are coryneform rods, slightly curved and club shaped, arranged singly or in pairs in L or V formations (Fig 7). It is strictly aerobic and nutritionally fastidious, requiring a specialised media such as NCP-88. When plated on to NCP-88, colonies of CMS are visible within 5 days at 25°C, and measure 0.5-1.5 mm in diameter after 7-10 days. The colonies are round to irregular with entire margins, white to cream in colour, raised, and usually mucoid and glistening (Fig 8). After 10-12 days of incubation, colonies become pale yellow (de la Cruz *et al*. 1992).

The bacterium can hydrolyse soluble starch, and utilise acetate, citrate and succinate. It can produce acid from the oxidation of mannitol and sorbitol. It cannot produce H_2S from the decomposition of organic sulphur compounds or from the reduction of sulphate under anaerobic conditions. It is unable to produce and maintain stable acid end products from glucose fermentation (de la Cruz *et al*. 1992).

4.1.1 Isolation/culture techniques

Equipment and Media

- 20-200 µL & 200-1000 µL pipettes and tips
- Autoclave
- Autoclaved mortar and pestles
- Balance
- Bunsen burner
- Centrifuge tubes
- Fridge
- Glass spreaders
- Incubator at 25°C
- Laminar flow
- Petri dishes
- Sterile sand
- Sterile scalpel blades
- Syringe with 22 µm filter

NCP-88; Semiselective agar media (1 L)

- Nutrient agar 23.0 g
- Yeast extract 2.0 g
- K_2HPO_4 MW = 174.18 2.0 g
- KH_2PO_4 MW = 136.09 0.5 g
- $MgSO_4 \cdot 7H_2O$ MW = 246.48 0.25 g
- D-mannitol ($C_6H_{14}O_6$) MW = 182.17 5.0 g

Autoclave the media at 121°C at 110 kPa for 25 minutes.

After autoclaving, allow the medium to cool to 50°C and add filter sterilised solutions of the following inhibitors (per litre):

- 300 µL Polymyxin B-sulfate stock (7,900 units per milligram, 10 mg/mL stock)
- 800 µL of Nalidixic acid stock (Na-salt, freshly dissolved in 10 mM NaOH, 10 mg/mL)
- 2.0 mL of Cycloheximide stock (dissolved in 47.5% ethanol, 100 mg/mL stock)

Method

1. Weigh approximately 100 mg potato tuber tissue taken from the basal portion of the stem or the heal end of the potato tuber
2. Homogenise using a mortar and pestle with approximately 0.1 g of sterile sand in 2 mL of sterile distilled H_2O
3. Prepare serial dilutions to 10^{-4} by adding 100 µL to 900 µL sterile distilled water in sterile microcentrifuge tubes
4. Using a bent glass rod spread 100 µL of undiluted, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions onto NCP-88 media
5. Incubate at 25°C for 1-2 weeks
6. Colonies of CMS are visible within 5 days
7. After 7-10 days the colonies measure 0.5-1.5 mm in diameter, are round to irregular with entire margins, white to cream in colour, raised, and usually mucoid and glistening (Fig. 8)
8. After 10-12 days incubation, colonies become pale yellow

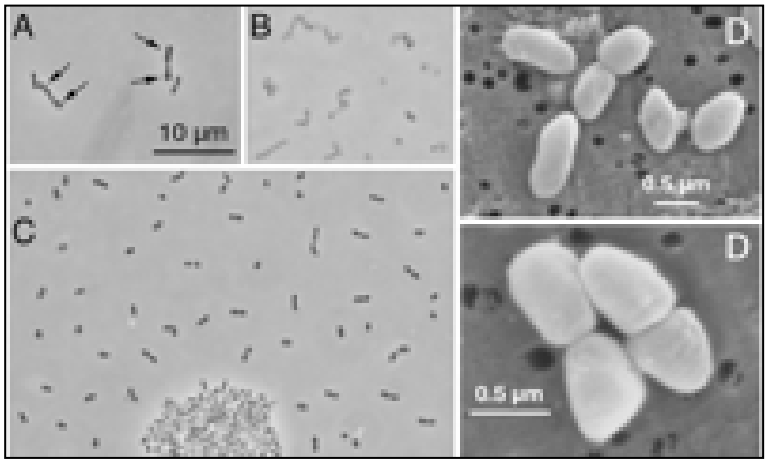

Figure 7 *Clavibacter michiganensis* subsp. cells http://aem.asm.org/content/vol69/issue5/images/small/am0531761004.gif

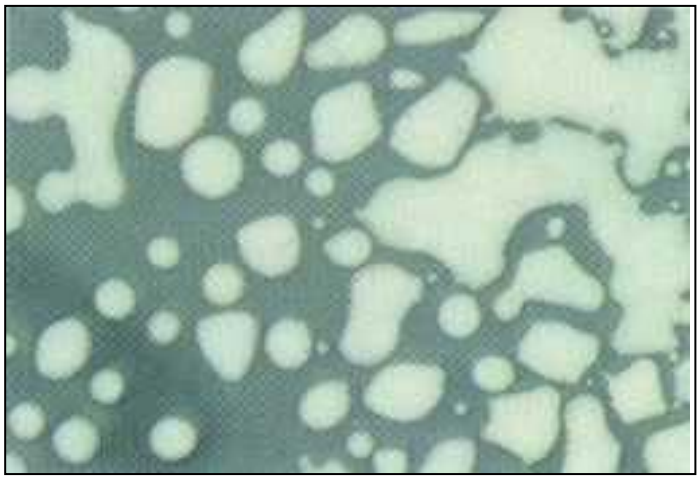

Figure 8 *Clavibacter michiganensis* subsp. *sepedonicus* colony morphology on NBY (nutrient broth yeast extract) medium. Growth on NBY is similar to growth on NCP-88 (Schaad *et al*. 1999)

4.2 *In Planta* methods

The eggplant bioassay was not verified.

4.2.1 Eggplant bioassay

The eggplant bioassay will confirm the presence of Cms in supernatant from infected potato tissue, by producing rapid symptoms after inoculation. The eggplant is also an excellent means of concentrating the bacterium to levels where isolation and purification are possible.

Wilted tissue may initially appear dark green or mottled but turns paler before becoming necrotic. Inter-veinal 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 Cms 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 *Pectobacterium carotovorum subsp*. *carotovorum* and *P. carotovorum* subsp. *atrosepticum*, *Phoma exigua* var. *foveata*, as well as large populations of saprophytic bacteria. Such wilts can be distinguished from those caused by Cms since whole leaves or whole plants wilt rapidly.

Under certain circumstances, in particular where growing conditions are not optimal, it may be possible for Cms 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 immunofluorescence staining (IF) test is considered positive, it may be considered necessary to test further. It is 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.

This eggplant bioassay procedure was not verified as part of the protocol.

Preparation of samples

Homogenise the heel ends until complete maceration has just been achieved in 0.05 M phosphate buffered saline (PBS pH 7.0) at a temperature of less than 30°C. Excessive maceration should be avoided.

Extract bacteria from the homogenate by one of the following methods:

Method 1:

a) Centrifuge the macerate at not more than 180 g for 10 minutes. Decant and discard the pellet.
b) Centrifuge supernatant at not less than 4 000 g for 10 minutes. Decant and discard the supernatant.

Method 2:

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 mm) 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.
d) Suspend the pellet in sterile 0.01 M phosphate buffer pH 7.2 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 or by lyophilisation. Divide the other part into halves using one half for the IF test and Gram stain and the other for the eggplant test.

It is imperative that all positive Cms controls and samples are treated separately to avoid contamination.

NB: Due to quarantine restrictions, some diagnostic labs may not be able to use a viable culture of Cms for control inoculations. However, heat-killed cells may be used as an IF control.

Eggplant propagation

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

2. Use eggplants at "leaf stage 3" when two to three leaves are fully uncurled. Eggplants should be grown in a glasshouse with the following environmental conditions:

    a. day length- 14 hours or natural day length if greater;

    b. temperature- day: 21 to 24°C, night: 15°C.

NB: Cms will not grow at temperatures >30°C.

Eggplant Assay

Distribute the potato pellet suspension (from 4.2.1) between at least 25 eggplants at leaf stage 3 (4.2.2) by one of the methods given below.

Slit inoculation 1

1. Support each pot horizontally (a block of expanded polystyrene with a piece 5 cm deep × 10 cm wide × 15 cm long, removed from one surface 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.

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.

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

4. Seal the cut with sterile Vaseline from a 2 mL syringe barrel.

Slit inoculation 2

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.

2. Using a sterile scalpel, make a diagonal (at an angle of approximately 5°) slit, 1.0 cm long and approximately two thirds of the stem thickness deep, starting the cut from the pellet drop.

3. Seal the cut with sterile Vaseline from a syringe barrel.

Syringe inoculation

1. Do not water eggplants for one day prior to inoculation to reduce turgor pressure.

2. Inoculate 25 eggplant stems with approximately 40 µL of potato pellet suspension (from 0) per plant, just above the cotyledons using a syringe fitted with a hypodermic needle (not less than 23G).

4.3 Serological methods

Note: *Serological tests available by Loewe (lateral flow device) and Agdia (ELISA) are available and will be incorporated into an updated protocol.*

The IF procedure was not verified.

4.3.1 Immunofluorescence (IF) testing

Prepare sample as per Eggplant bioassay method above.

Use antiserum to a known strain of Cms - ATCC 33113 (NCPPB 2137), or NCPPB 2140. 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. Cms (ATCC 33113 (NCPPB 2137), NCPPB 2140) (heat-killed) should be used as homologous antigen controls on a separate slide.

Procedure

1. Prepare three serial ten-fold dilutions (10^1, 10^2, 10^3) of the final pellet in distilled water.
2. Pipette a measured standard volume of each pellet dilution sufficient to cover the window (approximately 25 µL) or Cms suspension (approximately 10^6 cells/mL) to windows of a multi-spot slide.
3. Cover appropriate windows with Cms antiserum at the recommended dilutions, 0.01 M PBS pH 7.2 (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.
4. Incubate in a humid chamber at ambient temperature for 30 minutes.
5. Rinse with 0.01 M PBS pH 7.2. Wash for five minutes in three changes of 0.01 M PBS pH 7.2.
6. Carefully remove excess moisture.
7. Cover each window with FITC conjugate at the same dilution (and volume of antibody applied) used to determine the titre and incubate in a dark humid chamber at ambient temperature for 30 minutes.
8. Rinse and wash as before.
9. 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 cover glass.
10. Examine with a microscope fitted with an epifluorescent light source and filters suitable for working with FITC A magnification of 400 to 1,000 X 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.

NB. There are several problems which may be encountered with the immunofluorescence test. Background populations of fluorescing cells with atypical morphology and cross-reacting saprophytic bacteria with size and morphology similar to Cms 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 IF test should be retested using a different antiserum.

The technical limit of detection of this method is 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 Cms but should be confirmed with the eggplant assay. A negative immunofluorescence test is identified for any sample where morphologically typical fluorescing cells are not found and 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 positive' for Cms. The eggplant test is required for all samples considered as potentially positive.

4.4 Molecular methods

New procedures are available and will be incorporated into the protocol at a future date. These include:

1. *Real-time PCR of Schaad et al. (1999). Recently Vreeburg et al. (2016) have recommended the use of real-time PCR due to its increased sensitivity over other methods.*

[Vreeburg, R. A. M., Bergsma-Vlami, M., Bollema, R. M., Haan, E. G., Kooman-Gersmann, M., Smits-Mastebroek, L., & Janse, J. D. (2016). Performance of real-time PCR and immunofluorescence for the detection of *Clavibacter michiganensis* subsp. *sepedonicus* and *Ralstonia solanacearum* in potato tubers in routine testing. *EPPO Bulletin*, 46(1), 112-121.]

2. *DNA sequencing (Richert et al. 2005; Waleron et al. 2011). Q-bank is also a good database that provides methodology for sequence identification http://www.q-bank.eu/Bacteria/DefaultInfo.aspx?Page=Protocol Clavibacter.*

[Richert K, Brambilla E, Stackebrandt E. (2005). Development of PCR primers specific for the amplification and direct sequencing of gyrB genes from microbacteria, order Actinomycetales. *Journal of Microbiological Methods* 60, 115-23.

Waleron, M., Waleron, K., Kamasa, J., Przewodowski, W., & Lojkowska, E. (2011). Polymorphism analysis of housekeeping genes for identification and differentiation of *Clavibacter michiganensis* subspecies. *European Journal of Plant Pathology*, 131(2), 341-354.]

4.4.1 Polymerase chain reaction (PCR) detection using potato extracts

The two primer pairs used for the detection of Cms are listed below in Table 1. The primer pair, PSA-1/PSA-R (Pastrik 2000) is used to detect Cms, whereas the generic bacterial primer pair fD2/rD1 amplifies the bacterial 16SrDNA gene (Weisburg *et al*. 1991) as an internal control which tests the integrity of the extracted DNA template. It is recommended that all primers are used at a concentration of 100 ng/µL. Using the optimized multiplex PCR protocol, Pastrik was able to detect artificially added *C. michiganensis* subsp. *sepedonicus* in potato core fluid in the range of 2–20 CFU per PCR reaction mixture. Note Pastrik used internal control primers targeting the 18S rRNA gene which amplified host plant DNA rather than bacterial DNA.

Table 1 Primers used in the detection of Cms

Primer	Sequence (5'-3')	Target	PCR product
PSA-1	CTC CTT GTG GGG TGG GAA AA	CMS intergenic spacer region (16S-23S rDNA) (Pastrik 2000)	502 bp
PSA-R	TAC TGA GAT GTT TCA CTT CCC C		
fD2	AGA GTT TGA TCA TGG CTC AG	16S rDNA gene (Weisberg *et al*. 1991)	~1600 bp
rD1	AAG GAG GTG ATC CAG CC		

Potato tissue DNA extraction for PCR

Alternatively, a Promega Wizard DNA extraction kit, Qiagen DNeasy extraction kit, or similar can be used according to manufacturer's instructions.

Modified SCP

For 1000 mL Final Concentration

- Disodium succinate ($C_4H_4Na_2O_7$) 1 g 3.7mM
- Trisodium citrate ($C_6H_5Na_3O_7$) 1 g 3.9mM
- Dibasic potassium phosphate (K_2HPO_4) 1.5 g 8.6mM
- Monobasic potassium phosphate (KH_2PO_4) 1 g 7.3mM
- PVP40 50 g 1.25mM

Autoclave and add ascorbic acid (0.02M) and adjust to pH 7 just prior to use. The stock buffer, without ascorbic acid, can be stored frozen (-20^{o}C) for up to 6 months. The buffer with ascorbic acid can be stored at room temperature.

PBS/BSA

a) 10X PBS (1000 mL) Final Concentration

- Sodium chloride (NaCl) 80 g 1.4M
- Monobasic potassium phosphate (KH_2PO_4) 2 g 14.7mM
- Disodium phosphate (Na_2HPO_4) 11.5 g 81.0mM
- Potassium chloride (KCl) 2 g 26.8mM

Autoclave. Store at room temperature.

b) PBS/BSA - 1x PBS plus 0.2% BSA. Store at 4^{o}C.

CTAB buffer (+ 0.2% mercaptoethanol) (100 ml)

- 1M Tris, pH 7.5 ($H_2NC(CH_2OH)_3$) 20 mL
- 5M Sodium chloride (NaCl) 28 mL
- 500mM EDTA, pH 8.0 ($[CH_2.N(CH_2.COOH).CH_2COON_9]_2.2H_2O$) 4 mL
- CTAB ($C_{19}H_{42}NBr$) 2 g
- β-Mercaptoethanol (optional) 200 µL

Mix and make up to 100 mL with dH_2O. Store at room temperature.

Other reagents

- Chloroform:isoamyl alcohol - 24:1 mix of chloroform to isoamyl alcohol. Store at room temperature.

- Isopropanol - 100% isopropanol stored at 4°C.
- Ethanol - 80% ethanol. Store at room temperature.
- Water - Sterile distilled H_2O. Store at room temperature.

Extraction method

1. Place CTAB buffer in 60°C water bath
2. Weigh approximately 700 mg of plant tissue (tuber, stem and lower leaf mid vein)
3. Homogenise in 5 mL of modified SCP grinding buffer with autoclaved mortar and pestle, and using approximately 0.1 g sterile sand
4. Strain homogenate through sterile cheesecloth and transfer 500 µL to a sterile 2 mL centrifuge tube, or trim pipette tip with sterile scalpel blade and transfer 500 µL to a 2 mL sterile centrifuge tube (repeat for duplication of test)
5. Centrifuge at 12000 RPM for 5 minutes
6. Discard supernatant and re-suspend the pellet in 500 µL of PBS/BSA with gentle pipetting
7. Immediately add 800 µL pre-warmed CTAB buffer + 0.2% mercaptoethanol
8. Vortex and incubate the centrifuge tube at 60°C for 20 minutes, with occasional mixing
9. Add 600 µL chloroform:isoamyl alcohol (24:1), vortex vigorously, then centrifuge at 12000 RPM for 5 minutes
10. Transfer supernatant to a sterile 2 mL centrifuge tube
11. Add equal volume of cold isopropanol, mix well and leave on ice (or in freezer) for 10 minutes
12. Centrifuge at 12000 RPM for 10 minutes
13. Rinse pellet with 500 µL 80% ethanol
14. Centrifuge at 12000 RPM for 5 minutes, remove all ethanol with pipette, and air dry pellet by placing tube on its side.
15. Re-suspend pellet in 200 µL sterile dH_2O

4.4.2 Method

PCR reagents

PCR controls

- Positive control - DNA extract from potato infected with Cms
- Alternatively, a "plasmid control" that has the appropriate region of the Cms genome cloned into a plasmid
- Negative plant control - DNA extract from uninfected plant tissue of the same species as that used for the positive control.
- Negative buffer control - an aliquot of the PCR Master Mix without template.

5 x TBE buffer

Per 1 litre

- Tris ($C_4H_{11}NO_3$) 54 g
- Boric acid (H_3BO_3) 27.5 g
- 0.5M EDTA ($[CH_2.N(CH_2.COOH).CH_2COONa]_2.2H_2O$) pH 8.0 20 mL

-

Store at room temperature.

100 x TE buffer

Per 1 litre

- Tris ($C_4H_{11}NO_3$) 21.14 g
- 0.5M EDTA ($[CH_2.N(CH_2.COOH).CH_2COONa]_2.2H_2O$) 37.22 g

Adjust pH to 8.0± 0.2. Store at room temperature.

- 1% Agarose gel with ethidium bromide
- Use a 1% DNA grade agarose (w/v) gel made with 0.5x TBE solution, and stained with 0.03 µg/mL ethidium bromide.

6x loading dye

Final volume 100 mL

- 1 x TE 10 mL
- Glycerol 50 mL
- Bromophenol blue 100 mg

PCR Method

1. Label sterile 0.2 mL centrifuge tubes
2. Prepare "Master Mix" on ice in a sterile microcentrifuge tube
3. The “Master Mix” usually contains buffer, forward and reverse primers, dNTPs, Taq polymerase and nuclease-free water
4. Prepare the “Master Mix” according to the Taq polymerase manufacturer’s recommendations
5. Ensure that the final volume for each reaction is 24 µL
6. Add 24 µL of Master Mix to each PCR tube
7. Add 2 µL sdH_2O to the negative control tube, 2 µL test template to each sample’s respective tube, and 2 µL potato DNA infected with Cms into positive control tube.
8. PCR reaction under these conditions: 95°C 1 min, 30 cycles of [94°C 45 sec, 50°C 30 sec, 72°C 30 sec], 72°C 10 min], hold at 25°C
9. Mix 10 µL each PCR sample with 5 µL loading dye
10. Load samples onto a 1% agarose gel containing ethidium bromide
11. Electrophorese in 1 X TBE at 100V
12. Visualise and photograph gel on UV transilluminator.

A positive test is the amplification of a 502 bp product (Fig 9). This amplicon can be sequenced to confirm results.

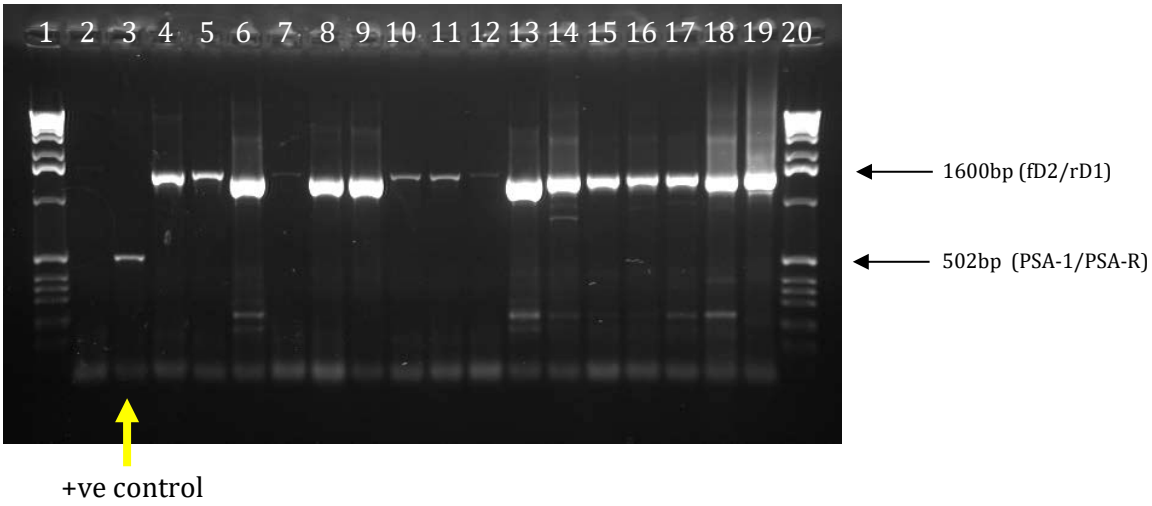

Figure 9 Example of electrophoresis gel showing PCR products generated with multiplex PCR using primer pairs PSA-1/PSA-R and fD2/rD1.

Below is the list of DNA extracts tested, including controls.

Lane 1 DNA molecular weight marker X, 0.07-12.2 kb (Roche™)
Lane 2 Negative buffer control
Lane 3 Positive Cms plasmid control
Lane 4 *Clavibacter michiganensis* subsp. *michiganensis* (NSW)
Lane 5 *Clavibacter michiganensis* subsp. *michiganensis* (VIC)
Lane 6 *Solanum tuberosum* (tuber) (Riverina, VIC)
Lane 7 *S. tuberosum* (tuber) (Toolangi, VIC)
Lane 8 *S. tuberosum* (tuber) (Olinda, VIC)
Lane 9 *S. sodomaeum* (leaf)
Lane 10 *S. melongena* (leaf)
Lane 11 *Lycopersicon esculentum* (leaf)
Lane 12 *Capsicum frutescens* (leaf)
Lane 13 *S. tuberosum* (tuber) infected with *Alternaria solani*
Lane 14 *S. tuberosum* (tuber) infected with *Streptomyces scabies*
Lane 15 *S. tuberosum* (tuber) infected with *Helminthosporium solani*
Lane 16 *S. tuberosum* (tuber) infected with *Spongospora subterranae*
Lane 17 *S. tuberosum* (tuber) infected with *Rhizoctonia solani*
Lane 18 *S. tuberosum* (tuber) infected with *Fusarium* wilt
Lane 19 *S. tuberosum* (tuber) infected with *Ralstonia solanacerarum*
Lane 20 DNA molecular weight marker "X", 0.07-12.2 kb (Roche™)

5 CONTACTS FOR FURTHER INFORMATION

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V1 of this protocol was reviewd by EJ Cother (then NSW Dept of Agriculture) and Megan Romberg (then MAF Biosecurity New Zealand). V2 was reveiwed by Rob Taylor, Plant Health & Environment Laboratory, Ministry for Primary Industries, New Zealand.

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8 APPENDICES

8.1 Visual examination for ring rot symptoms

Wash tubers in running tap water and remove the epidermis around the heel end of each a disinfected scalpel or potato peeler; disinfection may be achieved by dipping the implement in 70% ethanol and flaming.

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

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.

8.2 Tissue sampling for DNA extractions and bacterial isolations

8.2.1 Sampling methods

Because symptoms of ring rot are variable and sometimes masked by other diseases, Cms can be confirmed only by laboratory tests, including a pathogenicity test on eggplant and a PCR and serological test (De Boer and McNaughton 1986).

Late season potato sampling for Cms testing

Tuber sampling can occur at two stages, late in the growing season when the bacterium has moved into developing tubers, and toward the end of the storage period (Babadoost,1990). Internal and external symptoms may be hard to distinguish when tubers are inspected late in the growing season or at harvest, as the disease has not had a chance to establish sufficiently. However, in a severely infected crop, tubers may show symptoms. The disease continues to develop within the tuber during storage and will often show typical symptoms after a few months (Lelliott and Stead 1987).

1. Select several tubers from symptomatic plants in the field (late season) or from the storage facility.

2. Place tubers in a cardboard box lined with absorbent paper or collect 1 cm cores as described

3. Label bag with grower's name, variety name, seed or ware and current date.

4. Send to a diagnostic laboratory immediately after the material is collected.

Mid-season potato sampling for detection of Cms

The bacteria are most highly concentrated in the basal portions of the stem (Fig. 10), with fewer in the lower leaves and even less in the upper stem. By removing a single lateral stem from the symptomatic plant, all three areas can be sampled, thereby improving the probability of Cms identification.

The optimum tissue to sample for the detection of Cms is the basal portion of the stems or the heal ends of potato tubers (Fig. 10). When extracting from leaves, the highest numbers of bacteria are to be found in the midveins (Christie *et al*. 1993). To sample from the basal portion of the stem, take the lateral stem and remove a 1 cm segment at the point where pigmentation starts (soil level) (Fig. 11). It is important that the stem segment is green, as nonpigmented segments are difficult to prepare. This 1 cm segment should weigh approximately 0.5 g to 1.0 g.

To identify the heal end (stem end) of a tuber look for the remnants of the stolon, or for a scar which is left when the stolon is removed. The rose end (bud end) will have a concentration of eyes. When sampling the heal end of tubers it is best to remove a core sample from the stolon attachment site. Each core should weigh between 0.5 g to 1.0 g and include as much vascular tissue as possible.

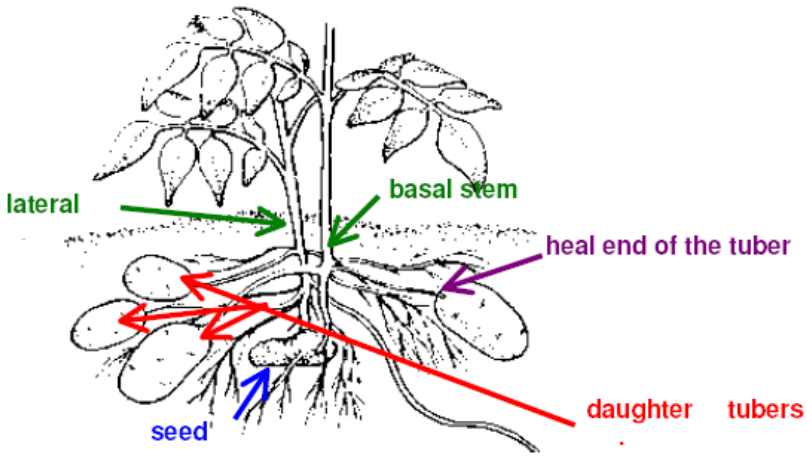

Figure 10 Diagram of a potato plant parts relevant to tissue sampling for Cms detection

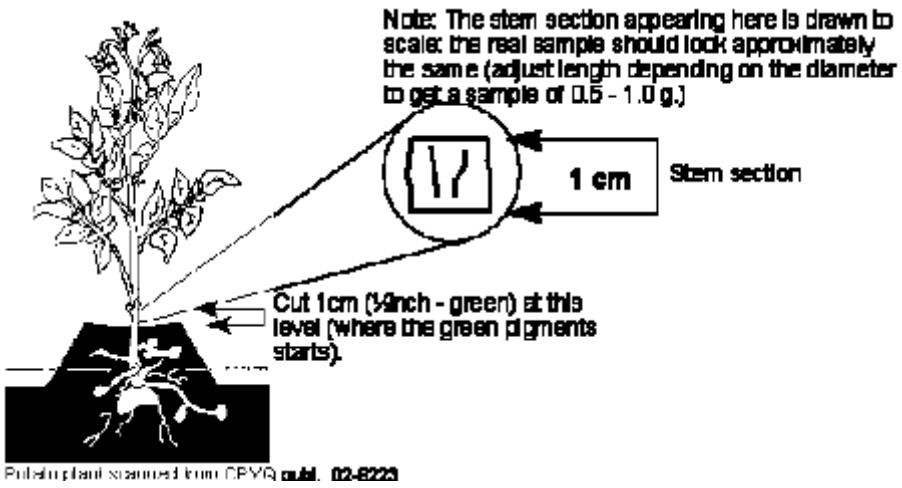

Figure 11 Method for collecting a stem section from a potato plant.

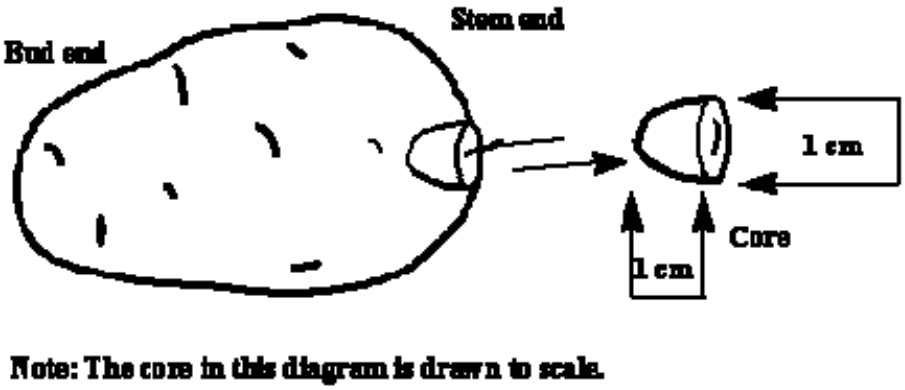

Figure 12 Sampling a 1cm core from a potato tuber.

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