ANNEX I

TEST SCHEME FOR DIAGNOSIS, DETECTION AND IDENTIFICATION OF THE RING ROT BACTERIUM, *CLAVIBACTER MICHIGANENSIS* (Smith) Davis *et al.* ssp. *SEPEDONICUS* (Spieckermann et Kotthoff) Davis *et al.*

SCOPE OF THE TEST SCHEME

Appendix 6

Validated PCR protocol and reagents

1. Multiplex PCR protocol with internal PCR control (Pastrik, 2000)

1.1. Oligonucleotide primers

Forward primer PSA-1	5′- ctc ctt gtg ggg tgg gaa aa -3′
Reverse primer PSA-R	5′- tac tga gat gtt tca ctt ccc c -3′
Forward primer NS-7-F	5′- gag gca ata aca ggt ctg tga tgc -3′
Reverse Primer NS-8-R	5’- tcc gca ggt tca cct acg ga -3’

Expected amplicon size from *C. m.* subsp. *sepedonicus* template DNA = 502 bp (PSA-primer set).

Expected amplicon size from the 18S rRNA internal PCR control = 377 bp (NS-primer set).

1.2. PCR reaction mix

Reagent	**Quantity per reaction**	**Final concentration**
Sterile UPW	15,725 µl	
10x PCR buffer[a] (15 mM $MgCl_2$)	2,5 µl	1x (1,5 mM $MgCl_2$)
BSA (fraction V) (10 %)	0,25 µl	0,1 %
d-nTP mix (20 mM)	0,125 µl	0,1 mM
Primer PSA-1 (10 µM)	0,5 µl	0,2 µM
Primer PSA-R (10 µM)	0,5 µl	0,2 µM
Primer NS-7-F (10 µM)[b]	0,1 µl	0,04 µM
Primer NS-8-R (10 µM)[b]	0,1 µl	0,04 µM
Taq polymerase (5 U/µl)[a]	0,2 µl	1,0 U
Sample volume	5,0 µl	
Total volume	25,0 µl	

a Methods were validated using *Taq* polymerase from Perkin Elmer (AmpliTaq or Gold) and Gibco BRL.

b Concentration of primers NS-7 F and NS-8-R were optimised for potato heel end core extraction using the homogenisation method and DNA purification according to Pastrik (2000) (see section 6.1.a) and 6.2). Re-optimisation of reagent concentrations will be required if extraction by shaking or other DNA isolation methods are used.

1.3. PCR reaction conditions

Run the following programme:

1 cycle of:	(i)	3 minutes at 95 °C (denaturation of template DNA)
10 cycles of:	(ii)	1 minute at 95 °C (denaturation of template DNA)
	(iii)	1 minute at 64 °C (annealing of primers)
	(iv)	1 minute at 72 °C (extension of copy)
25 cycles of:	(v)	30 seconds at 95 °C (denaturation of template DNA)
	(vi)	30 seconds at 62 °C (annealing of primers)
	(vii)	1 minute at 72 °C (extension of copy)
1 cycle of:	(viii)	5 minutes at 72 °C (final extension)
	(ix)	hold at 4 °C.

Note:

This programme is optimised for use with an MJ Research PTC 200 thermal cycler. Modification of the duration steps of cycles (ii), (iii) (iv), (v), (vi) and (vii) may be required for use with other models.

1.4. Restriction enzyme analysis of amplicon.

PCR products amplified from *C. m.* subsp. *sepedonicus* DNA produce a distinctive restriction fragment length polymorphism with enzyme *Bgl* II after incubation at 37 °C for 30 minutes. The restriction fragments obtained from *C. m.* subsp. *sepedonicus*-specific fragment are 282 bp and 220 bp in size.