[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 II

DETAILED METHODS FOR DETECTION OF *RALSTONIA SOLANACEARUM* IN POTATO TUBERS AND POTATO, TOMATO OR OTHER HOST PLANTS WITH SYMPTOMS OF BROWN ROT OR BACTERIAL WILT

1. Symptoms (see website http://forum.europa.eu.int/Public/irc/sanco/Home/main)

1.1. Symptoms on potato

The potato plant. The early stage of infection in the field is recognised by wilting of the leaves towards the top of the plant at high temperatures during the day with recovery at night. In early stages of wilting leaves remain green, but later yellowing and brown necrosis develops. Epinasty also occurs. Wilting of one shoot or whole plants becomes rapidly irreversible and results in the collapse and death of the plant. The vascular tissue of transversely cut stems from wilted plants usually appears brown and a milky bacterial ooze exudes from the cut surface or can be expressed by squeezing. When a cut stem is placed vertically in water, threads of slime will stream from the vascular bundles.

The potato tuber. Potato tubers must be cut transversely close to the heel (stolon end) or longitudinally over the stolon end. The early stage of infection is recognised by a glassy yellow to light brown discolouration of the vascular ring from which a pale cream bacterial ooze emerges spontaneously after some minutes. Later, the vascular discolouration becomes a more distinct brown and necrosis can extend into the parenchymatous tissue. In advanced stages, infection breaks outwards from the heel end and the eyes from which bacterial slime may ooze causing soil particles to adhere. Reddish-brown slightly sunken lesions may appear on the skin due to collapse of vascular tissues internally. Secondary development of fungal and bacterial soft rots is common in the advanced stages of the disease.

1.2. Symptoms on tomato

The tomato plant. The first visible symptom is the flaccid appearance of the youngest leaves. Under favourable environmental conditions for the pathogen (soil temperatures of approximately 25 °C; saturated humidity), epinasty and wilting of one side or of the whole plant follows within a few days leading to total plant collapse. Under less favourable conditions (soil temperature below 21 °C), less wilting occurs, but large numbers of adventitious roots may develop on the stem. It is possible to observe watersoaked streaks from the base of the stem which is evidence of necrosis in the vascular system. When the stem is cut crosswise, discoloured brown vascular tissues exude white or yellowish bacterial ooze.

1.3. Symptoms on other hosts

Solanum dulcamara and S. nigrum plants. Under natural conditions, wilting symptoms are rarely observed in these weed hosts unless soil temperatures exceed 25 °C or inoculum levels are extremely high (e.g. as for *S. nigrum* growing adjacent to diseased potato or tomato plants). When wilting does occur, the symptoms are as described for tomato. Non-wilting *S. dulcamara* plants growing with stems and roots in water may show internal light brown discolouration of vascular tissues on transverse section of the stem base or underwater stem parts. Bacteria may ooze from cut vascular tissues or form threads of slime if the cut stem is placed vertically in water, even in the absence of wilting symptoms.

2. Rapid screening tests

Rapid screening tests may facilitate presumptive diagnosis but are not essential. Use one or more of the following validated tests:

2.1. Stem streaming test

(See Section VI.A.1.)

2.2. Detection of poly-ß-hydroxybutyrate (PHB) granules

Characteristic PHB granules in the cells of *R. solanacearum* are visualised by staining heat-fixed smears of bacterial ooze from infected tissue on a microscope slide with Nile Blue A or Sudan Black (See Section VI.A.2.).

2.3. Serological agglutination tests

(See Section VI.A.3.)

2.4. Other tests

Further appropriate rapid screening tests include the IF test (see Section VI.A.5.), FISH test (see Section VI.A.7.), ELISA tests (see Section VI.A.8.) and PCR tests (see Section VI.A.6).

3. Isolation procedure

(a) Remove ooze or sections of discoloured tissue from the vascular ring in the potato tuber or from the vascular strands in stems of potato, tomato or other wilting host plants. Suspend in a small volume of sterile distilled water or 50mM phosphate buffer (Appendix 4) and leave for 5 to 10 minutes.

(b) Prepare a series of decimal dilutions of the suspension.

(c) Transfer 50-100 µl of the suspension and dilutions to a general nutrient medium (NA, YPGA or SPA; see Appendix 2) and/or to Kelman's tetrazolium medium (Appendix 2) and/or a validated selective medium (e.g. SMSA; see Appendix 2). Spread or streak with an appropriate dilution plating technique. If useful, prepare separate plates with a diluted cell suspension of *R. solanacearum* biovar 2 as a positive control.

(d) Incubate the plates for two to six days at 28 °C.

— On the general nutrient media, virulent isolates of *R. solanacearum* develop pearly cream-white, flat, irregular and fluidal colonies often with characteristic whorls in the centre. Avirulent forms of *R. solanacearum* form small round non-fluidal, butyrous colonies which are entirely cream-white.

— On Kelman's tetrazolium and SMSA media, the whorls are blood red in colour. Avirulent forms of *Ralstonia solanacearum* form small round non-fluidal, butyrous colonies which are entirely deep red.

4. Identification tests for *R. solanacearum*

Tests to confirm identity of presumptive isolates of *R. solanacearum* are shown in Section VI.B.**]**