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EPPO STANDARD ON DIAGNOSTICS

PM 7/21 (3) *Ralstonia solanacearum*, *R. pseudosolanacearum* and *R. syzygii* (*Ralstonia solanacearum* species complex)

Specific scope: This Standard describes a diagnostic protocol for *Ralstonia solanacearum*, *Ralstonia pseudosolanacearum* and *Ralstonia syzygii*, i.e. Phylotype/sequevar strain in the *Ralstonia solanacearum* Species Complex (RSSC).[1]
It should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*.
Authors and contributors are given in the Acknowledgements section.
Specific approval and amendment: Approved in 2003–09. First revised in 2018–02. Second revision in 2021–12.

1 | INTRODUCTION

Ralstonia solanacearum (Smith 1896) Yabuuchi et al., 1996 is included in the EPPO A2 List of pests recommended for regulation and in many EPPO members' lists of regulated pests. It has been described by Fegan and Prior (2005) as distributing into four Phylotypes in a species complex. Each Phylotype comprises multiple phylogenetic and pathogenic variants differing in barcoding genes (including ITS, *hrp*B, *mut*S and *egl*), known as sequevars. Historically, the species complex has been recognized as a number of phenotypically diverse strains that were originally placed into five pathogenic races and five biovars (Buddenhagen et al., 1962; Hayward, 1964). Recently, it has been reclassified by Safni et al. (2014) into three distinct species: *R. solanacearum* (Phylotype II), *Ralstonia pseudosolanacearum* (Phylotypes I and III) and *Ralstonia syzygii* (Phylotype IV). *Ralstonia syzygii* comprises three subspecies: subsp. *syzygii* found on *Syzygium aromaticum* (clove), subsp. *celebesensis* found on banana and subsp. *indonesiensis* found on *Solanum tuberosum* (potato), *Solanum lycopersicum* (tomato), *Capsicum annuum* (chilli pepper) and *Syzygium aromaticum* (clove). These three species are covered by this diagnostic protocol under the taxonomy of 'Phylotype-sequevar', which clearly separates Phylotype I and III strains, and other epidemiologically distinct strains (called ecotype) in Phylotype II (causing potato brown rot or Moko disease).

Phylotype I strains are referenced to be of Asian origin, Phylotype II strains are referenced as of South American origin, whereas Phylotype III strains have evolved in Africa and Phylotype IV strains in Indonesia. Their importance is compounded by the large range of economically important hosts, which include *S. tuberosum*, *S. lycopersicum*, *S. melongena* (eggplant), *Musa* spp. (banana and plantain), *Nicotiana tabacum* (tobacco) and many ornamental plants. One genotype assigned to Phylotype IIB sequevar 1 (IIB-1), formerly referred to as race 3 biovar 2 and known as the causal agent of potato brown rot, is of particular importance to the EPPO region, having spread around the world from South America with the movement of infected seed potato and pelargonium cuttings. This genotype can cause wilting in areas with a temperate climate while most other genotypes are more adapted to tropical climates. This genotype is already present in the EPPO region, usually with few occurrences or restricted distribution, but has the potential to spread. It mostly attacks cultivated solanaceous plants, such as *S. tuberosum*, *S. lycopersicum*, *S. melongena* and *C. annuum*, as well as some solanaceous weeds, such as *Solanum nigrum* (black nightshade) and *Solanum dulcamara* (woody nightshade), without causing symptoms. In temperate EPPO countries, symptomless infection of *S. dulcamara* growing with roots along banks of surface water bodies is an important factor in disease epidemiology (Elphinstone et al., 1998; Janse et al., 1998; Wenneker et al., 1999). It has also been found on non-solanaceous hosts, including *Pelargonium zonale* (horse-shoe pelargonium), and has spread from Africa and Central America via the international trade in young plants (Strider et al., 1981; Janse et al., 2002). The reported host range of the different species is listed in the EPPO Global Database (EPPO, 2021a, 2021b, 2021c, 2021d).

Within Phylotype II, Genotypes IIA-6, IIA-24, IIB-3 and IIB-4, and some strains from IIA-41, IIA-53 and IIB-25 were described as causing the Moko disease of *Musa* spp. and *Heliconia* (Albuquerque et al., 2014). These occur mainly in South and Central America and the Caribbean but are also reported on cooking banana genotypes ABB and BBB in the Philippines (causing so-called Bugtok disease). A variant of *R. solanacearum* Phylotype IIB-4, which is not pathogenic on banana,

[1]Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

named Phylotype IIB-4NPB strain, was found to affect cucurbits and *Anthurium* in Martinique and has also been found in Brazil, Costa Rica and Trinidad (Wicker et al., 2007). Other susceptible hosts for both Moko and NPB strains include *S. tuberosum*, *S. lycopersicum*, *C. annuum*, *S. melongena*, *Impatiens hawkerii* (New-Guinea impatiens), *Heliconia caribaea* (wild heliconia) and some weeds (*Portulaca oleracea*, *Cleome viscosa* and *Solanum americanum*). Plantain could also be latently infected following inoculation. In Portugal, a virulent strain of *R. solanacearum* Phylotype IIA-50 (broad host range, identified as biovar 1) was found on potato and caused rapid wilting of lower leaves, yellowing and reduced growth of the whole plant and could also cause symptoms on *S. lycopersicum*, *N. tabacum*, *S. melongena*, *Pelargonium* spp. and *Eucalyptus globulus* (Cruz et al., 2008); This same sequevar was also found in Uruguay (Siri et al., 2011). Some Phylotype I strains behave similarly to this virulent strain of *R. solanacearum* Phylotype IIA-50.

Strains that were previously designated as the broadest 'race 1' occur in tropical areas all over the world, attacking many hosts in over 50 plant families. Some of these strains have occasionally been introduced into the EPPO region with ornamental/herbal plants or plant parts of tropical origin and can cause disease under greenhouse conditions in temperate climates, for example in *Curcuma longa* (turmeric), *Anthurium*, *Epipremnum* or more recently *Rosa* spp. (Tjou-Tam-Sin et al., 2017). Within *R. pseudosolanacearum*, some strains with specific sequevars affect *Zingiber officinale* (ginger) (Prameela and Suseela Bhai, 2020) and *Morus alba* (mulberry) (Ren et al., 1981; He et al., 1983).

For the geographical distribution of the species see the EPPO Global Database online (EPPO, 2021a, 2021b, 2021c, 2021d).

Flow diagrams describing the diagnostic procedure for *R. solanacearum*, *R. pseudosolanacearum* and *R. syzygii* in potato tubers and potato plant samples (symptomatic and asymptomatic) and in water samples are presented in Figures 1 and 2, respectively.

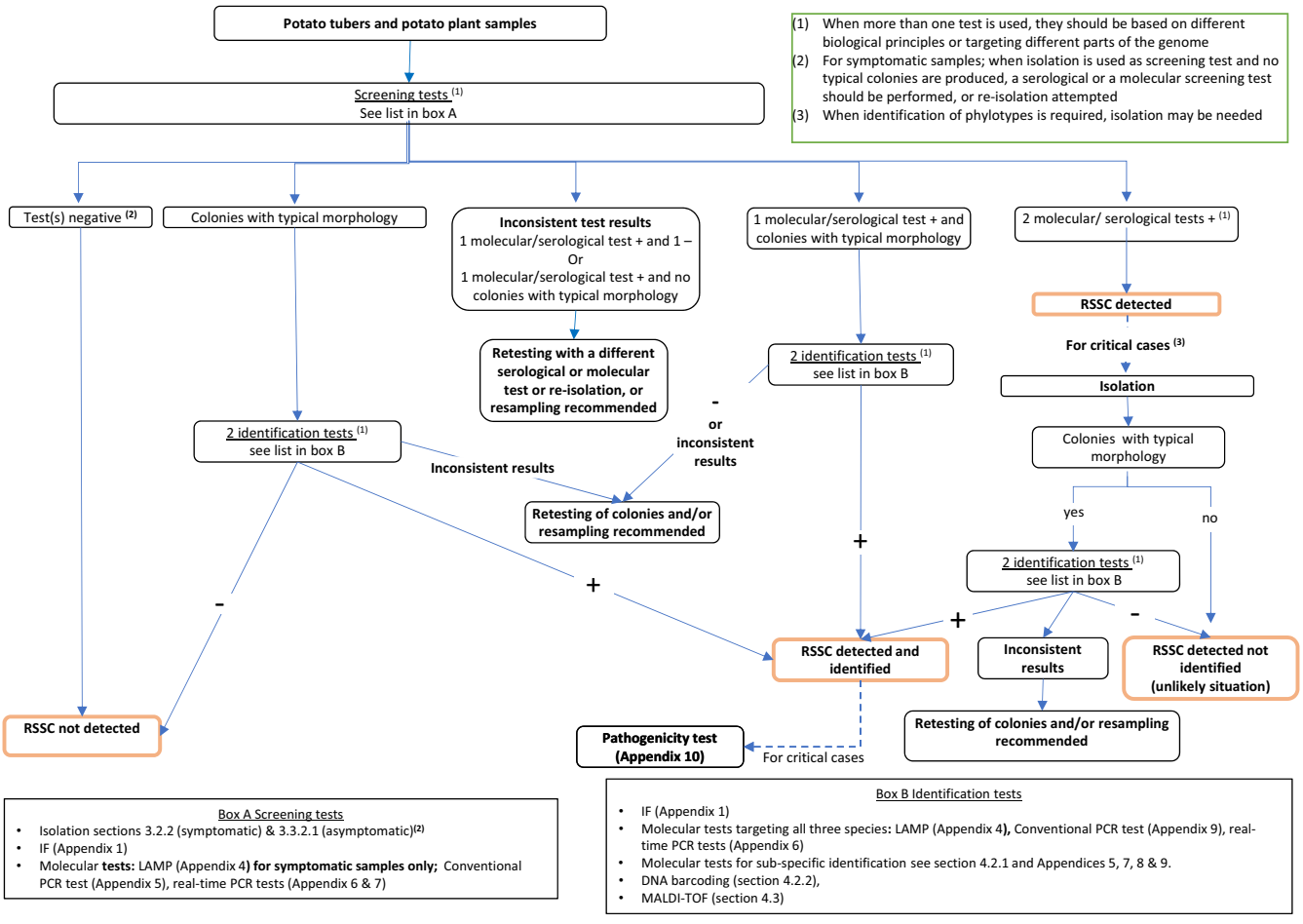

FIGURE 1 Flow diagram describing the diagnostic procedure for *Ralstonia solanacearum*, *Ralstonia pseudosolanacearum* and *Ralstonia syzygii* (*Ralstonia solanacearum* species complex, RSSC) in potato tubers and potato plant samples (symptomatic and asymptomatic). This flow diagram may also be implemented for other hosts, but specific validation data is limited (the current knowledge on the use of different tests with matrices other than potato is presented in Table S1 in the supplementary information)

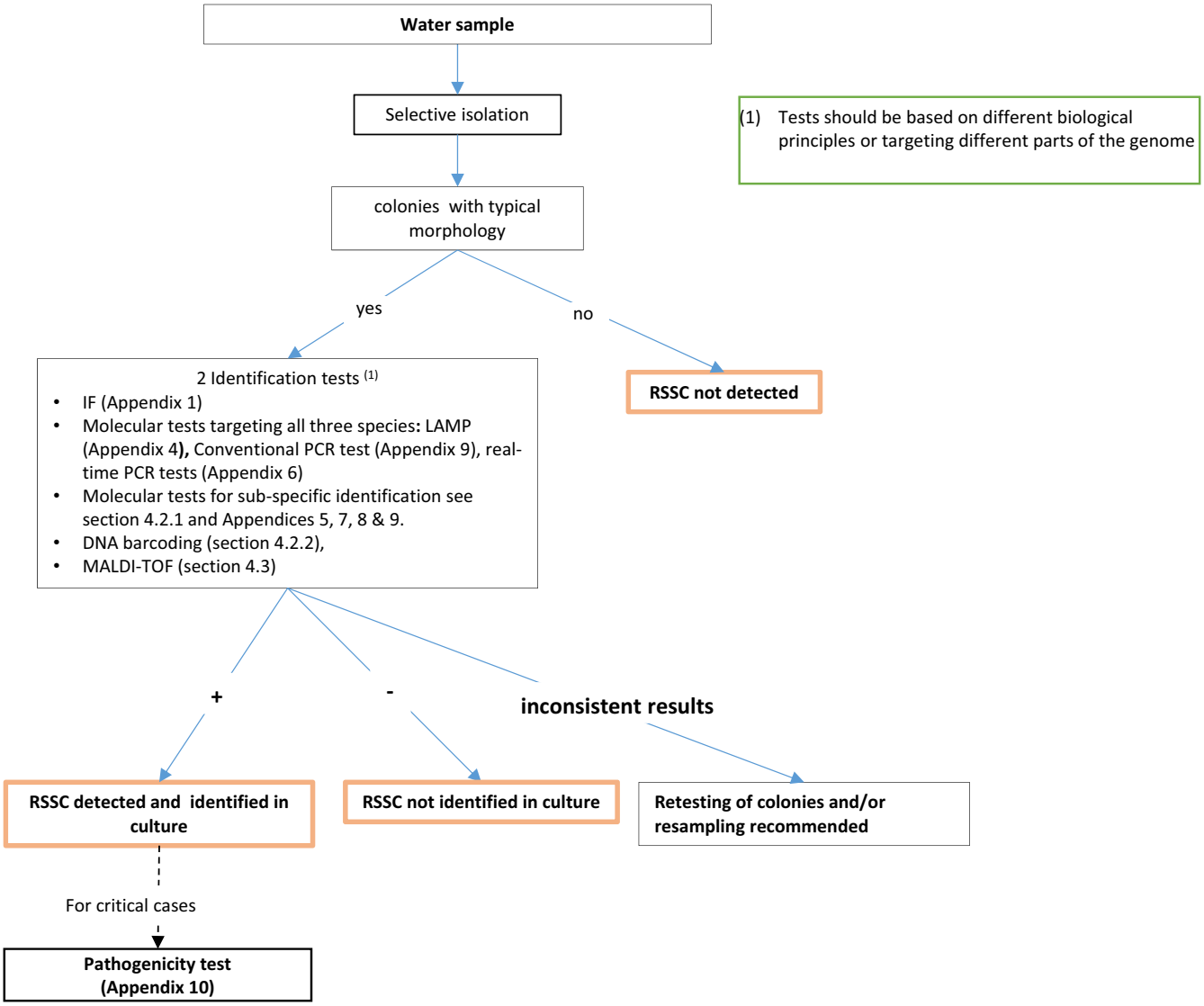

FIGURE 2 Flow diagram describing the diagnostic procedure for *Ralstonia solanacearum*, *Ralstonia pseudosolanacearum* and *Ralstonia syzygii* (*Ralstonia solanacearum* species complex, RSSC) in water samples

2 | IDENTITY

Name: *Ralstonia solanacearum* (Smith 1896) Yabuuchi et al., 1996 emend. Safni et al., 2014.
Synonyms: *Ralstonia solanacearum* (Smith 1896) Yabuuchi et al., 1996; *Burkholderia solanacearum* (Smith 1896) Yabuuchi et al., 1992; *Pseudomonas solanacearum* (Smith 1896) Smith, 1914; many other synonyms in older literature.
Taxonomic position: Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Burkholderiaceae.
EPPO Code: RALSSL.
Phytosanitary categorization: EPPO A2 List no. 58, EU Annex designation II/B.

Name: *Ralstonia pseudosolanacearum* Safni et al., 2014.
Synonyms: *Ralstonia solanacearum* (Smith 1896) Yabuuchi et al., 1996; *Burkholderia solanacearum* (Smith 1896) Yabuuchi et al., 1992; *Pseudomonas solanacearum* (Smith 1896) Smith, 1914; many other synonyms in older literature.
Taxonomic position: Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Burkholderiaceae.
EPPO Code: RALSPS.
Phytosanitary categorization: EPPO A2 List no. 401, EU Annex designation II/A.

Name: *Ralstonia syzygii* (Roberts et al. 1990) Vaneechoutte et al., 2004 emend. Safni et al., 2014.
Synonyms: *Ralstonia solanacearum* (Smith 1896) Yabuuchi et al., 1996; *Burkholderia solanacearum* (Smith 1896) Yabuuchi et al., 1992; *Pseudomonas solanacearum* (Smith 1896) Smith, 1914; many other synonyms in older literature.
Taxonomic position: Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Burkholderiaceae.
EPPO Code: RALSSY.
Phytosanitary categorization: EPPO A1 List no. 400. EU Annex designation II/A (for *Ralstonia syzygii* subsp. *celebesensis* and *Ralstonia syzygii* subsp. *indonesiensis*).

FIGURE 3 Foliar symptoms on potato caused by *Ralstonia solanacearum* (Phylotype IIB-1). Courtesy Defra, Crown Copyright

FIGURE 4 Bacterial ooze from potato eye. Courtesy J. Janse, PD Wageningen (NL)

3 | DETECTION

3.1 | Symptoms

Symptoms are described for the main hosts. Information on symptoms is also available in the EPPO Datasheets (EPPO, 2021a, 2021b, 2021c, 2021d) for the relevant *Ralstonia* species.

3.1.1 | *Solanum tuberosum*

Foliar symptoms include rapid wilting of leaves and stems, usually first visible in single stems (Figure 3) at the warmest time of day. Eventually, plants fail to recover and become yellow and then necrotic. As the disease develops, a streaky brown discoloration may be observed on stems above the soil line, and the leaves may have a bronze tint. Epinasty of the petioles may occur. A white, slimy mass of bacteria often exudes from cut or broken vascular bundles. On tubers, external symptoms may or may not be visible, depending on the state of development of the disease. Infection eventually results in bacterial ooze emerging from the eyes and stolon end attachment of infected tubers (Figure 4). Soil may adhere to the tubers at the eyes. Cutting the diseased tuber will reveal a browning and eventual necrosis of the vascular ring and immediately surrounding tissues. A creamy fluid exudate usually appears spontaneously on the vascular ring of the cut surface a few minutes after cutting (Figure 5). In the case of ring rot the tuber has to be

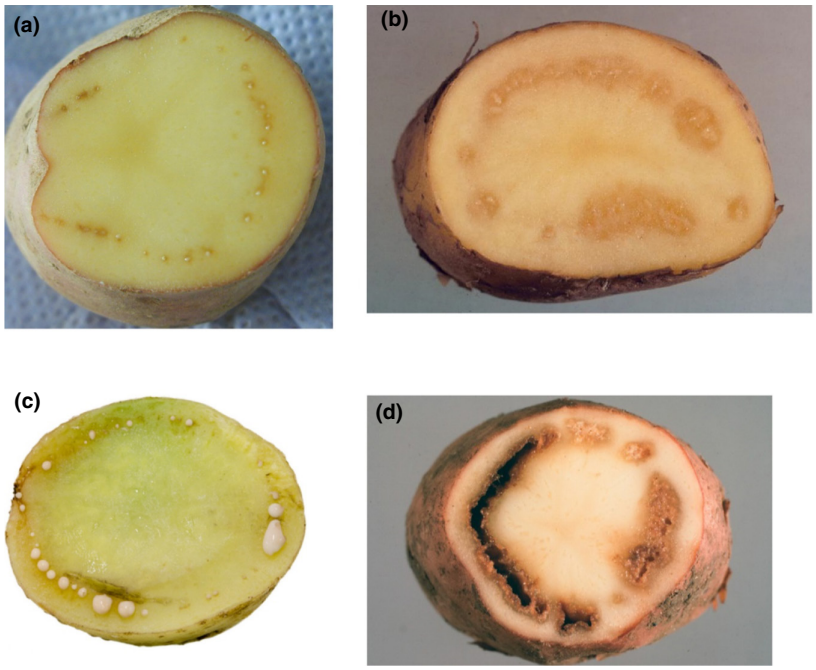

FIGURE 5 Symptoms of brown rot caused by Phylotype IIB-1 on cut tubers from early to advanced symptoms (a–d, respectively). (a) Courtesy J. van Vaerenbergh (ILVO, BE). (b), (d) Courtesy Defra, Crown Copyright. (c) Courtesy G. Cellier (Anses, FR)

FIGURE 6 Wilting of tomato caused by (a) *Ralstonia pseudosolanacearum* and (b) *Ralstonia solanacearum*. (c) Bacteria oozing from cut xylem vessels. (a) Courtesy G. Cellier (Anses, FR). (b), (c) Courtesy Defra, Crown Copyright.

FIGURE 7 Wilting of eggplant caused by *Ralstonia solanacearum* (Phylotype II). Courtesy M. Bergsma Vlami (NPPO-NL)

squeezed to press out a mass of yellowish macerated vascular tissue and bacterial slime. Lenticel infections have been described by Rodrigues-Neto et al. (1984). Plants with foliar symptoms may produce healthy and diseased tubers, while plants that show no signs of wilting may sometimes produce diseased tubers.

3.1.2 | *Solanum lycopersicum* and *S. melongena*

The youngest leaves are the first to be affected and have a flaccid appearance, usually at the warmest time of day. Wilting of the whole plant may follow rapidly if environmental conditions are favourable for the pathogen (Figures 6–8). Under less favourable conditions, the disease develops less rapidly, stunting may occur and large numbers of adventitious roots are produced on the stem. The vascular tissues of the stem show a brown discoloration and, if the stem is cut crosswise, drops of white or yellowish bacterial ooze may be visible (Figure 6c).

FIGURE 8 Wilting of eggplant caused by *Ralstonia pseudosolanacearum* (Phylotype I). Courtesy G. Cellier (Anses, FR)

FIGURE 9 Southern wilt of tobacco caused by *Ralstonia solanacearum* Phylotype not known. Courtesy R. J. Reynolds Tobacco Company Slide Set, R. J. Reynolds Tobacco Company, https://bugwood.org/

3.1.3 | *Nicotiana tabacum*

One of the main symptoms is unilateral wilting of leaves and their premature yellowing (Figure 9). Leaves on one side of the plant or even half a leaf may show wilting symptoms. In severe cases, leaves wilt without changing colour and stay attached to the stem. As in tomato, the vascular tissues show a brown discoloration when cut open. The primary and secondary roots may become brown to black.

3.1.4 | *Cucurbit* plants

Symptoms on cucurbits due to PIIB-4NPB develop rapidly from older to younger leaves that may wilt or not. Leaves turn yellow with necrotic lesions between or along major veins (as for *Anthurium*). Plants become flaccid and eventually collapse and die; there are no apparent symptoms on mature fruits (Figure 10).

3.1.5 | *Musa* spp.

Moko disease of banana caused by *R. solanacearum* is easily confused with the disease caused by *Fusarium*

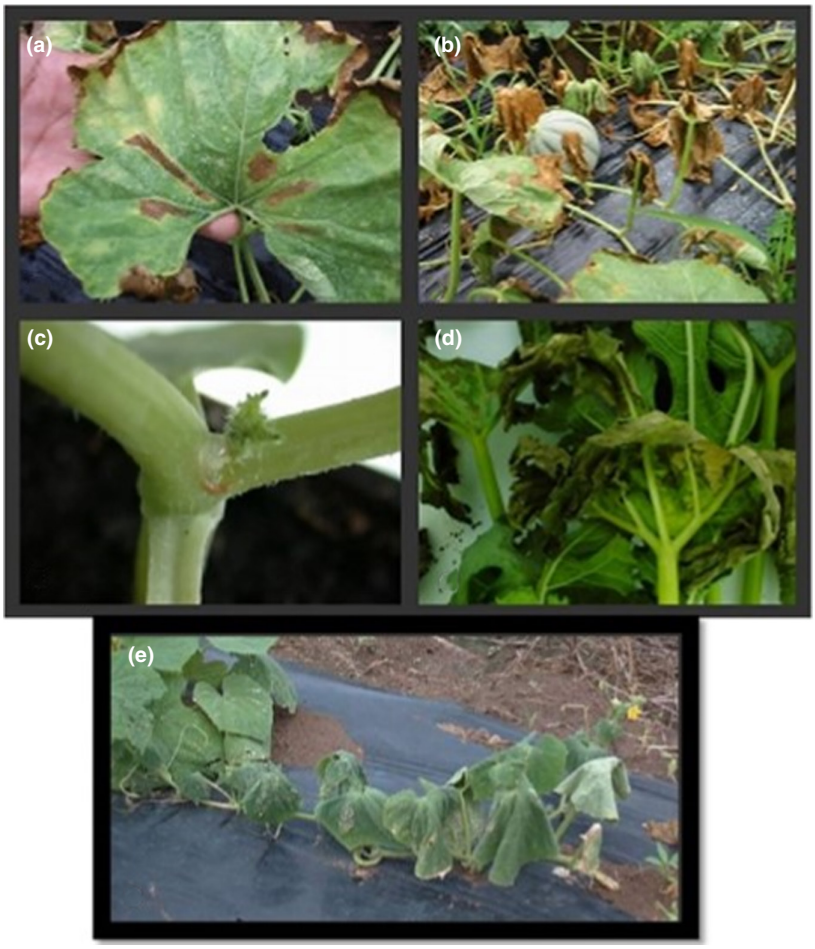

FIGURE 10 Symptoms caused by Phylotype IIB-4NPB on *Cucumis melo* (a, b and c), *Cucurbita pepo* (d) and *Cucumis sativus* (e) (from Wicker et al., 2002)

oxysporum f. sp. *cubense*. A clear distinction is possible when fruits are affected: brown dry rot is seen only in the case of Moko disease. On young and fast-growing plants, the youngest leaves turn pale-green or yellow and wilt (Figure 11). Within a week all leaves may collapse. Young suckers may be blackened, stunted or twisted. The pseudostems show brown vascular discoloration. It is now known that some Moko strains are also able to cause wilt on solanaceous plants, producing the same symptoms as brown rot strains in this type of host.

3.1.6 | Ornamental hosts

3.1.6.1. Pelargonium *(geranium)*

The first symptoms are wilting and subsequent chlorosis (often sectorial yellowing) of leaves (Figure 12a). Stems may blacken and eventually become necrotic. Internally, vascular browning is often visible. Leaves later become brown and necrotic as the whole plant desiccates, collapses and dies (Figure 12b).

3.1.6.2. Curcuma longa

The first symptoms of *R. pseudosolanacearum* (Phylotype I) in *Curcuma* are wilting and subsequent chlorosis of leaves. Stems, including the flower stems (stalks), may acquire a brownish to black discoloration and become necrotic (Figure 13). Similar symptoms may be seen in the roots, including the rhizomes of the plant. Internal vascular browning is often visible. Under favourable environmental conditions, wilting of the whole plant may follow rapidly.

3.1.6.3. Anthurium

Greasy, water-soaked lesions (on the lower leaf surface) turn necrotic with greasy margins (on the upper leaf surface). When the disease becomes systemic, these lesions,

FIGURE 11 Symptoms caused by Moko strain Phylotype IIB-4 on young banana plants (control on the left). Courtesy G. Cellier (Anses, FR)

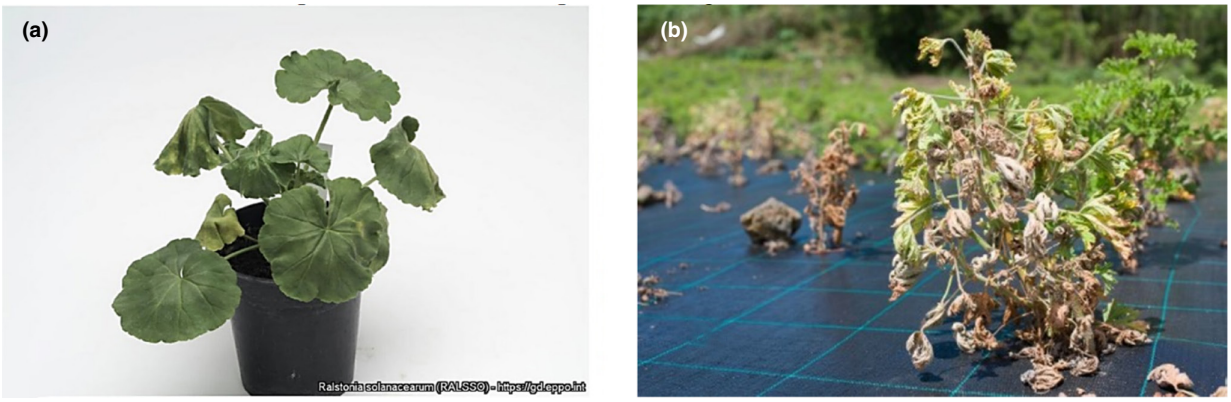

FIGURE 12 Wilting symptoms on *Pelargonium* infected with (a) *Ralstonia solanacearum* and (b) *Ralstonia pseudosolanacearum* (Phylotype III). (a) Courtesy Defra, Crown Copyright. (b) Courtesy G. Cellier (Anses, FR)

FIGURE 13 *Curcuma* plant infected with *Ralstonia pseudosolanacearum* Phylotype I showing a brown discoloration of the base of the stem and of the roots. Courtesy M. Bergsma Vlami (NPPO-NL; left and middle) and J.D. Janse (right)

FIGURE 14 Symptoms caused by *Ralstonia solanacearum* on *Anthurium* spp. Courtesy Coranson-Beaudu and Wicker (Cirad, FR)

generally originating from the insertion point of the leaf with the petiole, develop following the main and secondary veins in a full or partial glove-shape. External infections (disseminated by water) may develop from any natural opening, such as hydathodes. Leaves may turn yellow depending on the severity of the systemic invasion, and the stem may rot with abundant bacterial ooze. The plant eventually collapses and dies (Figure 14).

3.1.6.4. Rosa *spp.*

Initial wilting of young shoots and flower stalks in *Rosa* spp. (Figure 15) is followed by yellowing and early abscission of leaves, stunting, dieback with black necrosis of pruned branches and in some cases purulent discharge of creamy white slime on cut wounds in the stem (Figure 16). Typical symptoms following heavy infections of *R. pseudosolanacearum* (Phylotype I-33) include necrosis of the stems and intense brown discoloration at the stem base (Tjou-Tam-Sin et al., 2017) (Figure 16).

3.1.7 | Possible confusions

Wilting symptoms caused by *Ralstonia* spp. may be confused with those caused by other wilt pathogens, for example *Clavibacter sepedonicus*, *Fusarium* spp., *Verticillium* spp., *Dickeya* spp. on potato, *C. michiganensis* subsp. *michiganensis* on tomato and *Xanthomonas hortorum* on

FIGURE 15 *Rosa* sp. flower stalk with necrotic wilted leaves caused by *Ralstonia pseudosolanacearum* Phylotype I. Courtesy N. Tjou-Tam-Sin (NPPO-NL)

FIGURE 16 *Rosa* sp. (tea rose) dieback with black necrosis of pruned branches and discharge of slime on cut wounds in the stem, *Ralstonia pseudosolanacearum* Phylotype I. Courtesy N. Tjou-Tam-Sin (NPPO-NL)

Pelargonium. Rapid presumptive tests are described in Section 3.2.1.

3.2 | Detection in symptomatic plant material

Detection is by either isolation (Section 3.2.2), IF (Section 3.2.3.1) or molecular PCR tests (Section 3.2.3.2). **Depending on the specificity of the test, *Ralstonia solanacearum*, *R. pseudosolanacearum* and *R. syzygii* can be considered not detected in a sample based on one screening test**. A rapid presumptive test is also described

FIGURE 17 Bacterial ooze from a tomato cut stem suspended in water Courtesy Defra, Crown Copyright

in Section 3.2.1. In critical cases (EPPO, 2017), a positive result from two of these screening tests should be further supported by isolation (if not performed as a screening test) and subsequent identification of the isolated bacterium (Section 4). When identification of phylotypes is required, isolation may also be needed.

3.2.1 | Rapid presumptive diagnostic tests

Bacterial slime oozes spontaneously from the cut surface of infected stems when suspended in water (Figure 17). Such oozing is generally not observed with other bacterial or fungal pathogens and this test is of presumptive diagnostic value, if positive. Immunoassay field test kits, based on lateral flow device technology (Danks & Barker, 2000), are also available (see Appendix 1).

3.2.2 | Isolation from symptomatic host plants

3.2.2.1. Sample preparation for isolation

Sampling may include removal of bacterial ooze or sections of discoloured tissue from the vascular ring in a potato tuber or from the vascular strands in stems/pseudostems of wilting host plants. The sample should then be suspended in a small volume of sterile distilled water or phosphate buffer (PB; 0.05 M) for 5–10 min.

3.2.2.2. Isolation

Isolation from symptomatic material can be performed using general non-selective nutrient media such as nutrient agar (NA), yeast peptone glucose agar (YPGA), sucrose peptone agar (SPA) (Lelliott & Stead, 1987) or Kelman's tetrazolium medium (Kelman, 1954). Some strains may produce a brown diffusible pigment on some media (Figure 18a). Since secondary infections and high populations of saprophytic bacteria are often present, isolation on a semi-selective medium is usually recommended. The semi-selective medium as modified by Elphinstone et al. (1996) (mSMSA) or Sequeira medium (Granada & Sequeira, 1983 modified in Poussier et al., 1999) have been widely used for isolation from symptomatic plant material.

Suspensions should be prepared and aliquots (50–100 µL) transferred to plates of a general nutrient medium (NA, YPGA or SPA; Lelliott & Stead, 1987) and/or to Kelman's tetrazolium medium (Kelman, 1954) and/or semi-selective mSMSA/Sequeira medium (Appendix 2) by spreading or streaking, using an appropriate dilution plating technique. Separate plates can be prepared with diluted cell suspensions of reference strains of the relevant *Ralstonia* spp. as positive control(s). The plates should be incubated for 2–6 days at 28°C.

Colony characteristics (Figure 18). On general nutrient media, virulent isolates develop pearly cream-white, flat, irregular and fluidal colonies (Figure 18a) often with characteristic whorls in the centre. On these media most avirulent forms of *Ralstonia* spp. form small, round, non-fluidal butyrous colonies, which are entirely cream-white.

On Kelman's tetrazolium (Figure 18d) and mSMSA (Figure 18b)/Sequeira medium (Figure 18c), the whorls are blood red in colour. On these media avirulent forms

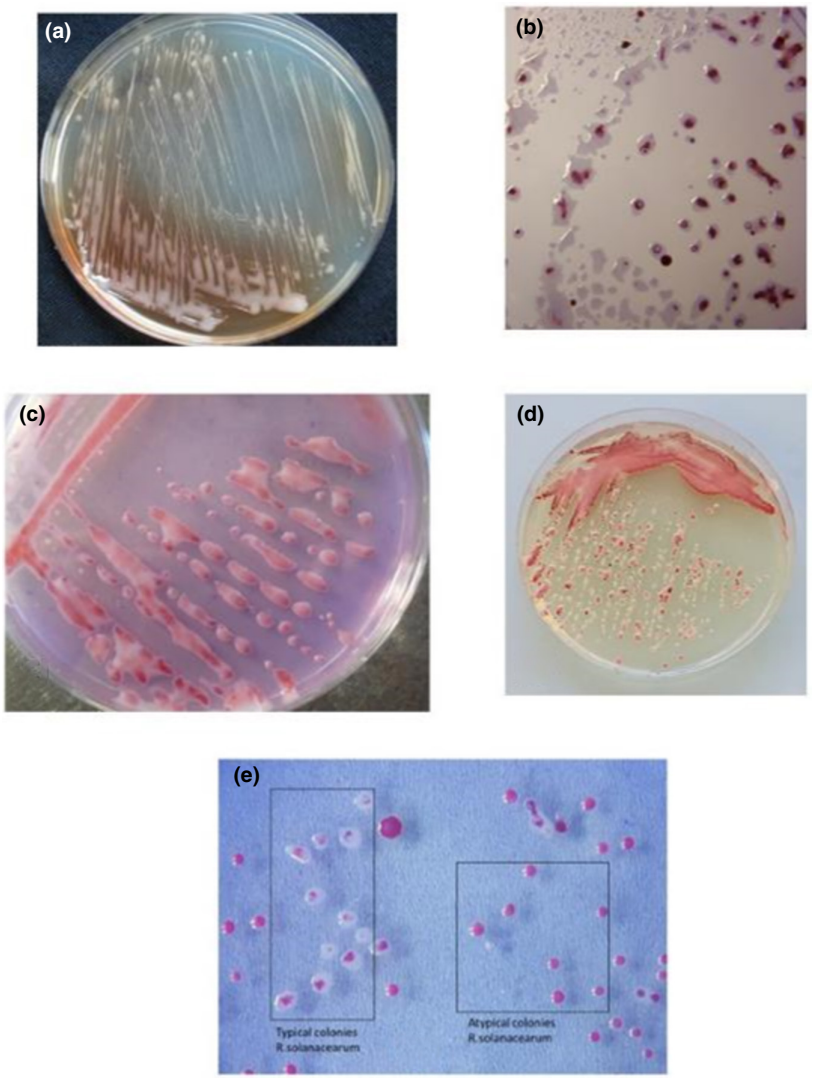

FIGURE 18 Phylotype IIB-1 strain growing on (a) SPA medium, (b) SMSA medium, (c) Sequeira medium, (d) *Ralstonia pseudosolanacearum* on Kelman's media and (e) typical/atypical colonies isolated on mSMSA from water samples. (a), (b) and (c) Courtesy J. van Vaerenbergh (ILVO, BE). (c) and (d) Courtesy G. Cellier G (Anses, FR)

of *Ralstonia* spp. form small, round, non-fluidal butyrous colonies which are entirely deep red and difficult to distinguish amongst other saprophytic bacteria which may be co-isolated. Typical colonies are shown in Figure 18e.

Note that colony morphology may vary with the matrix from which it is isolated.

Isolation of R. syzygii subspecies.

Preliminary work carried at the National Reference Centre (NVWA, NL) has shown that mSMSA can be used for the isolation of *R. syzygii* subsp. *indonesiensis* and subsp. *celebesensis* but not for subsp. *syzygii*. *R. syzygii* subsp. *syzygii* did not grow on SMSA or on mSMSA supplemented with yeast extract.

3.2.3 | Other screening tests

3.2.3.1. Immunofluorescence test

Instructions to perform an immunofluorescence (IF) test are provided in the EPPO Standard PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria*. It is essential to verify the specificity of the antibodies. Sources of validated antibodies are given in Appendix 1. The IF test is usually performed on undiluted or concentrated (see Sections 3.3.1.1 and 3.3.1.2) plant extracts and 10-fold dilutions of these in 10 mM PB (pH 7.2) (see Appendix 2).

3.2.3.2. Molecular tests

Several molecular tests have been developed for the detection and identification of all RSSC Phylotypes. For information on the Phylotypes detected by each test see Table 1. The following tests, routinely used for screening plant material, are described in full in this Standard.

- A loop-mediated isothermal amplification (LAMP) test (Lenarčič et al., 2014) was recently described for diagnosis of *Ralstonia* spp. in symptomatic plants and can be used on site (see Appendix 4).
- Conventional PCR tests, the most widely validated and routinely used screening tests for detection of *R. solanacearum* Phylotype II and *R. pseudosolanacearum* Phylotype I in latently infected potato tubers (Pastrik et al., 2002), are described in Appendix 5.
- TaqMan real-time PCR (Weller et al., 2000) is described in Appendix 6 and can be used to detect all four Phylotypes.
- The NYtor real-time TaqMan PCR test (Vreeburg et al., 2018) is described in Appendix 7 and can be used to detect *R. solanacearum* and *R. pseudosolanacearum*. This test can be used in multiplex to detect *Clavibacter sepedonicus*.
- Duplex PCR (Cellier et al., 2015) for *R. solanacearum* Phylotype IIB-4NPB and Moko strains (sequevars IIB-3, IIB-4, IIA-6, IIA-24, IIB-25, IIA-41 and IIA-53) in *Musa* spp. Is described in Appendix 8.

The selection of the tests to be performed should be made taking into account the Phylotype likely to be present on the sample.

3.2.3.3. Other tests

A number of other tests have been developed but are no longer commonly used. They are consequently not described in this protocol. These include bioassay (Janse, 1988), tests based on fluorescent *in situ* hybridization (FISH; Wullings et al., 1998) and enzyme-linked immunosorbent assay (ELISA; Caruso et al., 2002).

3.3 | Detection in asymptomatic plant material

Screening is performed using an IF test, selective plating on mSMSA and/or a molecular test. **Depending on the analytical specificity and sensitivity of the test *Ralstonia solanacearum*, *R. pseudosolanacearum* and *R. syzygii* can be considered not detected in a sample based on one screening test.** In critical cases (EPPO, 2017), a positive result from two different screening tests should be further supported by isolation (Section 3.2.2.2) and identification of the bacterium (Section 4). The procedures described in this protocol have been standardized and validated in many EU member states through interlaboratory comparisons (e.g. Van Vaerenbergh et al., 2017), although most validation was done on potato tubers.

3.3.1 | Sampling and sample preparation

Screening methods have been validated based on a composite sample size of 200 potato tubers or up to 200 stems from potato or other host plants and pieces randomly collected from the population to be tested. More intensive sampling requires tests on additional samples of 200 tubers. The maximum number of tubers or stems that can be processed in one test is 200, as a higher number may lead to inhibition of the tests or difficulties in interpreting the results. The procedure can be conveniently applied to samples of fewer than 200 tubers or stems.

3.3.1.1. Potato tubers

Potato tubers can be first washed and air dried, if necessary, to remove any excess soil which may contain saprophytic/opportunistic bacteria that may cause false-positive results in the IF test. After removing a small area of peel with a sterile knife from the heel (stolon) end of each tuber, small cores (e.g. 0.2–0.5 g) of the exposed vascular tissue can be removed, keeping the amount of non-vascular tissue to a minimum.

After covering the 200 vascular tuber cores from each sample in sterile 50 mM PB, pH 7.0 (see Appendix 2), the bacteria can be extracted from the tissue by either:

TABLE 1 Molecular tests for detection and identification of *Ralstonia* species and Phylotypes

Reference	Details of test	Primers	*Ralstonia* species and Phylotypes identified
Tests recommended in the protocol			
Weller et al. (2000) or Vreeburg et al. (2016) Appendix 6	Real-time TaqMan® PCR: 16S rRNA gene sequence	RS-I-F/RS-II-R/RS-P (or RSP-55T)	*R. solanacearum* (Phylotype II) *P. pseudosolanacearum* (Phylotypes I and III) *R. syzygii*[a]
Weller et al. (2000) Appendix 6	Real-time TaqMan® PCR: from subtractive hybridization	B2-I-F/B2-II-R/B2-P	*R. solanacearum* (Phylotypes IIB-1, IIB-2 and some other Phylotype IIB isolates of undetermined sequevar)
Pastrik et al. (2002) Appendix 5	Conventional PCR: 16–23S rRNA spacer sequence	RS-1-F/RS-1-R RS-1-F/RS-3-R	*R. solanacearum* (Phylotype II) *R. pseudosolanacearum* (Phylotype I and III[b])
Test based on Fegan and Prior (2005)	Conventional multiplex PCR Phylotypes (Pmx) 16-23S rRNA spacer sequence	Nmult21:1F/Nmult22:RR Nmult21:2F/Nmult22:RR Nmult23:AF/Nmult22:RR	*R. pseudosolanacearum* (Phylotype I) *R. solanacearum* (Phylotype II) *R. pseudosolanacearum* (Phylotype III)
and		Nmult22:InF/Nmult22:RR	*R. syzygii*[a] (Phylotype IV)
Opina et al. (1997) Appendix 9	Conventional PCR: conserved *IpxC* gene sequence	759/760	*R. solanacearum* (Phylotype II) *R. pseudosolanacearum* (Phylotypes I and III) *R. syzygii*[a]
Lenarčič et al. (2014) Appendix 4	LAMP: *egl* gene sequence	F3-Rs/B3-Rs/FIP-Rs/Bip-Rs/ Floop-RS/Bloop-RS	*R. solanacearum* (Phylotype II) *R. pseudosolanacearum* (Phylotypes I and III) *R. syzygii* (strains JV1010 from Indonesia and deviating strain from tomato from Australia, CIRAD RUN14 = ACH732 are not detected)
Cellier et al. (2015) Appendix 8	Conventional duplex PCR: putative *KfrA* and chemotaxis-related protein sequences	93F/93R 5F/5R	Phylotype II Moko strains (all) and Phylotype IIB-4NPB Phylotype IIB-4NPB only
Vreeburg et al. (2018) Appendix 7	Multiplex real-time TaqMan PCR	Rsol_F Rsol_R Rsol_P1 Rsol_P2	*R. solanacearum* (Phylotype II) *P. pseudosolanacearum* (Phylotypes I and III)
Other tests			
Seal et al. (1992)	Conventional PCR: from subtractive hybridization	PS96-H/PS96-I	*R. solanacearum* (Phylotype II) *R. pseudosolanacearum* (Phylotype I)
Seal et al. (1993)	Conventional PCR: 16S rRNA gene sequence	OLI-1/Y-2	*R. solanacearum* (Phylotype II) *R. pseudosolanacearum* (Phylotypes I and III) *R. syzygii*[a]
Fegan et al. (1998)	Conventional PCR: from subtractive hybridization	630/631	*R. solanacearum* (Phylotypes IIB-1, IIB-2 and some other Phylotype IIB isolates of undetermined sequevar)
Boudazin et al. (1999)	Conventional PCR: 16S rRNA gene sequence	D2/B	*R. solanacearum* (Phylotype II) *R. syzygii*[a]

TABLE 1 (Continued)

Reference	Details of test	Primers	*Ralstonia* species and Phylotypes identified
Pastrik and Maiss (2000)	Conventional PCR: 16S rRNA gene sequence	PS1/PS2	*R. solanacearum* (Phylotype II)
			R. pseudosolanacearum (Phylotypes I and III)
			R. syzygii[a]
Ozakman & Schaad, 2003	Real-time TaqMan PCR: from subtractive hybridization	RSC-F/RSC-R/RSC-P	*R. solanacearum* (Phylotypes IIB-1, IIB-2 and some other Phylotype IIB isolates of undetermined sequevar)
Schönfeld et al. (2003)	Conventional PCR: *fliC* gene sequence	Rsol_fliC_for/Rsol_fliC_rev	*R. solanacearum* (Phylotype II)
			R. pseudosolanacearum
			R. syzygii[a]
			Phylotypes covered not all confirmed
Prior and Fegan (2005)	Conventional multiplex PCR Moko (Mmx) from subtractive hybridization.	Mus35-F/Mus35-R	*R. solanacearum* Moko (Phylotype IIB-3)
		Mus20-F/Mus20-R	*R. solanacearum* Moko (Phylotypes IIB-4 and IIB-4NPB)
		Mus06-F/Mus06-R	*R. solanacearum* Moko (Phylotype IIB-4 (PB))
		SI28-F/SI28-R	*R. solanacearum* Moko (Phylotype IIB-6)
Plant Health Australia (2006)	Conventional multiplex PCR: New multiplex-PCR Moko (Mmx) from subtractive hybridization	IS24-F/IS24-R	*R. solanacearum* Moko (Phylotype IIB-3)
		Mus20-F/Mus20-R	*R. solanacearum* Moko (Phylotypes IIB-4 and IIB-4NPB)
		SI28-F/SI28-R	*R. solanacearum* Moko (Phylotype IIB-6)
		VC46-F/VC46-R	*R. solanacearum* Moko (Phylotype IIB-24)
Guidot et al. (2009)	Conventional PCR: from genome comparisons	Multiple primers	*R. solanacearum* (Phylotypes IIB-1 and IIB-2)
Kubota et al. (2011)	LAMP and conventional PCR: from in silico genome comparisons	Various primers	*R. solanacearum* (Phylotypes IIB-1 and IIB-2)
Kubota et al. (2011)	Conventional PCR: from genome comparisons	BDB2400-F/BDB2400-R	*R. syzygii* subsp. *celebesensis* (Phylotype IV-10 blood disease bacterium strains)
Ha et al. (2012)	Real-time PCR with SYBR green detection: phage protein gene (RRSL_02403) sequence	RRSL_2403F/RRSL_2403R	*R. solanacearum* (Phylotypes IIB-1 and IIB-2)
Massart et al. (2014)	Real-time PCR: 16–23S rRNA spacer sequence	Multiraso-F/Multiraso-R/ Multiraso-P	*R. solanacearum* (Phylotype IIB and some Phylotype IIA strains)
Stulberg et al. (2015)	Conventional multiplex PCR: from genome comparisons	Various primers	*R. solanacearum* (Phylotypes IIB-1 and IIB-2 only)

[a]Subspecies targeted not known. When testing for *R. syzygii* with these tests, additional evaluation is needed.
[b]Data generated by the EURL for Pest of Plants on Bacteria.

a. rotary shaking (50–100 rpm) for 4 h below 24°C or for 16–24 h refrigerated, or
b. mechanical homogenization in a sealed bag using a suitable grinding apparatus (e.g. a Homex 6 homogenizer) or rubber mallet.

After decanting the supernatant, it can be clarified either by slow-speed centrifugation (at not more than 180 *g* for 10 min at 4–10°C) or by vacuum filtration (40–100 µm), washing the filter with additional (approximately 10 mL) extraction buffer. The bacterial fraction

can then be concentrated by centrifugation at 7000 *g* for 15 min (or 10 000 *g* for 10 min) at 4–10°C and discarding the supernatant without disturbing the pellet. After resuspending the pellet in 1.5 mL of 10 mM PB, pH 7.2 (Appendix 2) and in case further testing is required, a proportion of the extract (e.g. 500 µL) should be stored with 10–25% (v/v) sterile glycerol at –16°C to –24°C (weeks) or at –68°C to –86°C (months). The remainder of the resuspended pellet should preferably be kept refrigerated (at approximately 4°C) when not processed immediately and used in the following screening tests, which should be optimized before use to enable detection of 10^3–10^4 cells mL^{-1} of a reference strain of the appropriate *Ralstonia* sp. added to a negative sample of resuspended pellet as a positive control.

3.3.1.2. Other asymptomatic host plants

Plant material should be preferably processed immediately or within 72 h if kept refrigerated. Stored stem/pseudostem samples should be refreshed prior to testing by a cross-section at each end to expose freshly cut xylem vessels.

Banana plants

Testing for banana plants is performed on individual plants. The first rolled leaves of the pseudostem should be removed to avoid contamination. The sample to be tested should consist of 2.0 g of freshly excised pseudostem material.

Other plants

Detection of latent *Ralstonia* populations in other symptomless plants (e.g. potato, tomato, eggplant, *Pelargonium* spp., *Curcuma* spp., *Anthurium* sp., *Epipremnum* spp., *Rosa* spp., *S. dulcamara*) is commonly done on composite samples. For outdoor grown plants, pathogen detection will be most reliable during warm growing conditions (>15°C), although natural infections can be detected all year round in the perennial *S. dulcamara* growing in watercourses in temperate regions. Screening tests can be applied to composite samples containing up to 200 stem segments taken from a representative sample of the plant population under investigation. Stem segments (approximately 1–2 cm long) are removed using a disinfected knife from the base of each main stem or lowermost side-shoot just above the soil level. For *S. dulcamara* or other host plants growing in water, 1–2 cm sections are removed from underwater stems or stolons with aquatic roots. Visual examination for internal symptoms (vascular staining or bacterial ooze) can be done at this stage. For in *vitro plants*, stem segments of up to 2 cm from the base of the plant are collected. Any stem segments with symptoms should be set aside and tested separately (see Section 3.2).

Extraction of the bacteria

Stem segments can be briefly disinfected with 70% ethanol, rinsed with sterile water and immediately blotted dry on absorbent paper. After covering the stem segments from each sample in sterile 50 mM PB, pH 7.0 (see Appendix 2), the bacteria can be extracted from the tissue by:

a. rotary shaking (50–100 rpm) for 4 h below 24°C or for 16–24 h refrigerated or
b. mechanical homogenization in a sealed bag using a suitable grinding apparatus (e.g. a Homex 6 homogenizer) or rubber mallet, or
c. particularly for banana plants, samples can be ground in a sterile mortar or chopped with sterile scalpel in 5 mL of extraction buffer, leaving for approximately 10 min before performing the test.

Further clarification of the extract or concentration of the bacterial fraction is not usually required but may be achieved by filtration and/or centrifugation as described in Section 3.3.1.1.

The neat or concentrated sample extract should then be tested immediately or within 2 h if kept at room temperature. If necessary, the remaining extract can be stored at 4–10°C during the testing period, although this may affect the reliability of pathogen isolation. Preferably store the remaining extract with 10–25% (v/v) sterile glycerol at −16 to −24°C or at −68 to −86°C in case further testing is required.

3.3.2 | Screening tests

3.3.2.1. Isolation

When isolation is used as a screening test, it requires careful preparation of the samples to minimise the number of non-target organisms and maximise the possibility of isolating the target organism; spiked plant sample extract controls should be systematically included.

Serial dilutions of the re-suspended pellets or plant extracts should be prepared and aliquots (50–100 µL) spread on plates of semi-selective mSMSA (Appendix 2). The plates should be incubated for 2–6 days at approximately 28°C.

For colony description see Section 3.2.2.2.

The Sequeira medium may be used for other hosts than potato, but selectivity needs to be verified. For bananas (or where the potential presence of *R. solanacearum* Moko strains is suspected), isolation media should be amended with 1 g/L of yeast extract (see Section 3.2.2.2).

It should be noted that isolation may fail when tubers have been refrigerated.

3.2.2.2. Other screening tests

All screening tests described under Section 3.2.3 can be performed, with the exception of the LAMP test (Lenarčič et al., 2014), which has not yet been validated for detection in asymptomatic plant material.

3.4 | Detection in other matrices

3.4.1 | Surface or recirculation water, sewage/industrial effluents

Surface water should be ideally sampled when water temperatures are at or above 15°C. At selected sampling points, surface water can be collected by filling sterile tubes or bottles, ideally at a depth below 30 cm and in the vicinity of any known host plants. For industrial or sewage effluents, samples should be collected from the point of effluent discharge.

It is advisable to take duplicated samples of at least 50 mL, taken at a minimum of three different moments in time per sampling point. Samples should be transported in cool (range 10–15°C) and dark conditions and tested preferably within 24 h.

Dilution plating on semi-selective mSMSA medium or Sequeira medium (Appendix 2) should ensure that any background saprophytic colony-forming populations are diluted out. Plates should be spread with 50–100 µL of sample for each dilution and incubated at 28°C. They should then be observed for typical *R. solanacearum* colonies after 48 h and daily thereafter for up to 6 days.

To improve the likelihood of detecting *Ralstonia* spp. In water, it is recommended to first concentrate bacterial populations using one of the following methods:

1. Centrifugation of 30–50 mL of subsamples at 10 000 *g* for 10 min (or 7000 *g* for 15 min) preferably at 4–10°C, discarding the supernatant and resuspending the pellet in 1 mL of sterile water.
2. Membrane filtration (1 L through a maximum pore size of 0.45 µm) followed by washing the filter in 5–10 mL of 10 mM PB and retention of the washings. This method is suitable for larger volumes of water containing low numbers of saprophytes. If the samples are turbid, this may block the filtration process and saturate the filter. A 1/10 dilution of the sample in sterile water is then highly recommended.

Dilution plating of the concentrated samples can then be performed as above (Section 3.3.2.1).

Concentration is usually not advisable for samples of sewage/industrial effluents since increased populations of competing saprophytic bacteria will inhibit detection of *Ralstonia* spp.

3.4.2 | Detection in soil

Although some survival of the bacteria in soil is reported (see relevant EPPO datasheets; EPPO, 2021a, 2021b, 2021c, 2021d), testing soil is not recommended as it is considered erratic due to the highly dispersed and low population of bacteria in soil. Testing known weed hosts or crop hosts and their volunteers growing in the soil is considered more reliable.

4 | IDENTIFICATION

When isolation is performed, identification should be done using at least two tests, based on different biological principles or targeting two different parts of the genome for molecular tests. Relevant tests are described below. For critical cases (EPPO, 2017), when a positive identification is made, it is recommended to perform a pathogenicity test to confirm infection in the sample.

4.1 | IF

Instructions to perform an IF test are provided in the EPPO Standard PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria*. Commercially available polyclonal antibodies may be subject to false-positive reactions. Sources of validated antibodies are given in Appendix 1. It is essential to verify the phylotype specificity of the antibodies.

4.2 | Molecular methods

4.2.1 | Molecular tests

Several molecular tests are available for rapid identification of the three *Ralstonia* species (see Table 1). It is recommended to use more than one primer set for reliable identification, including at least one test which is known to detect all three species. The choice of other PCR tests will also depend on which species is suspected. The following PCR tests, for which validation data is available, are described in full in this protocol.

Molecular tests targeting all three species:

- A LAMP test Lenarčič et al. (2014), described in Appendix 4
- A real-time TaqMan® PCR test (Weller et al., 2000), described in Appendix 6
- A conventional PCR test (Opina et al., 1997) performed as part of a Phylotype-specific multiplex PCR test, described in Appendix 9.

PCR for subspecific identification:

- Conventional PCR tests (Pastrik et al., 2002), for specific identification of either *R. solanacearum* (Phylotype II) or *R. pseudosolanacearum* (Phylotype I) strains, described in Appendix 5
- Nytor real-time TaqMan PCR test Vreeburg et al. (2018) for specific identification of either *Ralstonia solanacearum* or *R. pseudosolanacearum*, described in Appendix 7

- A duplex PCR (Cellier et al., 2015) for *R. solanacearum* Phylotype IIB-4NPB and Moko strains (sequevars IIB-3, IIB-4, IIA-6, IIA-24, IIB-25, IIA-41 and IIA-53) in *Musa* spp. Described in Appendix 8.
- Phylotype-specific multiplex conventional PCR tests (Fegan & Prior, 2005), described in Appendix 9.

A microarray that allows a multiplex characterization of a given *R. solanacearum* strain within 17 major phylogenetic/ecotype groups has been developed (Cellier et al., 2017). This custom microarray represents a significant improvement in the epidemiological monitoring of *R. solanacearum* strains worldwide, and it has the potential to provide insights for phylogenetic incongruence of RSSC strains based on the host from which the bacterium has been isolated and may be used to indicate potentially emergent strains.

4.2.2 | DNA barcoding

Comparisons of sequenced PCR products amplified from selected housekeeping gene loci allow accurate differentiation of Phylotypes and sequevars within the three *Ralstonia* species from their closest relatives. The EPPO Standard PM 7/129 *DNA barcoding as an identification tool for a number of regulated pests* Appendix 2 (EPPO, 2021a, 2021b, 2021c, 2021d) describes a procedure based on the combination of the two genes to identify the *Ralstonia solanacearum* species complex: *16S* ribosomal DNA (*16S* rDNA) is used for the identification of the genus, and *egl* for the identification of the *Ralstonia solanacearum* species complex, i.e. *Ralstonia solanacearum*, *Ralstonia pseudosolanacearum* and *Ralstonia syzygii*. This protocol is adapted from Wicker et al. (2007). General procedures for sequencing are described in Appendix 7 and 8 of PM 7/129 (EPPO, 2021a, 2021b, 2021c, 2021d). Sequences are available in https://qbank.eppo.int/bacteria/organisms.

4.3 | Matrix-assisted laser desorption/ionization time of flight mass spectrometry

A matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) method for proteomic analysis has been described by Prior et al. (2016) and Bilt et al. (2018). This allows rapid, reliable and robust identification of strains of the three *Ralstonia* species. Database entries (mass spectra profiles, MSPs) specific for the three species were created prior to the routine identification of isolates from plant and water samples. For their routine identification, all individual isolates were included in duplicate by directly depositing harvested 2-day-old bacterial cells from NA plates onto a stainless plate, without having to perform any extraction step. All spectra were obtained in linear positive-ion mode with an *m/z* range of 2000–20 000 Da. Validation of the database entries (MSPs) of reference isolates for the three *Ralstonia* and related species has been performed (Bilt et al., 2018), demonstrating that this method can be successfully used for routine identification.

4.4 | Other tests

4.4.1 | Genomic fingerprinting tests

Ralstonia strains can be reliably identified by matching their unique BOX-PCR genomic fingerprints to those of reference strains of similar identity (see EPPO PM 7/100 *rep*-PCR *tests for identification of bacteria*).

4.4.2 | Fatty acid analysis

Methods for identification using fatty acid methyl ester (FAME) analysis are described by Janse (1991) and in Anonymous (2006).

4.4.3 | General phenotypical characteristics

Differential biochemical characteristics that can help to distinguish the plant pathogenic *Ralstonia* species and subspecies are described in Safni et al. (2014) and Prior et al. (2016).

4.4.4 | Pathogenicity test

The procedure for the pathogenicity test is described in Appendix 10.

5 | REFERENCE MATERIAL

NCPPB [(1)] **325**^{T} = CFBP[(2)] 2047^{T} = **LMG**[(3)] **2299**^{T} (*R. solanacearum* type strain, Phylotype IIA-7, (race 1, biovar 1).

CFBP 3857 = **NCPPB 4156** [*R. solanacearum* Phylotype IIB-1 (race 3, biovar 2)].

NCPPB 2314 = CFBP 1412 [*R. solanacearum* Phylotype IIB-4 (race 2, biovar 1)].

NCPPB 1029^{T} = LMG 9673^{T} [*R. pseudosolanacearum* type strain, Phylotype III-19 (race 1, biovar 1)].

NCPPB 3996 = CFPB 3928 [*R. pseudosolanacearum* Phylotype I-18 (race 1, biovar 3)].

NCPPB 4029 = CFBP 4615 [*R. pseudosolanacearum* Phylotype I (race 4, biovar 4)].

NCPPB 4011 = CFBP 4617 [*R. pseudosolanacearum* Phylotype I-12 (race 5, biovar 5)].

NCPPB 3446^{T} = LMG 10661^{T} = DSMZ[(4)] 7385^{T} [*R. syzygii* subsp. *syzygii* type strain (Phylotype IV)].

LMG 27703^T = DSMZ 27478^T = **CFBP 7288^T** [*R. syzygii* subsp. *indonesiensis* type strain (Phylotype IV)].

LMG 27706^T = DSMZ 27477^T [*R. syzygii* subsp. *celebesensis* type strain (Phylotype IV)].

Depending on the intended use (isolation, pathogenicity test) it is important that the reference strain displays a virulent morphotype on mSMSA. Strains that do so are indicated in bold. These strains also have confirmed virulence in tomato plants.

1. National Collection of Plant Pathogenic Bacteria (NCPPB), Fera, Sand Hutton, York YO411LZ, UK; www.ncppb.fera.defra.gov.uk
2. Collection Française de Bactéries associées aux Plantes (CFBP), CIRM, IRHS-INRA, 42 Rue Georges Morel, CS60057, 49071 Beaucouzé Cedex, France; https://www6.inra.fr/cirm/CFBP-Bacteries-associees-aux-Plantes
3. Belgian Co-ordinated Collections of Micro-organisms (BCCM)/LMG Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent, K. L. Ledeganckstraat 35, B-9000 Gent, Belgium; http://bccm.belspo.be/
4. Leibniz Institute DSMZ–German Collection of Microorganisms and Cell Cultures, Inhoffenstraße 7B, 38124 Braunschweig, Germany; www.dsmz.de/contact.html

6 | REPORTING AND DOCUMENTATION

Guidance on reporting and documentation is given in EPPO Standard PM 7/77 *Documentation and reporting on a diagnosis*.

7 | PERFORMANCE CHARACTERISTICS

When performance characteristics are available, these are provided with the description of the test. Validation data is also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended that this database is consulted as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

8 | FURTHER INFORMATION

Further information on this organism can be obtained from:

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9 | FEEDBACK ON THIS DIAGNOSTIC PROTOCOL

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share, please contact diagnostics@eppo.int.

10 | PROTOCOL REVISION

A periodic review process is in place to identify the need for revision of Diagnostic Protocols. Protocols identified as needing revision are marked as such on the EPPO website.

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This protocol was originally drafted by JD Janse formerly Plant Protection Service NL.

The first revision was prepared by JG Elphinstone, formerly Fera Science Ltd, United Kingdom; M Bergsma-Vlami, Plant Protection Service, Netherlands; J van Vaerenbergh, ILVO Belgium; G Cellier, Anses, France – Reunion Island; F Poliakoff, formerly, Anses, France.

The second revision was prepared by A Aspin Fera Science Ltd, M Bergsma-Vlami & TM Raaymakers Plant Protection Service, Netherlands; J van Vaerenbergh, ILVO Belgium; S Paillard Anses, France, F Poliakoff, formerly, Anses, France; R Vreeburg NAK, Netherlands.

The protocol was reviewed by the Panel on Diagnostics in Bacteriology.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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APPENDIX 1 - VALIDATED ANTIBODIES FOR SEROLOGICAL TESTS

1. Field test kits

Validated lateral flow device (LFD) field test kits for rapid presumptive field diagnosis include:

- Pocket diagnostic® (PD51119; Abingdon Health, York, GB) with monoclonal antibody Y9 raised against *Ralstonia solanacearum* (Phylotype II) reference strain NCPPB 4156 from potato.
- ImmunoStrip® (ISK 33900; Agdia Inc., Elkhart, ID, US) with monoclonal antibody Ps-1 raised against *Ralstonia pseudosolanacearum* reference strain A3380 from peanut.

Performance characteristics available

Analytical sensitivity

- Pocket diagnostic® (PD51119) detected down to (1.0–3.0) × 10^5 cfu mL^{-1} of 5 reference strains of *R. solanacearum* (Phylotype II) in water. Data from Fera.
- ImmunoStrip® (ISK 33900) detection limit in plant extract = 5 × 10^5 cfu mL^{-1}. Data from Agdia.

Analytical specificity

- Pocket diagnostic® (PD51119) showed 100% inclusivity with 29 reference strains of *R. solanacearum* (Phylotype II), 16 reference strains of *R. pseudosolanacearum* (Phylotypes I and III) and 6 reference strains of *R. syzygii* (Phylotype IV) and 100% exclusivity with 15 reference strains of related *Ralstonia* spp. (n = 3), other bacterial pathogens of potato (n = 7) or bacteria known to cross-react with polyclonal antibodies to *R. solanacearum* (n = 5). Data from Fera.
- Monoclonal antibody Ps-1 used in ImmunoStrip® (ISK 33900) showed 100% inclusivity with 73 reference strains belonging to *R. solanacearum* (Phylotype II) and *R. pseudosolanacearum* (Phylotype I) and 100% exclusivity with 73 reference strains from 5 pathovars of *Pseudomonas syringae*, 10 strains of other *Pseudomonas* spp. and 24 strains of four other genera (Alvarez et al., 1993). Data from Agdia.

Diagnostic sensitivity

- Pocket diagnostic® (PD51119) combined sensitivity of 100% compared with real-time TaqMan PCR of Weller et al. (2000), tested on healthy (n = 21) and infected (n = 18) stems of tomato, eggplant and *Solanum dulcamara* and potato tuber samples (200 tubers each) with (n = 4) and without (n = 19) latent infections. Data from Fera.

Diagnostic specificity

- Pocket diagnostic® (PD51119) combined specificity of 87.2% (5 false-positive results with tomato, eggplant and *S. dulcamara* stems). Data from Fera.
- ImmunoStrip® (ISK 33900) diagnostic specificity unknown but no cross-reaction reported with potato, tomato or *Pelargonium* tissues. Unreliable for testing crown and root tissue of *Helleborus*. Data from Agdia.

2. Immunofluorescence

Procedures for performing the immunofluorescence test are described in full in EPPO Standard PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria*. Antibodies for use in this test, which have been validated in EUPHRESCO interlaboratory comparisons (Van Vaerenbergh et al., 2017), include polyclonal antibodies from:

- Loewe Biochemica, Sauerlach, DE (07356).
- Plant Print Diagnostics S.L., Valencia, ES (1546-H/IVIA).
- Prime Diagnostics, Wageningen, NL (B-Rsol_C).

Performance characteristics available

- Loewe Biochemica (07356):

Manufacturers' analytical specificity data shows 100% inclusivity (based on testing of 45 isolates of *R. solanacearum* and 5 isolates of *R. pseudosolanacearum*) and 100% exclusivity (based on testing of 24 isolates of 7 other plant pathogenic, including 12 isolates of pathogens which can affect potato, tomato or *Pelargonium*).

Vreeburg et al. (2016) obtained the following performance data using Loewe Biochemica goat polyclonal antibodies (07356) on spiked extracts prepared from 200 healthy potato heel end cores.

Analytical sensitivity: 100% accurate down to 10^5 cfu mL^{-1} but only 50% and 33% accurate at 10^4 and 10^3 cfu mL^{-1}, respectively. No detection at 10^2 cfu mL^{-1}.

Repeatability: 100% (n = 8).

Reproducibility: 100% (n = 8).

Diagnostic sensitivity: 100%, based on testing 187 routine potato samples (200 tubers each) of which 14 had tested positive and 173 had tested negative according to the full procedure annexed to EU Directive 2006/63/EC.

Diagnostic specificity: 98.3% based on the same samples as described above.

- Plant Print Diagnostics S.L. (1546-H/IVIA):

Manufacturers' analytical specificity data shows 100% inclusivity (based on testing 71 isolates of *R. solanacearum* and *R. pseudosolanacearum*) and 100% exclusivity (based on testing of 56 isolates of 7 genera of other plant pathogenic bacteria).

- Prime Diagnostics (B-Rsol_C):

Manufacturer's analytical specificity data shows 100% inclusivity (based on testing 28 isolates of *R. solanacearum* or *R. pseudosolanacearum*) and 100% exclusivity (based on testing 1 isolate each of 4 other potato pathogens and 1 isolate of *Burkholderia cepacia*). No false-positive results were found when testing healthy plant extracts, including potato leaves or tuber peel, *Pelargonium* leaves or stems, and rose leaves or stems.

APPENDIX 2 - BUFFERS AND MEDIA

All buffers and media are sterilized by autoclaving at 121°C for 15 min, except when stated otherwise. When expecting problems with fungal contamination it is recommended to add antifungal compounds (such as cycloheximide 50–100 mg L^{-1} or nystatin 35 mg L^{-1}).

1. Buffers

Phosphate buffer (PB; 50 mM, pH 7.0) for the extraction and dilution of bacteria from infected tissues

Na_2HPO_4	4.26 g
KH_2PO_4	2.72 g
Distilled water	1.0 L

Additional components may be useful, as follows:
Lubrol flakes (deflocculant) 0.5 g L^{-1}
DC silicone antifoam (antifoam agent) 1.0 mL L^{-1}
tetrasodium pyrophosphate (antioxidant) 1.0 g L^{-1}
Polyvinylpyrrolidone-40000 (PVP-40; binding of PCR inhibitors) 50 g L^{-1}.

Phosphate buffer (10 mM PB, pH 7.2) for resuspension of pelleted extracts.

$Na_2HPO_4 \cdot 12H_2O$	2.70 g
$NaH_2PO_4 \cdot 2H_2O$	0.40 g
Distilled water	1.0 L

GuHCl buffer:

Guanidine hydrochloride	50.95 g
Citric acid monohydrate	0.35 g
Citric acid trisodium (dihydrate)	0.02 g
Polyvinylpyrrolidone 40	2.00 g
EDTA	0.49 g
Triton X-100	0.33 mL
Molecular grade water	To make up to 100 mL

Guanidine hydrochloride is dissolved by first making a slurry of guanidine hydrochloride in 16.7 mL of 96% ethanol before adding the water; pH before autoclaving is 2 to 2.4.

2. Generic media

2.1. Nutrient agar (NA) (Lelliott & Stead, 1987)

Peptone	5.0 g
Yeast extract	3.0 g
NaCl	0.5 g
Microbiological-grade agar	15.0 g
Distilled water	1.0 L

pH is adjusted to 7.2.

2.2. Sucrose peptone agar (SPA) (Lelliott & Stead, 1987)

Sucrose	20.0 g
Peptone	5.0 g
K_2HPO_4	7.0 g
Microbiological-grade agar	12.0 g
Distilled water	1.0 L

pH is adjusted to 7.2.

2.3. Yeast peptone glucose agar (YPGA) (Lelliott & Stead, 1987)

Yeast extract	5.0 g
Bacto Peptone	5.0 g
Glucose	10.0 g
Microbiological-grade agar	15.0 g
Distilled water	1.0 L

pH is adjusted to 7.2.

2.4. Kelman's tetrazolium medium (Kelman, 1954)

Bacto Peptone	11.0 g
Glycerol	6.3 g
Microbiological-grade agar	18.0 g
Distilled water	1.0 L

After autoclaving, cool to 47°C ± 2°C and add 25 mg of 2,3,5-triphenyl-2*H*-tetrazolium chloride and 80 µL Tilt (propiconazole).
pH is adjusted to 7.2.

3. Semi-selective isolation media

3.1. Modified semi-selective medium from South Africa (SMSA) (Elphinstone et al., 1996) (mSMSA)

Bacto Peptone (BD)	10.0 g
Glycerol	5 mL
Bacto Agar (BD)	15.0 g
Casamino acids (BD)	1.0 g
Yeast extract (BD)[a]	1.0 g
Deionized distilled water	1 L

Abbreviations: BD, Becton, Dickinson and Company.
[a] For isolation of *Ralstonia solanacearum* Moko strains or the Phylotype II sequevar 4 (NPB) strain, 1 g of yeast extract should be added to the basal medium (1 L) before autoclaving.

After autoclaving, cool to 45–50°C and add sterilized aqueous stock solutions of the following ingredients to obtain the specified final concentrations:

2,3,5-Triphenyl-2*H*-tetrazolium chloride (Sigma-Aldrich T8877)	0.050 g
Crystal violet (Sigma-Aldrich C0775)	0.005 g
Chloramphenicol (water soluble; Sigma-Aldrich C3175)	0.005 g
Penicillin G (benzylpenicillin sodium salt; Sigma-Aldrich P8431)	825 U
Polymyxin B (sulphate salt; Sigma-Aldrich P1004)	600 000 U
Bacitracin (Sigma-Aldrich B0125)	1250 U
Final pH is usually about 6.5 and does not need adjusting.	

Comments

1. Oxoid Agar No. 1 can be used in place of Bacto Agar (Becton, Dickinson and Company). In this case growth of *R. solanacearum* will be slower or may not occur. Typical colonies of *Ralstonia* spp. may take 1–2 days longer to form and the red coloration may be lighter and more diffuse than on Bacto Agar.
2. Increasing the Bacitracin concentration to 2500 U L^{-1} may reduce populations of competing bacteria without affecting growth of the *Ralstonia* spp. (Elphinstone et al., 1996).
3. Store media and stock solutions of antibiotics at 4°C in the dark and use within 1 month.
4. If alternative sources of antibiotics or crystal violet are used it may be necessary to first dissolve in small quantities of ethanol or methanol prior to making up stock solutions with distilled water.
5. Plates should be free from surface condensation before use.
6. Avoid excess drying of plates.
7. Quality control can be performed after preparation of each new batch of medium by plating a suspension of a reference culture of a relevant *Ralstonia* species and observing formation of typical colonies after incubation at 28°C for 2–6 days.
8. Cycloheximide (e.g. from Sigma C-7698) 50 mg L^{-1} can be added to inhibit yeasts and fungi

Performance characteristics available

Matrices tested: potato tubers, *Pelargonium* petioles, surface water.

Analytical sensitivity: From potato tuber samples 3×10^2 cfu mL^{-1}, from water samples 3×10^1 cfu mL^{-1} and from *Pelargonium* petioles 6.5×10^2 cfu mL^{-1}.

Analytical specificity of 25 strains of different race/biovar combinations, all formed typical colonies on SMSA. Of 37 nontarget bacteria found on potato tubers, water and ornamental plants, some growth was observed on SMSA by *Robbsia andropogonis*, *Burkholderia cepacia* and *Ralstonia pickettii*; however, colony morphology was not typical (all 37 bacteria gave negative results).

Repeatability: 100%.

Reproducibility: 100%.

Diagnostic sensitivity: 100% (cf. TaqMan® PCR of Weller et al., 2000).

Diagnostic specificity: 100% (cf. TaqMan® PCR of Weller et al., 2000).

3.2. Sequeira semi-selective medium (Granada & Sequeira, 1983; modified by Poussier et al., 1999) modified by G. Cellier (Anses) (pers. comm.)

Bacto Peptone	11.0 g
Glycerol	6.3 g
Bacto Agar	18.0 g
Yeast extract	1.0 g
Deionized distilled water	1 L

After autoclaving, let the medium cool to 45–50°C and add sterilized aqueous stock solutions of the following ingredients to obtain the specified final concentrations:

2,3,5-Triphenyl-2H-tetrazolium chloride	0.025 g
Crystal violet	0.002 g
Chloramphenicol	0.005 g
Penicillin G	20 U
Polymyxin B	0.01 g (60 000 U)
Adjust pH to	7.2

The medium should be kept at a temperature of <20°C in dark conditions for no more than 3 months.

APPENDIX 3 - DNA EXTRACTION

Other extraction methods than the ones described below may be used. They should be shown to reliably extract amplifiable target DNA from a known negative plant extract to which a reference strain of *R. solanacearum* between 10^3 and 10^4 cfu mL^{-1} has been added as a positive control. This can be evaluated by performing a verification as described in PM 7/98.

DNA should preferably be stored at approximately −20°C.

1. DNA extraction from plant material

1.1. DNA extraction kits

Three different extraction kits have been compared by the EURL for pests of plants on bacteria for potato extracts following the manufacturer's instructions: DNeasy Plant Mini Kit by Qiagen, the Easy-DNA™ gDNA Purification Kit by Invitrogen[5] and the QuickPick™ SML Plant DNA Kit by Bio-Nobile. The kit by Bio-Nobile was used with the KingFisher Flex (ThermoFisher, MA, USA) for

[5]When testing is performed as a duplex also targeting *Clavibacter sepedonicus* 1 mg per 100 µL of lysozyme is added in the lysis buffer and heating is to 37°C for 30 min, as validated by Pastrik & Maiss (2000).

automation purposes. The molecular test used was the real-time PCR of Weller et al. (2000) with the adjusted probes of Vreeburg et al., 2016. The evaluation was performed on potato extracts and all Phylotypes were included. These kits have been shown to perform at an equivalent level at 5×10^3 cfu mL^{-1} (EURL, pers. comm.). Magnetic beads based QuickPick™ SML Plant DNA kits have also been used to extract DNA from symptomatic plants of tomato, eggplant and pelargonium (Dreo et al., 2014), see Appendix 4.

An evaluation of the analytical sensitivity and selectivity of the real-time PCR (Weller et al., 2000) with the adjusted probe of Vreeburg et al., 2016 for the detection of *R. solanacearum* (Phylotype II) and *R. pseudosolanacearum* (Phylotype I) was performed for seven plant matrices other than potato tubers by the NRC, NPPO-NL using the QuickPick™ SML Plant DNA Kit by Bio-Nobile. The kit by Bio-Nobile was used with the KingFisher Flex (ThermoFisher) for automation purposes. The matrices were anthurium, eggplant, begonia, pelargonium, pepper (*Capsicum annuum*), rose and tomato.

The analytical sensitivity for anthurium, rose, pelargonium and tomato was found to be 1.6×10^4 cfu mL^{-1}, whereas for paprika (*Capsicum annuum*) and eggplant it was 3.2×10^3 cfu mL^{-1} and for begonia 6.4×10^2 cfu mL^{-1}. When an additional DNA purification step using polyvinylpolypyrrolidone was done, a lower analytical sensitivity was reported, but only for pelargonium and rose (see https://dc.eppo.int/validation_data/validationlist).

1.2. Vreeburg et al. (2018)

An alternative DNA extraction protocol has been published in Vreeburg et al. (2018).

Allow the solid particles in the resuspended pellet to settle to the bottom and mix 100 µL of the supernatant with 11 µL of lysozyme solution (25 mg mL^{-1} lysozyme (Sigma Aldrich Zwijndrecht, the Netherlands) in 100 mM Tris (ThermoFisher Scientific, Waltham, MA, USA) with 10 mM EDTA, pH 8.0.

Incubate by shaking at 37°C for 30 min.

Add 11 µL of 10% w/v SDS, 15 µL of 5 M NaCl and 20 µL of GuHCl buffer (Appendix 2). Incubate by shaking at approximately 95°C for 15 min and then cool on ice. Next, add ice-cold MPC protein precipitation solution (EpiCentre, Madison WI, USA), mix and keep on ice for 5 min, followed by centrifugation for 10 min at 3500 *g* at 4–20°C. Transfer the supernatant to a tube or deep-well block prefilled with 225 µL (per well) of isopropanol and 20 µL of SNAP bead solution (Stratec Molecular, Birkenfeld, Germany). Wash the beads three times with 400 µL of 70% ethanol and elute with 100 µL of ultrapure water at 65°C.

2. DNA extraction from colonies

For crude DNA extraction from presumptive *Ralstonia solanacearum* species complex cultures and from cultures of reference strains, suspend approximately 1 µL of cell material (e.g. using a 1-µL loop) or one colony in 100 µL of sterile distilled water. Heat in closed microvials at approximately 95°C or 100°C for a minimum of 10 min. A freezing step before the heating may be performed.

Alternatively, a cell suspension in 0.05 mM NaOH can be prepared: 100 µL of the cell suspension in closed tubes is heated at approximately 95°C for approximately 5 min.

The lysate can be stored at approximately −20°C.

Extraction kits may also be used.

APPENDIX 4 - LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) (LENARČIČ ET AL., 2014)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. The following LAMP test is used for on-site detection as well as laboratory detection of *R. solanacearum* (Phylotype II), *R. pseudosolanacearum* (Phylotypes I and III) *and R. syzygii* (some strains not detected; Table 1) in symptomatic plant material. The test can be applied to bacterial exudates of symptomatic plant material. The test also performed well on a limited set of artificially and naturally contaminated samples of potato tubers prepared according to Directive 2006/63/EC, detecting 10^5 cells mL^{-1} of potato tuber extract; however, further data is needed to confirm its reliability for latent testing.

1.2. The test was developed by Lenarčič et al. (2014).

1.3. The test targets the endoglucanase (*egl*) gene.

1.4. Oligonucleotides: primer sequences are given in 5′–3′ orientation with positions on the *egl* sequence of the GMI100 strain (GenBank accession number DQ657595) in brackets:

F3_RS_*egl*	5′-GAGCAACTACATCTATCCGTC-3′ (330–350)
B3_RS_*egl*	5′-CATCAGCCCGAAGATGAC-3′ (637–654)
FIP_RS_*egl*	5′-ACAGCTCGTTCGCGTCGACGACAGC GCGACCTACTA-3′ (354–371, 446–463)
BIP_RS_*egl*	5′-GGTTCGTCAACGCCGTGAGATCACG TTGCCGTAGTAG-3′ (476–493, 540–558)
FLoop_RS_*egl*	5′-GTTCATGCCCTTGTTCTTG-3′ (372–390)
BLoop_RS_*egl*	5′-GCTCGATCCGCACAACTA-3′ (516–533)

The validation data has been produced using standard purified primers; however, HPLC-purified primers are now generally advised for LAMP.

1.5. The test has been successfully performed on three different machines, SmartCycler (Cepheid, Sunnyvale, CA, US), Genie II (Optigene Ltd, Horsham, GB) and Roche Light Cycler 480 (Roche Applied Science), with comparable results. Because of the differences in melting curve analysis among the instruments, the melting temperature (T_m) range can differ and was observed to be approximately 1°C lower in GenieII compared with SmartCycler and LightCycler 480.

1.6. Software allowing fluorescence acquisition in real-time and melting curve analysis should be used. The specific instrument manual should be consulted. Note: software for cycling (real-time PCR devices) should be programmed to measure the fluorescence during the amplification and the melting curve analysis.

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1. The test can be applied to pure cultures, bacterial exudates and extracts from small pieces of symptomatic plant material after heating (see point 2.1.2), and to plant material after a DNA extraction (see 2.1.3 below).

2.1.2. Heating. A heating time of 2 min at 95°C was repeatedly successful for the confirmation of tuber infections (six tubers tested) and other symptomatic plant material. For heating, collect approximately 2–10 µL of bacterial exudates or tuber vascular tissue or a piece of stem from symptomatic host plant (e.g. a 1-cm piece cut vertically or in small pieces). Put the material into 100 µL of sterile water or phosphate buffer and briefly vortex. Heat in a waterbath or heat in an incubator set at 95–98°C for 2 min. Inoculation loops can be used at all stages of preparation.

2.1.3. DNA extraction from plants. For on-site use on symptomatic tubers no DNA extraction is performed. Magnetic-bead-based QuickPick SML Plant DNA kits (Bio-Nobile, Turku, FI) automated with the KingFisher mL system (Thermo Labsystem) can be used to extract and purify DNA from extracts from 200 potato tubers and symptomatic plants of tomato, eggplant and pelargonium. The extraction is done from 100-µL extracts according to Pirc et al. (2009), with a minor modification (440 µL of lysate is used for purification).

2.1.4. DNA should preferably be stored at approximately −20°C.

2.2. LAMP Master mix.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	7.9	N.A.
Isothermal master mix (Optigene Ltd)	2×	12.5	1×
F3_RS_*egl*	100 µM	0.05	0.2 µM
B3_RS_*egl*	100 µM	0.05	0.2 µM
FIP_RS_*egl*	100 µM	0.5	2 µM
BIP_RS_*egl*	100 µM	0.5	2 µM
FLoop_RS_*egl*	100 µM	0.5	2 µM
BLoop_RS_*egl*	100 µM	0.5	2 µM
Subtotal		22.5	
Genomic DNA sample (boiled bacterial suspension or DNA isolated from plant extract)		2.5	
Total		25	

2.2.1. LAMP conditions: 60°C for 30 min; 80 melting curve analysis: 98°C to 80°C, 0.05°C s^{-1}.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction (not applicable when crude homogenates are tested) and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

For on-site applications only NAC and PAC are used.

3.2. Interpretation of results

To assign results from PCR-based tests the following criteria should be followed:

Verification of the controls

- NAC (and if relevant NIC) should produce no fluorescence.
- The PAC (and if relevant PIC) amplification curve should be exponential. T_m should be characteristic of the *Ralstonia* species (see below).

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve and has a characteristic T_m. Note that the determined T_m values vary among phylogenetically distinct strains. Also, T_m values depend on the instruments and temperature profile used to generate melting curve analysis. Consequently, the specific T_m should be verified in each laboratory. In the amplification of strains using SmartCycler (Cepheid) the T_m values were 94.6°C (±0.2) for Phylotype I (Asia), 94.5°C (±0.4) for Phylotype III (Africa), 93.8°C (±0.2) for Phylotype IIA and 93.7°C (±0.2) for Phylotype IIB. Positive samples of exudates from infected potato tubers (R3Bv2 strain) analysed on GenieII exhibited a T_m of 92.1 ± 0.11°C.
- A test will be considered negative if it produces no fluorescence or the T_m is not characteristic of the *Ralstonia* species.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

4.1. Analytical sensitivity data

- On pure cultures:

Sensitivity was assessed in triplicate on pure culture suspension on a single strain that represents each Phylotype:

- Phylotype I (GBBC1172): 10^4 cells mL^{-1}
- Phylotype IIA (RUN 30): 10^6 cells mL^{-1}
- Phylotype IIB (GBBC729): 10^5 cells mL^{-1}
- Phylotype III (LMG 2296): 10^4 cells mL^{-1}
- Phylotype IV (RUN 71): 10^6 cells mL^{-1}

- On spiked potato tubers:

10^5 cells per mL of plant extract, based on three serial dilutions of *R. solanacearum* in extracts from potato tubers, which had previously tested negative for *Ralstonia*.

- On infected symptomatic plants:

All symptomatic material tested gave positive results. The following symptomatic material was tested:

- Plants with wilting symptoms (2–4 days after inoculation): bacterial ooze at the site of inoculation was collected using a sterile plastic inoculation loop and suspended in 100 µL of sterile water.
- Bacterial ooze from seven symptomatic tubers was tested.

4.2. Analytical specificity data

The test was 98% accurate (one isolate of *R. syzygii* from Australia gave a false-negative result, no false positives). Tested on 88 strains of target *Ralstonia* spp., 13 nontarget and potentially cross-reacting strains and 13 other bacterial pathogens that can be present on *Ralstonia* spp. host plants: *Ralstonia pickettii*, *R. mannitolilytica*, *Burkholderia caryophylli*, *B. cepacia*, *B. plantarii*, *B. andropogonis*, *B. glumae*, *Paenibacillus polymyxa*, *Pseudomonas marginalis* pv. *marginalis*, *Enterobacter* sp., *Ochrobacterium anthropic*, *Pectobacterium atrosepticum*, 3 strains of *Pectobacterium* spp., *Dickeya chrysanthemi* 3 strains of *Dickeya solani*, *D. dianthicola*, *D. dieffenbachiae* and *Clavibacter sepedonicus*.

In addition, Lenarčič et al. (2014) detected all 84 isolates tested of the *R. solanacearum* species complex (including 41 *R. solanacearum*, 35 *R. pseudosolanacearum* and 8 *R. syzygii* isolates) with the exception of 1 genetically distinct isolate of *R. syzygii* (RUN 14) from Australia. Of 26 nontarget bacteria, all tested negative with the exception of 1 isolate of *Ralstonia mannitolilytica* (CFBP 6737).

4.3. Data on repeatability

Repeatability: 100% detection for dilutions with at least 10^4 copies of *Ralstonia solanacearum* DNA or more. The test was repeated 10 times on *R. solanacearum* GBBC1172 strain at 10^8 cells mL^{-1}. Time of positivity (Tp) was 12.7 ± 0.49 min, with T_m of 94.1 ± 0.32°C. Lower dilution (10^4 cells mL^{-1}) was repeated three times, with times to positivity for strain GBBC1172 as follows: 23.2, 23.0 and 16.2 min.

4.4. Data on reproducibility

The test was performed successfully on three different machines: SmartCycler (Cepheid), Genie II (Optigene Ltd) and Roche Light Cycler 480 (Roche Applied Science). Results are comparable. However, due to different ways of obtaining T_m by the GenieII machine, the range of T_m is about 1°C lower than the T_m obtained with the SmartCycler and LightCycler 480.

4.5. Analytical selectivity

No cross-reactivity of different hosts/cultivars/tissues was observed. DNA from healthy plant extracts was tested:

potato (43 samples/21 cultivars), tomato (4), eggplant (3), pelargonium (6) and *Solanum dulcamara* (6).

4.6. Diagnostic sensitivity

100%: All 50 samples tested with the *egl* LAMP test showed results in agreement with the diagnostic status of the sample (samples above the limit of detection of the method). Seven real diagnostic samples that were previously confirmed to be positive for *R. solanacearum* were positive using the *egl* LAMP test.

4.7. Diagnostic specificity

100% evaluated on 43 samples of healthy potato extract (confirmed by real-time PCR and immunofluorescence) were negative using the *egl* LAMP assay.

Extracts of healthy plants of other hosts [tomato (4), eggplant (3), pelargonium (6) and *S. dulcamara* (6)] were all negative.

APPENDIX 5 - CONVENTIONAL PCR TESTS FOR DETECTION AND IDENTIFICATION OF SPECIFIC *RALSTONIA SOLANACEARUM* (PHYLOTYPE II) AND *RALSTONIA PSEUDOSOLANACEARUM* (PHYLOTYPE I) (PASTRIK ET AL., 2002)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. The test was originally described for the detection of *Ralstonia* spp. (mostly corresponding to current Phylotypes I and II only) in potato tuber tissue extracts and for identification of *Ralstonia* isolates in Phylotypes I and II. Primer RS-1-F is generic for all strains, whereas the specificity of the reverse primers was selected for amplification of *R. solanacearum* Phylotype II strains (RS-1-R) only or *R. pseudosolanacearum* Phylotype I strains (RS-3-R) only. Further data has been generated by the EURL Pests on plants – Bacteria and show that the primer (RS-3-R) also amplifies *R. solanacearum* Phylotype III.

1.2. The test was developed by Pastrik et al. (2002).

1.3. The test targets 16–23S rRNA intergenic spacer (ITS) sequences. Primers NS-5-F and NS-6-R (Dams et al., 1988; White et al., 1990) amplify 18S rRNA target DNA co-extracted from plant samples and can be used in multiplex or simplex as an internal positive control (IPC).

1.4. Oligonucleotides:

Forward primer	RS-1-F	5′-ACTAACGAAGCAGAGATGCATTA-3′	718 bp
Reverse primer	RS-1-R	5′-CCCAGTCACGGCAGAGACT-3′	
Reverse primer	RS-3-R	5′-TTCACGGCAAGATCGCTC-3′	716 bp
Forward primer	NS-5-F	5′-AACTTAAAGGAATTGACGGAAG-3′	
Reverse primer	NS-6-R	5′-GCATCACAGACCTGTTATTGCCTC-3′.	310 bp

2. Methods

2.1. Nucleic acid extraction

See Appendix 3.

PCR tests can be performed on undiluted and diluted DNA extracts. Testing diluted DNA extracts (1/2 or 1/5) may help to avoid false-negative PCR results due to polymerase inhibitors co-extracted from plant samples. DNA may be prepared in advance and stored frozen prior to use.

DNA should preferably be stored at approximately −20°C.

2.2. Polymerase chain reaction

2.2.1. Phylotype II-specific PCR with internal control

2.2.1.1. Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	12.625	N.A.
PCR buffer[a] with 15 mM $MgCl_2$	10×	2.5	1×
dNTPs (e.g. Boehringer, Mannheim, DE[a])	20 mM	0.125	0.1 mM
BSA (fraction V) (e.g. Serva, Germany)	10%	0.25	0.1%
Forward primer (RS-1-F)	10 µM	2	0.8 µM
Reverse primer (RS-1-R)	10 µM	2	0.8 µM
Forward primer (NS-5-F[b])	10 µM	0.15	0.06 µM
Reverse primer (NS-6-R[b])	10 µM	0.15	0.06 µM
Taq Polymerase (Gibco[a])	5 U μL^{-1}	0.2	1 U
Subtotal		20	
Genomic DNA extract		5	
Total		25	

[a] The above test was originally optimized for the MJ Research PTC 200 thermocycler with Gibco BRL Taq Polymerase and buffer. Provider companies might not exist anymore. Further optimization is recommended for use with other reagents and systems.

[b] Concentrations of primers NS-5-F and NS-6-R were optimized for potato heel end core extraction and DNA purification using the methods according to Pastrik and Maiss (2000). Re-optimization of reagent concentrations will be required if extraction by shaking or another DNA extraction method is used.

2.2.1.2. PCR conditions: an initial 5-min incubation at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 58°C and 45 s at 72°C, followed by a final extension of 5 min at 72°C.

2.2.2. Phylotype I-specific PCR with internal control

2.2.2.1. Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	11.925	N.A.
PCR buffer with 15 mM $MgCl_2$ [a]	10×	2.5	1×
dNTPs (e.g. Boehringer[b])	20 mM	0.125	0.1 mM
BSA (fraction V) (e.g. Serva, Germany)	10%	0.25	0.1%
Forward primer (RS-1-F)	10 µM	1	0.4 µM
Reverse primer (RS-3-R)	10 µM	1	0.4 µM
Forward primer (NS-5-Fc)	10 µM	0.15	0.06 µM
Reverse primer (NS-6-Rc)	10 µM	0.15	0.06 µM
Taq Polymerase (Gibcoa)	5 U μL^{-1}	0.2	1 U
Subtotal		20	
Genomic DNA extract		5	
Total		25	

[a] The above method was originally optimized for a MJ Research PTC 200 thermocycler with Gibco BRL Taq Polymerase and buffer. Provider companies might not exist anymore. Further optimization is recommended for use with other reagents and systems.

[b] Provider as indicated in the publication, but this company does not exist anymore. Further optimization is recommended for use with other dNTPs.

2.2.2.2. PCR conditions: an initial 5-min incubation at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 58°C and 45 s at 72°C, followed by a final extension of 5 min at 72°C.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification, preferably of a sample of uninfected matrix or, if not available, clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample known to contain the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

The multiplex PCR (2.2.1 above) includes an additional IPC involving amplification of 18S rDNA co-extracted from the host plant.

3.2. Interpretation of results

Verification of the controls

- NIC and NAC: no band is visualized.
- PIC, PAC and when relevant IPC: a band of the expected size is visualized.

When these conditions are met

- A test will be considered positive if a band of 718 bp (RS-1-F and RS-1-R), 716 bp (RS-1-F and RS-3-R) and 310 bp (NS-5-F and NS-6-R) is visualized.
- A test will be considered negative if no band or a band of a different size than expected is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Mostly from Pastrik et al. (2002) unless indicated otherwise.

4.1. Analytical sensitivity

The minimum population of *R. solanacearum* NCPPB1331 detected following serial dilution in sterile water or potato extracts was 0.5 cfu mL^{-1} after DNA purification and concentration (Pastrik et al., 2002) whether performed as simplex (RS-1-F/RS-1-R primers only) or as multiplex with NS-5-F/NS-6-R primers as an IPC.

4.2. Analytical specificity

Testing was done on 29 NCPPB reference strains of 3 *Ralstonia* Phylotypes in the species complex from multiple

hosts and countries worldwide. RS-1-F/RS-1-R primers gave positive results with all 16 isolates of *R. solanacearum* (Phylotype II), and negative results with all 8 isolates of *R. pseudosolanacearum* (Phylotype I) and 5 isolates of *R. syzygii*. RS-1-F and RS-3-R primers gave negative results with all 16 isolates of *R. solanacearum* (Phylotype II) and 5 isolates of *R. syzygii*, and gave positive results with all 8 isolates of *R. pseudosolanacearum* (Phylotype I).

Negative results were obtained with both primer sets after testing 25 nontarget bacteria, including *Ralstonia eutropha* (3 isolates), *R. pickettii* (3 isolates), *Burkholderia andropogonis* (3 isolates), *B. caryophylli* (3 isolates), *B. cepacia* (4 isolates), *B. gladioli* (1 isolate), *B. glumae* (2 isolates), *B. plantarii* (2 isolates) and *Clavibacter sepedonicus* (4 isolates).

Further validation data carried out on JKI have shown a cross-reaction with *R. pickettii*.

4.3. Repeatability

Not available.

4.4. Reproducibility

In three Euphresco interlaboratory comparisons, 29, 23 and 39 laboratories obtained accurate results in an average of 97.5%, 98.1% and 98.6% of a total of 475 tests performed on spiked, naturally infected and healthy potato tuber samples using a variety of sources of Taq polymerase and thermocyclers (van Vaerenbergh et al., 2017).

4.5. Diagnostic specificity

Diagnostic specificity was 100%. All 13 potato samples naturally latently infected with *R. solanacearum* (confirmed by IF, isolation and tomato bioassay) tested positive with multiplex PCR using primers RS-1-F/RS-1-R and NS-5-F/NS-6-R. All 4300 healthy potato samples from 143 potato cultivars tested negative (Pastrik et al., 2002).

APPENDIX 6 - REAL-TIME TAQMAN® PCR TESTS FOR DETECTION AND IDENTIFICATION OF STRAINS OF ALL *RALSTONIA SOLANACEARUM*, *RALSTONIA PSEUDOSOLANACEARUM* AND *RALSTONIA SYZYGII* (PHYLOTYPES I–IV) AND *R. SOLANACEARUM* PHYLOTYPE IIB SEQUEVAR 1 (WELLER ET AL., 2000)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. The test is used for the detection and identification of all strains of *R. solanacearum*, *R. pseudosolanacearum*, *Ralstonia syzygii* (Phylotypes I–IV) and for specific detection and identification of *R. solanacearum* Phylotype II sequevar 1 (race 3) only.

1.2. The test was developed by Weller et al. (2000).

1.3. Real-time PCR primers and probes RS-I-F, RS-II-R and RS-P target specific 16S rRNA gene sequences conserved within all *R. solanacearum*, *R. pseudosolanacearum* and *R. syzygii* strains (Weller et al., 2000). Improved specificity for detection of all strains was demonstrated by Vreeburg et al. (2016) using a TaqMan® probe modified from the original probe described by Weller et al. (2000). Modified primers RS-I-FmAK/RS-II-RmAK (Körner et al., 2017) were also developed to improve the specificity of this test. Real-time PCR primers and probes B2-I-F, B2-II-R and B2-P target specific prophage sequence specific to strains of *R. solanacearum* Phylotype IIB-1 (race 3, biovar 2) (Fegan et al., 1998). Real-time PCR primers COX-F, COX-R and COX-P target plant cytochrome oxidase gene sequences co-extracted from plant samples and are used as internal positive controls (IPC) which can be used in a multiplex reaction with RS primers and probe.

1.4. Oligonucleotides:

Forward primer	RS-I-F[a]	5′-GCATGCCTTACACATGCAAGTC-3′
Reverse primer	RS-II-R[a]	5′-GGCACGTTCCGATGTATTACTCA-3′
Forward primer	B2-I-F	5′-TGGCGCACTGCACTCAAC-3′
Reverse primer	B2-II-R	5′-AATCACATGCAATTCGCCTACG-3′
Forward primer	COX-F	5′-CGTCGCATTCCAGATTATCCA-3′
Reverse primer	COX-R	5′-CAACTACGGATATATAAGAGCCA AAACTG-3′

[a] The use of modified primers RS-I-FmAK/RS-II-RmAK in place of RS-I-F/RS-II-R was found to reduce/eliminate false-positive results with high Ct values, sometimes found to occur due to co-extraction from potato tuber tissue of DNA from bacteria of the genus *Advenella* (Körner et al., 2017).

Forward primer	RS-I-FmAK	5′-CATGCCTTACACATGCAAGTC-3′
Reverse primer	RS-II-RmAK	5′-CACGTTCCGATGTATTACTCA-3′

Probes:

Probe	RS-P[a]	5′-[FAM]-AGCTTGCTACCTGCCGGCGAGTG-[TAMRA]-3′
Probe	B2-P	5′-[VIC]-TTCAAGCCGAACACCTGCTGCAAG-[TAMRA]-3′
Probe	COX-P	5′-[VIC]-TGCTTACGCTGGATGGAATGCCCT-[TAMRA]-3′

[a] A modification of probe RS-P, containing a minor groove binding (mgb) unit was described by Vreeburg et al. (2016) and results in elimination of occasional false-positive results with high Ct values due to non-specific binding to non-target DNA extracted from potato tuber tissue.

Probe	RSP-55T	5′-[FAM]-AGCTTGCTACCTGCCGG-[NFQ-MGB]-3′

1.5. The test was originally optimized for the Applied Biosytems AmpliTaq™ and buffer with the ABI 7700 and 7900 sequence detector TaqMan® systems and the Cepheid Smartcycler system. Validation data from the article are under these conditions.

2. Methods

2.1. Nucleic acid extraction

See Appendix 3

PCR tests can be performed on undiluted and diluted DNA extracts. Testing diluted DNA extracts (1/2 or 1/5) may help to avoid false-negative PCR results due to polymerase inhibitors co-extracted from plant samples.

DNA should preferably be stored at approximately −20°C.

2.2. Real-time polymerase chain reaction (real-time PCR)

2.2.1. Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	12.875	N.A.
Real-time PCR buffer (Applied Biosystems)	10×	2.5	1×
$MgCl_2$ (Applied Biosystems)	25 mM	3.5	3.5 mM
dNTPs (Applied Biosystems)	2.5 mM	2	0.2 mM
Forward primer (RS-I-F, B2-I-F, COX-F)	10 µM	0.75	0.3 µM
Reverse primer (RS-II-R, B2-II-R, COX-R)	10 µM	0.75	0.3 µM
Probe 1 (RS-P, B2-P, COX-P, RSP-55T[a])	5 µM	0.5	0.1 µM
AmpliTaq Gold Polymerase[b] (Applied Biosystems)	5 U μL^{-1}	0.125	0.625 U
Subtotal		23	
DNA dilution		2	
Total		25	

[a] A modification of probe RS-P see 1.4

[b] Method originally optimized for Applied Biosytems AmpliTaq Gold™ and buffer with the ABI 7700 and 7900 sequence detector TaqMan® systems and the Cepheid Smartcycler system. Further optimization will be required for use with other reagents and systems.

2.2.2. PCR conditions

an initial 10-min incubation at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. Annealing time can be reduced to 30 s if using the Cepheid Smartcycler system.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following external controls should be included for each series of nucleic acid extractions and amplifications:

- Negative isolation control (NIC): 50 mM phosphate buffer (pH 7.0) used for extraction of the bacteria from plant tissue.
- Positive isolation control (PIC): 50 mM phosphate buffer (pH 7.0) spiked with a reference strain of *R. solanacearum*.
- Negative amplification control (NAC): molecular-grade water used in the PCR mix.
- Positive amplification control (PAC): DNA extract of *R. solanacearum* cells added to molecular-grade water or negative tissue extract at a concentration above the detection threshold (10^3–10^4 cells mL^{-1}).

3.2. Interpretation of results

Verification of the controls

- The PIC, PAC and IPC amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

4.1. Analytical sensitivity

When tested on pure cultures (4 isolates of *R. solanacearum* and 1 isolate of *R. pseudosolanacearum*) diluted in water, the lowest populations detected were between 10^3 and 10^4 cfu mL^{-1} using the RS-I-F/RS-II-R/RS-P assay and 10^5 cfu mL^{-1} using the B2-I-F/B2-II-R/B2-P assay (Weller et al., 2000).

When tested on 1:100 dilutions of extract from 200 potato heel-end cores after spiking with *R. solanacearum*, the lowest populations detected were 10^4 cfu mL^{-1} using the RS-I-F/RS-II-R/RS-P assay and 10^5 cfu mL^{-1} using the B2-I-F/B2-II-R/B2-P assay (Weller et al., 2000).

4.2. Analytical specificity

RS-I-F/RS-II-R/RS-P test: 100% inclusivity when tested at Fera on 60 NCPPB reference strains of *R. solanacearum* species complex from multiple hosts and countries worldwide, including 37 isolates of *R. solanacearum* (Phylotype II), 17 isolates of *R. pseudosolanacearum* (Phylotypes I and III) and 6 isolates of *R. syzygii*. There was 100% exclusivity with 30 nontarget bacteria, including serologically cross-reacting strains listed in EU Council Directive 2006/63/EC and other bacterial pathogens that

can be present on *Ralstonia* host plants and other closely related *Ralstonia* and *Burkholderia* species. One isolate obtained from sugarcane in Jamaica (NCPPB 928), identified as *Ralstonia* sp., tested positive.

False-positive results have also been obtained with an isolate identified as *Advenella kashmirensis* from rose when using the probe RS-P but not when using the alternative RSP-55T probe (Körner et al., 2017).

B2-I-F/B2-II-R/B2-P assay: 100% inclusivity when tested at Fera against all 20 *R. solanacearum* Phylotype II sequevar 1 (race 3) isolates collected worldwide. There was 100% exclusivity with 17 (nonrace 3) isolates of *R. solanacearum*, 17 isolates of *R. pseudosolanacearum*, 6 isolates of *R. syzygii* and with 30 nontarget bacteria, including serologically cross-reacting strains listed in EU Council Directive 2006/63/EC and other bacterial pathogens that can be present on *Ralstonia* host plants and other closely related *Ralstonia* and *Burkholderia* species.

False-positive results were obtained by *Dickeya dianthicola* strain GBBC 2039.

4.3. Repeatability

Vreeburg et al. (2016) demonstrated 100% repeatability ($n = 8$) when the RS-I-F/RS-II-R/RS-P assay was used in conjunction with a different but equivalent DNA extraction method to that described under 2.1 above.

4.4. Reproducibility

In two Euphresco interlaboratory comparisons, 19 and 24 laboratories obtained accurate results in an average of 95.4% and 93.5% of a total of 310 tests performed on spiked, naturally infected and healthy potato tuber samples using a variety of sources of TaqMan master mixes and thermocyclers (Van Vaerenbergh et al., 2017). Vreeburg et al. (2016) demonstrated 100% reproducibility.

4.5. Diagnostic sensitivity

Weller et al. (2000) reported that blind testing of extracts from 200 tuber samples successfully detected 4 known positive samples randomly distributed amongst 20 healthy samples of various varieties. All wilted plants of each host, which developed following inoculation with one of three isolates of *R. solanacearum* (NCPPB 325T, NCPPB 909 or NCPPB 4536), tested positive.

Vreeburg et al. (2016) demonstrated a diagnostic sensitivity of 100% using either the RS-I-F/RS-II-R/RS-P test or the RS-I-F/RS-II-R/RSP-55T test when testing 276 routine potato tuber samples previously confirmed by IF.

4.6. Diagnostic specificity

Weller et al. (2000) reported that no false-positive results were obtained from the healthy samples or from stem samples (10 plants each) of healthy tomato, eggplant, *Pelargonium* or *Solanum dulcamara*.

Vreeburg et al. (2016) demonstrated increased diagnostic specificity from 92.7% using the RS-I-F/RS-II-R/RS-P test to 100% using the RS-I-F/RS-II-R/RSP-55T test due to the elimination of false positives when testing 276 routine potato tuber samples.

APPENDIX 7 - NYTOR REAL-TIME TAQMAN PCR TEST FOR DETECTION AND IDENTIFICATION OF *R. SOLANACEARUM* (PHYLOTYPE II) AND *R. PSEUDOSOLANACEARUM* (PHYLOTYPES I AND III) (VREEBURG ET AL., 2018)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. The test was published by Vreeburg et al. (2018). It is used for the detection and identification of *R. solanacearum* and *R. pseudosolanacearum*. This real-time PCR is designed and validated as a multiplex test targeting *C. sepedonicus*, *R. solanacearum* and *R. pseudosolanacearum* in asymptomatic potato tubers.

1.2. The PCR was optimized for and the validation data was obtained with potato tuber heel end cores. It may be used in other matrices, but at least a verification should be conducted by the laboratories. Validation data was obtained using the DNA extraction method given in Appendix 3, Section 1.2.

1.3. The test targets the endoglucanase (*egl*) gene.

1.4. The positive internal control target is a conserved region of the ATP synthase β gene from *Solanum tuberosum*, amplified by the primers and probe: Stub_F, Stub_R and Stub_P.

1.5. Oligonucleotides:

Forward primer	Rsol_F	CGC GAA CGA GCT GTC
Reverse primer	Rsol_R	TCA CGT TGC CGT ART AG
Probe	Rsol_P1	FAM-CGG GTT CGT CAA CGC CGT GAC-BHQ1
	Rsol_P2	FAM-CGG GTT TGT CAA CGC CGT GAC-BHQ1
Forward primer	Stub_F	CGG ATA ATT CGT CCA ATC
Reverse primer	Stub_R	CCA GCA GTA GAT CCT TTA
Probe	Stub_P	[ATTO532]-CAA CCA TGC TTC AAC CTC GGA TC-[BHQ1][a]

[a] This PCR was designed and validated as a multiplex with *C. sepedonicus*.

1.6. The PCR was optimized for, and the validation data was obtained with an ABI 7500 real-time PCR system.
1.7. Software and analysis setting should be validated in the laboratory to meet the requirements of the test.

2. Methods

2.1. Nucleic acid extraction and purification
2.1.1. Tissue source: validated on tubers, may also be used for plant material or pure culture suspension
DNA extraction procedures from plants and potatoes are described in Vreeburg *et al.* (2018).
See Appendix 3, point 1.2
2.1.2. Storage temperature and conditions: DNA should preferably be stored at approximately −20°C
2.2. Real-time polymerase chain reaction(real-time PCR)
2.2.1. Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	1[a]	N.A.
iTaq Universal Probes Supermix (BioRad)	2×	10	1×
Forward primer (Rsol_F)	10 µM	1.6	0.8 µM
Reverse primer (Rsol_R)	10 µM	1.6	0.8 µM
Probe 1 (Rsol_P1)	2 µM	0.2	0.02 µM
Probe 2 (Rsol P2)	2 µM	0.2	0.02 µM
Forward primer (Stub_F)	10 µM	0.1	0.05 µM
Reverse primer (Stub_R)	10 µM	0.1	0.05 µM
Probe 3 (Stub_P)	1 µM	0.2	0.01 µM
Subtotal		15	
DNA dilution		5	
Total		20	

[a] This volume is changed to 0 µL per reaction when the NYtor PCR is run with the *C. sepedonicus* primers and probes. See Vreeburg et al. (2018).

2.2.2. PCR conditions: initial denaturation at 95°C for 3 min, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or, if not available, clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). DNA of *R. solanacearum* equivalent to a concentration of approximately 10^4 cfu mL^{-1}.

The NYtor real-time PCR uses an internal positive control (IPC) to monitor each individual sample separately. The positive internal control target is a chloroplastic gene of ATP synthase beta-subunit present in the potato DNA.

Alternative internal positive controls can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved nonpest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA).
- Amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls

Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results: to assign results from the PCR-based test the following criteria should be followed:

Verification of the controls

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Validation was carried out in accordance with PM 7/98 and validation data are published in Vreeburg et al. (2018).

4.1. Analytical sensitivity data

The test was developed and validated to classify 95% of samples with 5×10^3 cfu mL^{-1} positive, using a regression approach with a Ct cut-off value established in the laboratory.

4.2. Analytical specificity data

Inclusivity: 100% evaluated on 43 *R. solanacearum* isolates and 7 *R. pseudosolanacearum* isolates.

Exclusivity: 100% The exclusivity was evaluated on 44 nontarget strains, including potentially cross-reacting species and species that can be present on potato tubers.

4.3. Data on repeatability

100% for potato extracts spiked with 10^4 to 10^5 cfu mL^{-1} of different *R. solanacearum* strains.

4.4. Data on reproducibility

100% for potato extracts spiked with 10^4 to 10^5 cfu mL^{-1} of different strains when performed in one laboratory. This test was part of a test performance study (TPS) in 2018. In this TPS the NYtor test detected >95% of provided DNA samples isolated from extracts spiked with 1.2×10^4, 2.4×10^5 and 2.4×10^7 cfu mL^{-1}. Reproducibility including DNA extraction by the participating laboratories, using their own preferred extraction method, was 41% for 1.2×10^4, 56% for 2.4×10^5 and 78% for 2.4×10^7 cfu mL^{-1}.

APPENDIX 8 - DUPLEX PCR TEST FOR THE IDENTIFICATION OF *RALSTONIA SOLANACEARUM* PHYLOTYPE II STRAINS OF THE 4NPB AND MOKO ECOTYPES (CELLIER ET AL., 2015)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. This PCR is suitable for the identification of *R. solanacearum* Phylotype II strains of the 4NPB and Moko ecotypes. It can be used on cultures isolated from plant tissue and for direct detection in plant extracts.

1.2. The test is based on Cellier et al. (2015).

1.3. The target sequences are those of the RALUWv1_4260003 gene, coding for a putative KfrA protein (shared by strains of both the 4NPB and Moko ecotype), and the RAL70v1_1150031 gene (only found in the IIB-4NPB ecotype), coding for a chemotaxis-related protein.

1.4. Oligonucleotides:

Forward primer	93F	5′-CGC TGC GCG GCC GTT TCA C-3′;	477 bp (RALUWv1_4260003 gene)
Reverse primer	93R	5′-CGG TCG CGG CAT GGG CTT GG-3′	
Forward primer	5F	5′-GCG CGC GAG GCT GGT GAT GT-3′	661 bp (RAL70v1_1150031 gene)
Reverse primer	5R	5′-TGG GTT CGC AGG CGG ACA GC-3′	

1.5. The PCR amplification has been validated on a Veriti® thermal cycler (Applied Biosystems) and a GeneAmp PCR System 9700 thermal cycler (Thermo Fisher Scientific).

2. Methods

2.1. Nucleic acid extraction and purification (see Appendix 3)

DNA should preferably be stored at approximately −20°C.

2.2. Duplex PCR

2.2.1. Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	13.88	N.A.
G2® Hot Start Mix (Promega)	5×	5	1×
$MgCl_2$	25 mM	1.5	1.5 mM
dNTPs	10 mM	0.5	0.2 mM
Forward primer (93F, 5F)	100 µM	0.5 (each)	2 µM
Reverse primer (93R, 5R)	100 µM	0.5 (each)	2 µM
G2® Hot Start polymerase (Promega)	5 U $µL^{-1}$	0.125	0.625 U
Subtotal		23	
Homogenized plant material		2	
Total		25	

2.2.2. PCR conditions: 96°C for 5 min followed by 35 cycles of (94°C for 15 s, 70°C for 30 s, 72°C for 30 s) and a final step of 72°C for 10 min.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification, preferably of a sample of uninfected matrix or, if not available, clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample known to contain the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

3.2. Interpretation of results

To assign results from PCR-based tests the following criteria should be followed:

Duplex PCR tests

Verification of the controls

- NIC and NAC should produce no amplicon.
- PIC, PAC (and if relevant IC) should produce amplicons of the relevant size (depending on the target, endogenous or exogenous nucleic acid is used).

When these conditions are met

- A test will be considered positive for the Moko ecotype if amplicon of 477 bp is produced; it will be considered positive for the NPB ecotype if amplicons of 477 and 661 bp are produced.
- A test will be considered negative if it produces no band or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Performance characteristics were obtained with bacterial cultures and for analytical sensitivity on banana plant extracts. Data from ANSES, LSV, la Réunion.

4.1. Analytical sensitivity data

- Bacterial cultures

Analytical sensitivity calculated as 10^5 cfu mL^{-1} for both 93F/93R and 5F/5R pairs, and also for the duplex 93F/93R, 5F/5R PCR.

- Banana plant extracts

Analytical sensitivity calculated as 10^5 cfu mL^{-1} for both 93F/93R and 5F/5R pairs.

4.2. Analytical specificity data

- Bacterial cultures

Evaluated on 111 strains (40 targets strains and 71 non-target strains).

Inclusivity calculated as 92% for both 93F/93R and 5F/5R pairs.

Exclusivity calculated as 100% for both 93F/93R and 5F/5R pairs.

Accuracy calculated as 96% for the 93F/93R pairs and 100% for the 5F/5R.

- Banana plant extracts

Analytical sensitivity not evaluated.

4.3. Repeatability

- Bacterial cultures

Repeatability calculated as 95% for 93F/93R pairs and 100% for 5F/5R pairs.

- Banana plant extracts

Repeatability was 100% at 10^5 cfu mL^{-1}.

4.4. Reproducibility

- Bacterial cultures and banana plant extracts

Different PCR machines, operators, time and reagents were used for validation, showing no difference in the results.

APPENDIX 9 - MULTIPLEX CONVENTIONAL PCR TESTS FOR IDENTIFICATION OF PHYLOTYPES I–IV OF *RALSTONIA SOLANACEARUM*, *RALSTONIA PSEUDOSOLANACEARUM* AND *RALSTONIA SYZYGII* (OPINA ET AL., 1997; FEGAN & PRIOR, 2005)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. This multiplex conventional PCR is suitable for the identification of *Ralstonia solanacearum* (Phylotype II), *R. pseudosolanacearum* (Phylotype I or III) and *R. syzygii* (Phylotype IV). It has not been validated for direct detection in plant extracts.

1.2. The test is based on the publications of Fegan and Prior (2005) and Opina et al. (1997).

1.3. The target sequences for primers 759 and 760 (Opina et al., 1997) are those of the IpxC gene, coding for an in planta expressed protein (unique to strains of all four Phylotypes). The other primers are all targeted in the ITS intergenic region between the 16S and 23S rRNA genes (Fegan & Prior, 2005). The reverse primer Nmult22:RR is common to all four Phylotypes whereas the forward primers Nmult21:1F, Nmult21:2F, Nmult23:AF and Nmult22:InF are specific to Phylotypes I, II, III and IV, respectively.

1.4. Amplicon sizes in base pairs are:

- 280 bp or 282 bp for primers 759/760.
- 144 bp for primers Nmult21:1F/Nmult22:RR.
- 372 bp for primers Nmult21:2F/Nmult22:RR.
- 91 bp for primers Nmult23:AF/Nmult22:RR.
- 213 bp for primers Nmult22:InF/Nmult22:RR.

1.5. Oligonucleotides:

Primer	Sequence	Specificity
759	5′-GTCGCCGTCAACTCACTTTCC-3′	All 4 Phylotypes
760	5′-GTCGCCGTCAGCAATGCGGA ATCG-3′	All 4 Phylotypes
Nmult21:1F	5′-CGTTGATGAGGCGCG CAATTT-3′	Phylotype I
Nmult21:2F	5′-AAGTTATGGACGGTGGA AGTC-3′	Phylotype II
Nmult23:AF	5′-ATTACGAGAGCAATCGAA AGATT-3′	Phylotype III
Nmult22:InF	5′-ATTGCCAAGACGAGAGAA GTA-3′	Phylotype IV
Nmult22:RR	5′-TCGCTTGACCCTATAACGA GTA-3′	All 4 Phylotypes

1.6. The multiplex PCR has been validated on a GeneAmp PCR System 9700 thermal cycler (Thermo Fisher Scientific) and a PCR Express thermocycler (Hybaid, Teddington, GB).

2. Methods

2.1. Nucleic acid extraction and purification

For crude DNA extraction from presumptive *Ralstonia* isolates, suspend single colonies from each isolate and from cultures of reference strains in 100 µL of sterile distilled water. Heat in closed microvials at 95°C for 12 min. Transfer heated suspensions to ice and pulse centrifuge after cooling.

DNA should preferably be stored at approximately −20°C.

2.2. Multiplex PCR

2.2.1. Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	15.25	N.A.
PCR buffer	5×	5	1×
$MgCl_2$	25 mM	1.5	1.5 mM
dNTPs (Promega)	10 mM	0.5	0.2 mM
Forward primer (760)	100 µM	0.04	0.16 µM
Reverse primer (759)	100 µM	0.04	0.16 µM
Forward primer (Nmult21:1F)	100 µM	0.06	0.24 µM
Forward primer (Nmult21:2F)	100 µM	0.06	0.24 µM
Forward primer (Nmult23:AF)	100 µM	0.18	0.72 µM
Forward primer (Nmult22:InF)	100 µM	0.06	0.24 µM
Reverse primer (Nmult22:RR)	100 µM	0.06	0.24 µM
GoTaq G2 DNA polymerase (Promega)[a]	5 U $µL^{-1}$	0.25	1.25 U
Subtotal		23	
Target DNA		2	
Total		25	

[a] Further optimization will be required for use with other reagents and systems.

2.2.2. PCR conditions: 96°C for 5 min followed by 30 cycles of (94°C for 15 s, 59°C for 30 s, 72°C for 30 s) and a final step of 72°C for 10 min.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or, if not available, clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample known to contain the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

3.2. Interpretation of results

To assign results from this multiplex PCR-based test the following criteria should be followed:

Verification of the controls

- NIC and NAC should produce no amplicons.
- PIC and PAC should produce amplicons of the relevant size.

When these conditions are met

- A test will be considered positive if amplicons of the expected size(s) are produced.
- A test will be considered negative if it produces no band or bands of different sizes.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

4.1. Analytical specificity data

Compared with barcoding using 16S rRNA, *mutS* and *egl* gene targets, Fegan and Prior (2005) used multiplex PCR to correctly identify 55 of 56 (98.2%) *Ralstonia* reference strains representing the known diversity, including 47 strains of Phylotype II (*R. solanacearum*), 3 strains each of Phylotypes I and III (*R. pseudosolanacearum*) and 3 strains of Phylotype IV (*R. syzygii*). The Phylotype IV strain ACH0732 isolated from tomato in Australia was not identified using this test. Wicker et al. (2007) similarly correctly identified 100% of 77 isolates and reference strains, including 14 strains of Phylotype I (*R. pseudosolanacearum*), 57 strains of Phylotype II (*R. solanacearum*), 4 strains of Phylotype III (*R. pseudosolanacearum*) and 2 strains of Phylotype IV (*R. syzygii*).

4.2. Additional performance characteristics from Anses, FR (2008, formerly LNPV) for primers 759–760

Diagnostic sensitivity (evaluated with known reference cultures): 93.3% (3 strains not detected).

Diagnostic specificity (evaluated with known reference cultures): 96.7% (1 strain not detected).

APPENDIX 10 - PATHOGENICITY TEST

Pathogenicity of isolates is usually confirmed on the same host from which they were isolated, although tomato is usually also susceptible to those strains that are known to have a wide host range. Young healthy seedlings or cuttings should be raised in a greenhouse and preferably used at the second to fourth leaf stage. Prior to inoculation, plants should not be watered for 1–2 days to increase the uptake and translocation of the inoculated bacterial suspension in the plant vascular tissues. It is recommended that plants are kept in the same environmental conditions (25–30°C, ideally higher than 70% relative humidity and 14 h photoperiod) before and after inoculation to avoid stress, which could adversely affect the plant's response to pathogen inoculation.

The inoculum is prepared by culturing the bacteria for 24–48 h at 28°C on sucrose peptone agar or YPGA prior to suspending and diluting to the required concentration in sterile, distilled water. Bacterial density can be measured spectrophotometrically, adjusting the cell suspension to an optical density (OD) of 0.1 (λ = 660 nm), corresponding to a bacterial concentration of about 10^8 cells mL^{-1} and diluting 1:100 to 10^6 cells mL^{-1}.

Plant stems are inoculated just above the cotyledons using a syringe fitted with a hypodermic needle (not less than 23G). Alternatively, plants can be inoculated by making a vertical cut, approximately 0.5 cm long, to the centre of the stem between the cotyledons and the first true leaves (or in the base of the pseudostem in the case of monocotyledonous hosts), with a sterile scalpel. A drop (10–20 µL) of the pathogen suspension is then injected into the wound with a sterile syringe or pipette. After inoculating, the cut is sealed with sterile petroleum jelly. A minimum of five (up to 10) plants per isolate are inoculated. Five additional plants are inoculated with sterile water as a negative control and five other plants are inoculated with a reference strain of the appropriate *Ralstonia* species at the same concentration and with the same procedure as described above (as positive control).

Inoculated plants should be maintained in controlled conditions, with temperature around 28°C; in any case, care should be taken to avoid the temperature falling below 24°C, as wilting may be significantly delayed or may not appear at all. Plants should be incubated for 21–28 days and inspected regularly for the development of wilting. Symptoms usually appear between the 8th and 12th days after inoculation for potatoes but between the 3rd and 7th days after inoculation for tomato. Symptoms include partial or total wilting of leaflets and leaves, starting from those nearest to the inoculation cut. Epinasty is also a common symptom, with or without leaf wilting. Symptom development in *Musa* spp. usually takes longer than 3 weeks.

As soon as symptoms appear, the bacterium should be re-isolated from plants by taking a stem, pseudostem or petiole section above the inoculation point, placing it in a small volume (1–2 mL) of sterile distilled water or 50 mM phosphate buffer and soaking for 10–15 min. A loopful (10–20 µL) of the resulting suspension should then be streaked on YPGA and/or mSMSA medium and plates observed for the typical colonies (Section 3.2.2).

In the case of symptom development on both the positive control and at least one of the test plants, pathogenicity of an isolate is confirmed. In the case of no symptom development in any of the inoculated plants and the positive control is symptomatic, pathogenicity of isolates is not confirmed. In the case of no symptom development on both the test plants and the positive control, the pathogenicity assay is invalid and should be repeated, taking care to use a virulent reference strain as a control.

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ADDENDUM

Addendum for PM 7/21 (3) *Ralstonia solanacearum*, *R. pseudosolanacearum* and *R. syzygii* (*Ralstonia solanacearum* species complex)

In Table 1 a note should be added to the test 'Massart et al. (2014)' in the column 'Reference' under other tests. This note (which should be labelled c) should link to a new footnote: (c) The test from Massart et al. (2014) is described in Appendix 7 of PM 7/059 (2) *Clavibacter sepedonicus*.

The last 2 lines of this table should then read as follows (changes marked in bold).

Massart et al. (2014) Real-time[c]	Real-time PCR: 16–23S rRNA spacer sequence	Multiraso-F/Multiraso-R/ Multiraso-P	*R. solanacearum* (Phylotype IIB and some Phylotype IIA strains)
Stulberg et al. (2015)	Conventional multiplex PCR: from genome comparisons	Various primers	*R. solanacearum* (Phylotypes IIB-1 and IIB-2 only)

[a] Subspecies targeted not known. When testing for *R. syzygii* with these tests, additional evaluation is needed.

[b] Data generated by the EURL for Pest of Plants on Bacteria.

[c] **The test from Massart et al. (2014) is described in Appendix 7 of PM 7/059 *Clavibacter sepedonicus*.**

REFERENCES

EPPO (2022a) PM 7/59 (2) *Clavibacter sepedonicus*, *EPPO Bulletin* 52, 262–285.

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