[F1 ANNEX II

TEST SCHEME FOR DIAGNOSIS, DETECTION AND IDENTIFICATION OF *RALSTONIA SOLANACEARUM* (SMITH) YABUUCHI *ET AL.*

Textual Amendments

F1 Substituted by Commission Directive 2006/63/CE of 14 July 2006 amending Annexes II to VII to Council Directive 98/57/EC on the control of Ralstonia solanacearum (Smith) Yabuuchi et al..

SECTION VI

OPTIMISED PROTOCOLS FOR DETECTION AND IDENTIFICATION OF *R. SOLANACEARUM*

B. IDENTIFICATION TESTS

Identify pure cultures of presumptive *R. solanacearum* isolates using at least two of the following tests based on different biological principles.

Include known reference strains where appropriate for each test performed (see Appendix 3).

1. Nutritional and enzymatic identification tests

Determine the following phenotypic properties, which are universally present or absent in *R. solanacearum*, according to the methods of Lelliott and Stead (1987), Klement *et al.* (1990), Schaad (2001).

Test	Expected result
Fluorescent pigment production	–
Poly-ß-hydroxybutyrate inclusions	+
Oxidation/fermentation (O/F) test	O+/F–
Catalase activity	+
Kovac's oxidase test	+
Reduction of nitrate	+
Utilisation of citrate	+
Growth at 40 °C	–
Growth in 1 % NaCl	+
Growth in 2 % NaCl	–
Arginine dihydrolase activity	–
Gelatine liquefaction	–
Starch hydrolysis	–
Aesculin hydrolysis	–
Levan production	–

2. IF test

2.1. Prepare a suspension of approximately 10^6 cells per ml in IF buffer (Appendix 4).

2.2. Prepare a twofold dilution series of an appropriate antiserum (see website http://forum.europa.eu.int/Public/irc/sanco/Home/main).

2.3. Apply the IF procedure (Section VI.A.5.).

2.4. A positive IF test is achieved if the IF titre of the culture is equivalent to that of the positive control.

3. ELISA test

Note: If performing only 2 identification tests, do not use another serological test in addition to this method.

3.1. Prepare a suspension of approximately 10^8 cells per ml in 1X PBS (Appendix 4).

3.2. Perform an appropriate ELISA procedure with a specific monoclonal antibody to *R. solanacearum*.

3.3. A positive ELISA test is achieved if the ELISA reading obtained from the culture is at least half that obtained for the positive control.

4. PCR tests

4.1. Prepare a suspension of approximately 10^6 cells per ml in molecular grade sterile water.

4.2. Heat 100 µl of the cell suspension in closed tubes in a heating block or boiling waterbath at 100 °C for four minutes. The samples may then be stored at -16 to -24 °C until required.

4.3. Apply appropriate PCR procedures to amplify *R. solanacearum*-specific amplicons (e.g. Seal *et al.* (1993); Pastrik and Maiss (2000); Pastrik *et al.* (2002); Boudazin *et al.* (1999); Opina *et al.* (1997), Weller *et al.* (1999).

4.4. A positive identification of *R. solanacearum* is achieved if the PCR amplicons are the same size and have the same restriction fragment length polymorphisms as for the positive control strain.

5. FISH test

5.1. Prepare a suspension of approximately 10^6 cells per ml in UPW.

5.2. Apply the FISH procedure (Section VI.A.7.) with at least 2 *R. solanacearum*-specific oligo-probes (Appendix 7).

5.3. A positive FISH test is achieved if the same reactions are achieved from the culture and the positive control.

6. Fatty acid profiling (FAP)

6.1. Grow the culture on trypticase soy agar (Oxoid) for 48 hours at 28 °C.

6.2. Apply an appropriate FAP procedure (Janse, 1991; Stead, 1992).

6.3. A positive FAP test is achieved if the profile of the presumptive culture is identical to that of the positive control. The presence of characteristic fatty acids are 14:0 3OH, 16:0 2OH, 16:1 2OH and 18:1 2OH and absence of 16:0 3OH is highly indicative of a *Ralstonia* sp.

7. Strain characterisation methods

Strain characterisation using one of the following methods is recommended for each new case of isolation of *R. solanacearum*.

Include known reference strains where appropriate for each test performed (see Appendix 3).

7.1. Biovar determination

R. solanacearum is separated into biovars on the basis of the ability to utilise and/or oxidise three disaccharides and three hexose alcohols (Hayward, 1964 and Hayward *et al.*, 1990). Growth media for the biovar test is described in Appendix 2. The test can be successfully performed by stab inoculating the media with pure cultures of *R. solanacearum* isolates and incubating at 28 °C. If the media are dispensed into sterile 96 well cell culture plates (200 µl per well) colour change from olive green to yellow can be observed within 72 hours, indicating a positive test result.

	Biovar				
	1	**2**	**3**	**4**	**5**
Utilisation of:					
Maltose	–	+	+	–	+
Lactose	–	+	+	–	+
D (+) Cellobiose	–	+	+	–	+
Mannitol	–	–	+	+	+
Sorbitol	–	–	+	+	–
Dulcitol	–	–	+	+	–

Additional tests differentiate biovar 2 sub-phenotypes

	Biovar 2A(Worldwide distribution)	**Biovar 2A(Found in Chile and Colombia)**	**Biovar 2T(Found in tropical areas)**
Utilisation of trehalose	–	+	+
Utilisation of *meso*-inositol	+	–	+
Utilisation of D ribose	–	–	+
Pectolytic activity[a]	low	low	high

a See Lelliott and Stead (1987)

7.2. Genomic fingerprinting

Molecular differentiation of strains in the *R. solanacearum* complex can be achieved using several techniques, including:

7.2.1. Restriction fragment length polymorphism (RFLP) analysis (Cook *et al.*, 1989).

7.2.2. Repetitive sequence PCR using REP, BOX and ERIC primers (Louws *et al.*, 1995; Smith *et al.*, 1995).

7.2.3. Amplified fragment length polymorphism (AFLP) analysis (Van der Wolf *et al.*, 1998).

7.3. PCR methods

Specific PCR primers (Pastrik *et al*, 2002; see Appendix 6) can be used to differentiate strains belonging to division 1 (biovars 3, 4 and 5) and division 2 (biovars 1, 2A and 2T) of *R. solanacearum*, as originally defined by RFLP analysis (Cook *et al.*, 1989) and 16S rDNA sequencing (Taghavi *et al.*, 1996).**]**