Diagnostic Guide

Ralstonia solanacearum Species Complex: A Quick Diagnostic GuideRaymond O. García, Jim P. Kerns, and Lindsey Thiessen[†]

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Hosts: The *Ralstonia solanacearum* species complex is composed of several *R. solanacearum* strains with low relatedness (Prior and Fegan 2005) and has a wide host range. The bacterium affects over 200 plant species within 50 different families, mainly dicotyledonous, herbaceous members of the *Solanaceae* family (Denny 2006). A few monocots, weeds, crops, shrubs, and trees are affected as well (Denny 2006; Genin and Denny 2012). Some of the primary high-value crops susceptible to *R. solanacearum* are summarized in Table 1.

Pathogen: *R. solanacearum* was first described by Erwin F. Smith in 1896 and was first named *Bacillus solanacearum* (Smith 1896). In 1914, Smith changed its name to *Pseudomonas solanacearum* (Smith 1896) Smith 1914. *P. solanacearum* was included in the Approved Lists of Bacterial Names (Skerman et al. 1980), and a substantial amount of literature was published under this name. Later, Yabuuchi et al. (1992) transferred *P. solanacearum* and related nonfluorescent pseudomonads to a novel genus called *Burkholderia*. Subsequently, Yabuuchi et al. (1995) concluded that *Burkholderia solanacearum* was distinct enough from other members within the genus to be assigned in the newly proposed genus *Ralstonia*. To date, the pathogen's preferred scientific name is *Ralstonia solanacearum* (Smith 1896) Yabuuchi et al. 1995.

Taxonomy

R. solanacearum is a member of the nonfluorescent pseudomonads rRNA homology group II. The current *R. solanacearum* taxonomic state and infrasubspecific classification are shown in Tables 2 and 3, respectively, based on works by Prior et al. (2016), Remenant et al. (2011), and Safni et al. (2014) and the Taxonomy Browser tool on the National Center for Biotechnology Information website (<https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi>).

Symptoms and Signs

Specific symptoms of *R. solanacearum* can vary among the wide range of susceptible hosts; however, general characteristics are shared among the host complex, including wilting and yellowing of younger leaves. The wilt is often unilateral (Fig. 1A), meaning that it is restricted to one side of the plant or one side of the leaf midvein (Denny 2006). Because *R. solanacearum* is found in high cell densities on infected tissues, a common sign of the pathogen is a milky white bacterial ooze that accumulates at the surface of freshly

cut stems, rhizomes, or tubers (Denny 2006). In rare cases, when the cell density is low and the ooze does not form spontaneously, the “stem-streaming” test can be a useful diagnostic tool, especially in the field (Allen et al. 2001; Denny 2006; Momol and Champoiseau 2009). The stem-streaming test consists of placing a freshly cut stem segment in a clear vial containing water to induce the secretion of a viscous, white-creamy slime distinctive of *R. solanacearum* (Fig. 1B) (Momol and Champoiseau 2009). Additionally, symptom expression is directly affected by water availability and temperature. Generally, warmer temperatures above 28°C promote a faster symptom development than cooler temperatures (Denny 2006; Momol and Champoiseau 2009); however, symptoms may be slightly different across hosts and environmental conditions. A detailed description of the disease symptoms on representative hosts is provided below.

Tobacco. At the early stages of the disease cycle, the younger tobacco (*Nicotiana tabacum*) leaves are the first to wilt (Fig. 1C) (Denny 2006; Echandi 1991). As the disease progresses, the leaves turn yellow, and scorch-like symptoms may be expressed at the margins and between the veins, followed by a total collapse of the plant (Denny 2006). Stunting and leaf distortion may be observed in plants that do not die. Furthermore, primary and secondary roots may rot (Echandi 1991). When performing a cross section along the central axis, a reddish to brown discoloration of the vascular system, a hollow stem owing to the pith rotting, and lesions on the stem surface may be observed (Fig. 1D) (Denny 2006).

Tomato. Symptoms in tomato (*Solanum lycopersicum*) are similar to those observed on tobacco. These are first visible on the aboveground parts of the plant, specifically on the younger leaves (Fig. 1E) (Momol and Champoiseau 2009). However, the unilateral wilt may not be obvious, mainly owing to leaf size (Denny 2006). Under field conditions, wilt is often not observed until after flowering (McCarter 1991).

Potato. Wilting and chlorosis of the leaves located at the tip of the branches are the first visible symptoms of *R. solanacearum* infection on potato (*Solanum tuberosum*) (Denny 2006; Stevenson et al. 2001). Stunting and petiole epinasty may also occur (Denny 2006; Stevenson et al. 2001). Stem segments above the soil line may have dark brown streaks owing to necrosis and discoloration of the vascular system (Denny 2006; Stevenson et al. 2001). Secondary rotting, followed by death and desiccation, is common (Denny 2006). These symptoms may be observed at any phenological stage of the plant, and the rate at which they develop will be faster if the source of inoculum was an infected tuber used as vegetative material for propagation (Stevenson et al. 2001).

Belowground symptoms are important for identification of *R. solanacearum* on potatoes. Infected tubers can display a grayish brown discoloration of the vascular tissue and necrosis of the vascular ring and adjacent tissues (Denny 2006; Stevenson et al.

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2001). The periderm typically remains asymptomatic until the late stages of disease development (Denny 2006; Stevenson et al. 2001); however, in the case of infected young tubers, symptoms may be visible through the periderm if the bacterium is present at high cell

densities (Stevenson et al. 2001). Bacterial ooze may emerge from the eyes of the tuber, and when it dries, soil can adhere to the tuber, providing a “smutty” appearance (Fig. 1F) (Stevenson et al. 2001). Brown rot is often confused with ring rot caused by *Clavibacter michiganensis* subsp. *sepedonicus* (Denny 2006; Stevenson et al. 2001); however, these can be distinguished based on signs and symptomatology because the bacterial streaming and vascular discoloration are not as evident in ring rot as they are in brown rot (Stevenson et al. 2001).

Host Range

Strains of *R. solanacearum* have different characteristics, including host range (Hong et al. 2012), which led researchers to classify bacterial strains to subspecies or intraspecies levels, such as races and biovars. To date, five races and six biovars of *R. solanacearum* have been identified based on host specificity (Buddenhagen et al. 1962) and on the bacterium’s ability to acidify media containing different carbohydrate sources, reduce nitrate to nitrite, and denitrify from nitrate, respectively (Denny 2006; Hayward 1964). Despite being used for many years, race designation based on pathogenicity tests has proven to be unreliable (Genin and Denny 2012), because they are influenced by many factors such as host cultivar, growth conditions, inoculum concentration, and inoculation procedure (Denny 2006). Additionally, it does not correlate with phenotypic and ecological traits of the bacterium, allow accurate assessment of the genetic diversity at the infraspecific level, nor identify the evolutionary relationship between strains (Prior and Fegan 2005).

Owing to the inaccuracies of race and biovar classifications, current *R. solanacearum* infrasubspecific classification is based on phylotypes and sequevars (Hong et al. 2012). A phylotype is defined as a monophyletic cluster of strains revealed by phylogenetic analysis of sequence data, and phylotypes are equivalent to the subspecies level or even separate species (Fegan and Prior 2005). Four phylotypes have been identified based on sequence variation of the internal transcribed spacer region (Fegan and Prior 2005). A sequevar, also known as sequence variant, is defined as group of strains with a highly conserved sequence (Fegan and Prior 2005); currently, over 20 sequevars have been identified based on the partial endoglucanase gene (*egl*) sequence (Fegan and Prior 2005).

Race 1 is endemic in the Southeastern United States, has the broadest host range (Buddenhagen et al. 1962), and can be found across all the known phylotypes (Table 3). Phylotype II/sequevars 3, 4, and 6 (formerly race 2) affects triploid bananas (*Musa* spp.) and *Heliconia* spp., a tropical American relative of *Musa* spp. (Buddenhagen et al. 1962). Phylotype II/sequevars 1 and 2

TABLE 1
Common and scientific names of some of the primary high-value crops affected by *Ralstonia solanacearum*

Host common name	Scientific name	Disease common name ^a
Tobacco	<i>Nicotiana tabacum</i>	Granville wilt
Tomato	<i>Solanum lycopersicum</i>	Bacterial wilt
Potato	<i>Solanum tuberosum</i>	Bacterial wilt or brown rot
Pepper	<i>Capsicum</i> spp.	Bacterial wilt
Eggplant	<i>Solanum melongena</i>	Bacterial wilt ^b
Bananas	<i>Musa</i> spp.	Moko disease
Olive	<i>Olea europaea</i>	Bacterial wilt ^b
Ginger	<i>Zingiber officinale</i>	Bacterial wilt ^b
Geraniums	<i>Pelargonium</i> spp.	Southern wilt
Mulberry	<i>Morus</i> spp.	Bacterial wilt
Groundnut	<i>Arachis hypogaea</i>	Bacterial wilt

^a Disease common names as listed in the American Phytopathology Society (APS) Approved Revised Common Names of Plant Diseases website (<https://www.apsnet.org/publications/commonnames/Pages/default.aspx>).

^b Disease common names are not listed in the APS Approved Revised Common Names of Plant Diseases website; therefore, we referred to them as “bacterial wilt.”

TABLE 2
Ralstonia solanacearum current taxonomic state

Rank	Name
Domain	Bacteria
Phylum	Proteobacteria
Class	β-Proteobacteria
Order	Burkholderiales
Family	Burkholderiaceae
Genus	<i>Ralstonia</i>
Species ^a	<i>R. detusculanense</i> , <i>R. insidiosa</i> , <i>R. mannitolilytica</i> , <i>R. pickettii</i> , <i>R. pseudosolanacearum</i> , <i>R. solanacearum</i> , <i>R. syzygii</i>

^a To date, the genus *Ralstonia* is composed of seven recognized species.

TABLE 3
Ralstonia solanacearum current infrasubspecific classification

Phylotype	Sequevar	Biovar	Ecotype	Races	Species
I	12, 14, 16, 18	3, 4, 5	BW ^a of <i>Solanaceae</i> , ginger, mulberry	1, 4, 5	<i>R. pseudosolanacearum</i>
IIA	1, 2, 3, 4, 5, 6, 7, CIP10, CIP223, NCPPB3987	2-T, 1, 2	BW of <i>Solanaceae</i> , <i>Musa</i> spp.	1, 2, 3	<i>R. solanacearum</i>
IIB	1, 2, 3, 4, 5, 6, 7, CIP10, CIP223, NCPPB3987	2-T, 1, 2	Moko disease, NPB, ^b brown rot of potato, BW of tomato and geranium	1, 2, 3	<i>R. solanacearum</i>
III	19, 20, 21, 22, 23	2-T, 1	BW of <i>Solanaceae</i>	1	<i>R. pseudosolanacearum</i>
IV	8, 9, 10, 11	2-T, 1, 2	BW of <i>Solanaceae</i> , BDB of <i>Musa</i> spp., ^c Sumatra disease of clove	1	<i>R. syzygii</i>

^a BW = bacterial wilt.

^b Nonpathogenic to banana (NPB) are new pathogenic variants highly virulent to *Anthurium*, *Heliconia*, and cucurbits but not to bananas.

^c BDB = blood disease of banana.

FIGURE 1

Ralstonia solanacearum distinctive symptoms and signs on representative hosts: **A**, unilateral yellowing and wilting of tobacco plant in the field; **B**, the stem-streaming test; **C**, wilting of tobacco leaves in the field; **D**, reddish to brown discoloration of the vascular tissue on tobacco stem; **E**, wilting and yellowing of younger leaves of tomato in the greenhouse; and **F**, periderm smutty appearance of potato tubers.

(formerly race 3 strains, including the former race 3/biovar 2A) cause bacterial wilt on potato and tomato (Buddenhagen et al. 1962) (Table 3). Lastly, races 4 and 5 (currently classified within phylotype I/sequevars 12, 14, 16, and 18) infect ginger and mulberry trees, respectively (Table 3) (He et al. 1983; Persley et al. 1986).

A table summarizing most, if not all, the hosts from which *R. solanacearum* has been reported can be found at the CABI website (<https://www.cabi.org/isc/datasheet/45009>).

Geographic Distribution

R. solanacearum has a worldwide distribution, owing in part to its wide host range. The bacterium is a ubiquitous soil inhabitant of all continents and many islands between the Tropics of Cancer and Capricorn. In this region, the average annual rainfall exceeds 100 cm, the growing season is more than 6 months, the winter temperatures are higher than 10°C, the average summer temperatures surpass 21°C, and the average yearly temperature does not exceed 23°C (Denny 2006; Lucas 1975). Moraes (1947) first described the presence of strains capable of infecting potato even in temperate zones, where temperatures were $\leq 24^{\circ}\text{C}$. These strains

were later identified as race 3 biovar 2 (currently phylotype IIB/sequevar 1), also known as the “potato race.” Phylotype IIB/sequevar 1 is listed as a select agent by the USDA Agricultural Bioterrorism Protection Act of 2002.

Pathogen Isolation

R. solanacearum can be isolated from water, soil, or symptomatic tissues. Regardless of the sample source, the sampled materials should ideally reach the laboratory facilities within 24 h from the time of collection (Kinyua et al. 2014). Isolating the bacterium from infected plant tissues can provide a pure culture, because the bacterial ooze produced by *R. solanacearum* is usually composed of pure bacterial cells (Denny 2006). Thus, small pieces of tissue excised from younger lesions can be surface sterilized, homogenized in sterile distilled water or 0.05 M potassium phosphate buffer using a sterile mortar, streaked on standard low-ionic-strength bacteriological medium (e.g., modified Sequiera medium South Africa [SMSA] and triphenyl tetrazolium chloride [TZC]), and incubated for 48 h at 25 to 27°C (Table 4) (Denny 2006; Seleim et al. 2014). Some susceptible hosts will remain asymptomatic even when infected; SMSA should be used for these.

Water and soil samples tend to have low populations of *R. solanacearum*, not all cells may grow in vitro, and higher levels of saprophytic bacteria and/or contaminants are present (Denny 2006). Therefore, these sample sources should be cultured on SMSA (Denny 2006). Lastly, pure cultures of *R. solanacearum* can be generated using the dilution streaking technique and selecting a single colony.

Pathogen Identification

R. solanacearum can be identified or detected using culture-based methods such as semiselective media, serological methods such as immune-strip tests and enzyme-linked immunosorbent assay, biochemical tests such as BIOLOG, fatty acids and protein analyses, and nucleic acid-based methods (Bekele et al. 2011; Denny 2006; Seal et al. 1993; Seal and Elphinstone 1994; Tran et al. 2016).

Traditional and rapid methods used for initial screening under field conditions include the stem-streaming test and commercially available *R. solanacearum*-specific immunoassay strips (Agdia,

Elkhart, IN) (Denny 2006; Kinyua et al. 2014). Streaking the bacterium on SMSA or TZC is another culture-based method commonly employed for initial screening (Denny 2006; Kinyua et al. 2014). Screening the bacterium on TZC allows differentiation between virulent and nonvirulent strains. Virulent strains generate white colonies with pink centers (Fig. 2A), whereas colonies of nonvirulent strains are dark red (Fig. 2B). On SMSA, colonies appear white with pink centers, fluidal, and irregularly shaped, just as they look on TZC. Lastly, when grown on casamino acid–peptone–glucose (CPG), virulent strains are white to cream colored, irregularly round, fluidal, and opaque (Fig. 2C). In contrast, nonvirulent colonies are uniformly round, smaller, and butyrous (Fig. 2D).

Although the techniques mentioned above are generally reliable and easy to perform, each has unique limitations. Serological tests have minimum cell thresholds for detection, do not differentiate between dead and live cells, and the rate of false positives increases proportionally with the number of contaminants in the sample (Denny 2006; Narayanasamy 2011). Furthermore, none of the assays allow identification of infrasubspecific levels, such as phylotype. To address these limitations and to provide high levels of confidence in identification, highly sensitive and specific nucleic acid-based methods such as conventional polymerase chain reaction (PCR), qPCR, microarray, and loop-mediated isothermal amplification have been developed (Cellier et al. 2017; Tran et al. 2016). *R. solanacearum* universal, phylotype, and sequevar- and strain-specific primers are available and widely used (Cellier et al. 2017; Fegan et al. 1998; Fegan and Prior 2005; Kubota et al. 2011; Opina et al. 1997; Stulberg et al. 2015; Weller et al. 2000; Yun et al. 2010). Some of the most commonly used nucleic acid-based methods are summarized in Table 5.

Recently, Tran et al. (2016) conducted a study in which they compared the sensitivity, cost-effectiveness, and technical complexity of multiple assays employed for the detection and identification of *R. solanacearum*. Although time consuming and laborious, culture enrichment combined with dilution plating was the most sensitive of the assays considered, capable of detecting up to 10^1 CFU/ml. Also, the sensitivity of PCR-based methods was enhanced by using a polymerase with enhanced performance in the presence of plant inhibitors. Additionally, bacterial cells were killed within 60 min of application to Whatman FTA nucleic acid-binding cards, suggesting that samples on FTA cards can be safely transported from the field to the labs for diagnosis without presenting a biosecurity threat (Tran et al. 2016). Lastly, they concluded that conducting PCR-based methods from FTA cards was faster, easier, and sensitive enough to detect cell densities as low as 10^4 CFU/ml.

In conclusion, the reliability and specificity of the methodology employed will depend on the purpose of the identification (Denny 2006). For example, if the identification is for quarantine purposes or to confirm the introduction of the pathogen to areas where it was not present, then reliable and unequivocal methods should be employed (Denny 2006).

Pathogen Storage

Pure cultures of *R. solanacearum* can be stored at room temperature (20°C) in sterile tap, distilled, or deionized water (Champoiseau 2009). If stored in distilled or deionized water, cultures should be restreaked twice a year and repurified in the presence of nonmucoid colonies (Champoiseau 2009). Cryostorage in screw-capped vials containing CPG broth (500 μ l) amended with 20% glycerol (600 μ l) at -70°C is most convenient for long-term storage (Champoiseau 2009; Kinyua et al. 2014). To stock a strain, transfer from a 2-day-old CPG or TZC plate grown at 30°C (Champoiseau 2009). *R. solanacearum* can lose viability, or

TABLE 4 Recipes for the media commonly used for detection, isolation, and identification of <i>Ralstonia solanacearum</i>	
Medium	Per liter
Casamino acid–peptone–glucose (CPG) ^{a,b} (Kelman 1954)	
Casamino acid (casein hydrolysate)	1 g
Peptone	10 g
Glucose	5 g
For solid media add	
Agar	17 g
Triphenyl tetrazolium chloride (TTC or TZC) ^{a,b,c} (Kelman 1954)	
Casamino acid (casein hydrolysate)	1 g
Peptone	10 g
Glucose	5 g
For solid media add	
Agar	17 g
Modified Sequiera medium South Africa (SMSA) ^{a,b,c} (Elphinstone et al. 1996)	
Casamino acid (casein hydrolysate)	1 g
Peptone	10 g
Glycerol	5 ml
For solid media add	
Agar	17 g
After autoclaving add ^d	
Crystal violet*	5 mg
Polymyxin β sulfate*	100 mg
Bacitracin*	25 mg
Chloromycetin*	5 mg
Penicillin*	0.5 mg
When inhibition of fungal contaminants is desired, add	
Cycloheximide*	100 mg

^a pH should be adjusted to 6.5 to 7.0.

^b Sterilize at 121°C and 15 psi for 20 min.

^c Complement medium with 5 ml of a 1% TZC solution. TZC has to be added after autoclaving and letting the medium cool down to approximately 55°C . TZC stock solution can be filter sterilized or autoclaved for 5 min at 121°C and should be stored at 4°C or frozen.

^d Ingredients with an asterisk (*) next to them should be dissolved in 5 ml of 70% ethanol 30 min prior to use and added after autoclaving.

FIGURE 2

A, On triphenyl tetrazolium chloride (TZC), virulent strains produce a white with pink center, irregularly round, and fluidal colony (black arrow); **B**, avirulent strains produce a smaller, dark-red and regularly round colony on TZC; **C**, virulent strains are white or cream-colored, irregularly round, fluidal, and opaque colony on casamino acid-peptone-glucose (CPG); and **D**, avirulent strains are white or cream-colored, smaller, regularly round, and opaque colony on CPG.

cultures may become viable but nonculturable, if they are exposed to low temperatures (4°C) (van Elsas et al. 2001).

Pathogenicity Tests

For pathogenicity tests, soil drenching is one of the most common methods used for inoculation. Soil drenching or root inoculation often consists of wounding the roots and pouring 30 to 50 ml of a 10^8 CFU/ml (optical density [OD] at 600 nm wavelength = 0.1) bacterial suspension close to the crown of the plant (Hong et al.

2012). Strains are generally inoculated on 3- to 4-week old plants (four to five true leaves) (Hong et al. 2012). The inoculated plants are typically transferred to either a growth room with a 12-h light/dark cycle with an approximate temperature of 30°C (Hong et al. 2012) or a greenhouse. Stem injection is another common method used for inoculation during pathogenicity tests and is typically carried out by puncturing the stem with a needle and injecting 100 µl of bacterial suspension (OD = 0.1) (Winstead and Kelman 1952). For disease severity assessment, disease incidence is

TABLE 5
Available nucleic acid-based tools for the detection and identification of the *Ralstonia solanacearum* species complex

Reference	Method of assay	Taxonomic level	Primer pair name	Primer sequence
Opina et al. (1997)	PCR	Species complex	759/760	F: GTCGCCGTCAACTCACTTTCC R: GTCGCCGTGAGCAATGCGGAATCG
Fegan et al. (1998)	PCR	Phylotype IIB/ sequevar 1 (formerly race 3 biovar 2)	630/631	F: ATACAGAATTCGACCGGCAC R: AATCACATGCAATTCGCCTAC
Weller et al. (2000)	Multiplex, qPCR	Phylotype IIB/ sequevar 1 (formerly race 3 biovar 2)	B2-I-F B2-II-R	F: TGGCGCACTGCACTCAAC R: AATCACATGCAATTCGCCTACG TTCAAGCCGAACACCTGCTGCAAG
Fegan and Prior (2005)	Multiplex	Phylotype	B2-Probe Nmult21:1F – Phylotype I Nmult21:2F – Phylotype II Nmult23:AF – Phylotype III Nmult22:InF – Phylotype IV Nmult22:RR – All phylotypes	F: CGTTGATGAGGCGCGCAATTT F: AAGTTATGGACGGTGGAAGTC F: ATTACSAGAGCAATCGAAAAGATT F: ATTGCCAAGACGAGAGAAGTA R: TCGCTTGACCCTATAACGAGTA
Yun et al. (2010)	qPCR	Species complex	RSF RSR	F: GTGCCTGCCTCCAAAACGACT R: GACGCCACCGCATCCCTC
Kubota et al. (2011)	PCR	Phylotype IIB/ sequevar 1 (formerly race 3 biovar 2)	RRSL2403F RRSL2403R	F: TTGGCACGAGTCTGCACTAC R: CACATCAGGCGATACAGTGG
Stulberg et al. (2015)	Multiplex	Species complex Phylotype IIB/ sequevar 1 (formerly race 3 biovar 2)	RsSC-F RsSC-R RsSA-F RsSA-R	F: CCGAGCGCATATCGTTCACAC R: TTTGGCGTTCGGTTCGGAG F: CAACGATGCCTG GAAACTGACC R: TGGTCCGGGTTCAGGTAAATGTCAC
Cellier et al. (2017)	Microarray	17 major groups in the species complex	See reference for primer names	See reference for primer sequences

commonly used, but other methods such as wilt index are used as well (Winstead and Kelman 1952).

Recently, Singh et al. (2018) developed a pathogenicity assay performed under gnotobiotic conditions. Using the method developed, tomato seedlings showed wilting symptoms approximately 48 h postinoculation, and the pathogenicity assay could be completed within 2 weeks (Singh et al. 2018). Six- to 7-day-old tomato seedlings were root-dip inoculated in a bacterial suspension with an adjusted concentration of 10^9 CFU/ml and transferred to 2.0-ml sterile microcentrifuge tubes. Prior to the addition of 1 to 1.5 ml of sterile water, the root-dip-inoculated tomato seedlings were subjected to air exposure for approximately 5 min. All the inoculated seedlings along with controls were transferred to a growth chamber maintained at 28°C, 75% relative humidity, and a 12-h photoperiod (Singh et al. 2018). Lastly, seedlings were analyzed for disease progression from the next day onward until the seventh day postinoculation (Singh et al. 2018). The innovative pathogenicity assay is economic in terms of space, labor, and cost, as well as the required quantity of bacterial inoculum, and also useful for studying known virulence genes of *R. solanacearum* such as *hrpB*, *phcA*, and *pilT* (Singh et al. 2018).

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