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National Plant Disease Recovery System

Recovery Plan for *Ralstonia solanacearum* Race 3 Biovar 2 (Phylotype IIB, Sequevars 1 and 2) Causing Brown Rot of Potato, Bacterial Wilt of Tomato, and Southern Wilt of Geranium

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This recovery plan is one of several disease-specific documents produced as part of the National Plant Disease Recovery System (NPDRS) called for in Homeland Security Presidential Directive Number 9 (HSPD-9). The purpose of the NPDRS is to ensure that the tools, infrastructure, communication networks, and capacity required to mitigate the impact of high-consequence plant disease outbreaks are such that a reasonable level of crop production is maintained.

Each disease-specific plan is intended to provide a brief primer on the disease, assess the status of critical recovery components, and identify disease management research, extension, and education needs. These plans are not intended to be stand-alone documents that address the many and varied aspects of plant disease outbreaks, the multitude of decisions that must be made, and actions taken to achieve effective response and recovery. They will, however, help the United States Department of Agriculture (USDA) guide further efforts directed toward plant disease recovery.

Executive Summary

Bacterial wilt, an important disease of many crops, is caused by various subgroups of the bacterial pathogen *Ralstonia solanacearum*, formerly *Pseudomonas solanacearum*. Historically, strains of *R. solanacearum* were classified into races, based loosely on host range, and then into biovars, based on their differential ability to produce acid from a panel of carbohydrates. Due to its genetic diversity, *R. solanacearum* was designated as a species complex.

A revised classification system was proposed in 2005 based on DNA sequences and phylogenetic analysis. *R. solanacearum* strains were classified into four groups, called phylotypes I to IV, based on phylogenetic analysis. For a finer classification, strains of *R. solanacearum* were classified into sequevars based on the endoglucanase gene sequence. The strains historically classified as race 1 are nonmonophyletic and include phylotypes I, IIA, and III. Currently, phylotype II strains are designated *R. solanacearum*, phylotypes I and III strains have been moved to *R. pseudosolanacearum*, and phylotype IV strains are a subspecies within *R. syzygii.*

R. solanacearum race 1 (phy I, IIA, and III) naturally occurs in tropical and subtropical areas worldwide and typically has an extensive host range that includes important solanaceous crops. In the United States, *R. solanacearum* race 1 biovar 1 (phy IIA) is found in the southern states, where it causes bacterial wilt on a range of cultivated crops, such as pepper, tobacco, tomato, and, recently, blueberry, but rarely on potato. Potato production areas are located mainly in temperate climate areas. Furthermore, the endemic southeastern strains do not seem to persist in northern areas of the United States where potatoes are grown. Likely, bacterial wilt caused by these endemic strains has not spread to potato in other warmer areas of the United States because the seed potato system is isolated from the potato production system. In contrast, *R. solanacearum* race 3 biovar 2 (R3bv2) originated in South American tropical highlands and can survive and cause disease in temperate climates. R3bv2 causes bacterial wilt in solanaceous plants and geraniums. On potatoes, the disease is also known as brown rot of potato. In 2000, brown rot was estimated to affect 3.75 million acres in approximately 80 countries, with global damage estimates exceeding $950 million per year, which corresponds to more than $1,670 million per year in 2023. This bacterial strain is adapted to cooler temperatures and could be damaging to potato production regions of the United States. While race 1 causes loss to tomato crops in the southern United States, the economic impact of R3bv2 on potatoes and other host crops is unknown in temperate U.S. climates. If introduced, R3bv2 could affect 935,200 acres of potatoes, valued at $4.1 billion in 2021 (USDA-NASS 2021), with an export value of more than 17.9 million metric tons ($275.8 million) of fresh and seed potatoes in 2021 (USDA-FAS 2021).

It is very unlikely that a R3bv2 epidemic would exceed the global average potato loss to brown rot estimated at 3% by Savary et al. (2019), which would still be considerable with losses estimated at tens of millions of dollars per year. In Europe, where R3bv2

has been responsible for several outbreaks of potato brown rot over the last several decades, the pathogen is present in waterways and some soils, so it is important to maintain a tight surveillance system on sources of inoculum including water, wild hosts, seed potatoes, and ware potatoes (EU Commission Directorate General for Health and Food Safety 2017). Thanks to organized sampling in the EU, the overall incidence of R3bv2 had decreased by 2015 to 0.078% in potato ware and to 0% in seed potatoes.

Although R3bv2 is widely distributed in Asia, Africa, and South and Central America, and is found in some soils and waterways in Europe, it is not known to be established in the United States and Canada. R3bv2 was accidentally introduced into the United States on geranium cuttings produced in the highland tropics of Africa and Central America during 1999 and 2000. To manage this serious threat, R3bv2 was designated as a Federal Select Agent in the United States under the Agriculture Bioterrorism Protection Act of 2002. This Act requires private, state, and federal research laboratories or universities that possess, use, or transfer select agents to register with the USDA-Animal and Plant Health Inspection Service (APHIS)-Division of Agricultural Select Agents and Toxins (DASAT). Thanks to the rules implemented, USDA-APHIS and its state partners have successfully completed actions to eliminate R3bv2 from U.S. greenhouses. Notable events include several introductions that were successfully eradicated. In February 2003, the bacterium was identified in geranium cuttings imported from a facility in Kenya. The 2003 introduction resulted in the organism being identified in 127 individual greenhouses in 27 states. The organism was detected again in December 2003 and January 2004 in geranium cuttings that originated in Guatemala. No more positive identifications of R3bv2 were reported in the United States until 2020, when R3bv2 was confirmed on geraniums imported from Guatemala. In total, the response involved more than 650 facilities in 44 states. USDA-APHIS implemented random testing of imported *Pelargonium* plant material for *R. solanacearum* at its plant inspection stations beginning on September 27, 2021 (USDA-APHIS 2022), to further monitor and control introductions.

Management of bacterial wilt/brown rot is difficult due to lack of effective control measures and resistant plant cultivars. Chemical control, such as soil fumigants, has limited efficacy. In Florida, application of thymol, a plant-derived volatile compound, was shown to effectively reduce bacterial wilt on tomato caused by *R. solanacearum* race 1. Similarly, in Martinique, implementation and destruction of a cover crop planted with *Crotalaria spectabilis* was shown to significantly reduce bacterial wilt symptoms on tomato. Also, a systemic acquired resistance inducer, acibenzolar *S*-methyl, which triggers the salicylic acid pathway, was shown to improve resistance on two moderately resistant tomato varieties. However, the effect of these approaches to control R3bv2 on potatoes is unknown. Additionally, detection of the pathogen can be difficult due to the occurrence of latent (symptomless) infection in potato tubers and geranium cuttings. Only a few commercially acceptable and R3bv2-tolerant tomato cultivars are available that provide moderate levels of disease control, and their efficacy is limited geographically. In experimental field trials in Florida, transgenic resistance provided by a pattern recognition receptor elongation factor (EF-Tu)-receptor (EFR) from *Arabidopsis thaliana* inserted into a susceptible tomato variety reduced bacterial wilt incidence and severity under field conditions against a phylotype II strain prevalent in the southern United States. A prior study using EFR in a susceptible commercial potato variety and a breeding line showed similar trends against a R3bv2 strain in controlled greenhouse experiments. Thus, the best protection from losses to R3bv2 in the United States will be achieved mainly by exclusion through the effective use of statutory quarantines, effective sanitation standards for offshore geranium production, early detection, and eradication by host destruction and soil disinfestation. Development of effective disease management strategies and improvement of detection and monitoring tools are key components of this recovery strategy. Besides these recommended actions, other research, education, and extension priorities for effectively mitigating R3bv2 are summarized in this report.

Recommended Actions:

1) Develop disease management tactics to control bacterial wilt on potato, tomato, and geranium (phylotype I or IIA strains could be used as a model to advance this area) such as:
    a) Develop and/or screen additional chemicals (fumigants, plant defense inducers, plant essential oils and their active components, oxidizers, liming, etc.) and biological control products (bacteriophages and other biocontrol agents, anaerobic soil disinfection, etc.).
    b) Develop resistant plant varieties that are commercially viable using molecular and traditional techniques and evaluate the potential of transgenic and gene-edited plants.
    c) Exclude the pathogen from potato seeds, geranium cuttings, and tomato transplants and develop vegetative plant material certification schemes.
    d) Study the effects of cover crops, crop rotation, and mulches on pathogen dynamics and disease incidence.
2) Develop easy-to-use detection tools to reliably distinguish subgroups of *R. solanacearum* species complex, especially R. *solanacearum* R3bv2, from endemic strains of *R. solanacearum* or *R. pseudosolanacearum*. Special emphasis should be given to developing point-of-care (field-based) detection approaches for R3bv2.
3) Develop impact network analysis (INA) tools to identify key risk factors in production and movement of planting material (tubers, cuttings) from areas with endemic populations of R3bv2.
4) Educate extension agents, growers, crop consultants, and regulators in:
    a) Sampling, monitoring, and managing bacterial wilt in known hosts.
    b) The advantage of map-based tracking on a global scale, especially in countries from which planting material is imported to the United States.
    c) Information systems such as the Pest Information Platform for Education and Extension (PIPE).

I. Introduction

The first reports of bacterial wilt appeared in the late nineteenth century. The pathogen was observed causing disease on tomato, tobacco, peanut, and potato. Initially called "slime disease," it was found in subtropical regions including Asia, the Southeastern United States, and South America. In 1896 Erwin F. Smith demonstrated that slime disease was caused by a bacterium that he named *Bacillus solanacearum*. Several years later, he transferred the bacterium to the genus *Pseudomonas* (Janse 2012; OEPP/EPPO 2004). Based on 16S rRNA sequences, fatty acid composition and phenotypic characteristics, Yabuuchi et al. (1992) transferred seven species of *Pseudomonas*, including *P. solanacearum*, into the new genus *Burkholderia*. After additional work, Yabuuchi et al. (1995) transferred *Burkholderia solanacearum* to the new genus *Ralstonia.*

The genus *Ralstonia* is in the class *Betaproteobacteria* (Yabuuchi et al. 2005). Its members are aerobic, gram-negative rods with one to four polar flagella. *R. solanacearum* is oxidase positive and arginine dihydrolase negative. *R. solanacearum* strains produce intracellular poly-hydroxybutyrate crystals, a characteristic that can be used as a preliminary diagnostic (Denny and Hayward 2001).

A cool temperature, virulent variant, which was later categorized as race 3 biovar 2 (R3bv2), was first described by Moraes (1947) in Portugal. This variant was found in other Mediterranean countries and in mountains in the tropics. Further investigation led researchers to conclude that R3bv2 originated from potatoes in the Andes and was possibly introduced into the Mediterranean area during World War II (Janse 2012).

The USDA designated *R. solanacearum* R3bv2 as a select agent under the Agriculture Bioterrorism Act of 2002. A recent change in the select agent regulation has listed *R. solanacearum* as a select agent, and all strains of *R. solanacearum* are considered select agents until further testing can determine that they are not R3bv2 strains (CDC USDA 2023). In Europe, R3bv2 is considered an A2 quarantine pest by the European and Mediterranean Plant Protection Organization (EPPO). In Canada (Canadian Food Inspection Agency 2014) and China it is classified as a quarantine pathogen (Li et al. 2014). Table 1 shows countries that consider *R. solanacearum* a harmful organism.

II. Taxonomy – Subgroup Divisions

Bacterial wilt is an important soilborne vascular disease that is distributed worldwide, affecting over 50 plant families. The causal agent, *R. solanacearum*, is genetically diverse and has been considered a species complex that includes *R. solanacearum*, *R. pseudosolanacearum*, and *R. syzygii* (including the blood disease bacterium [BDB]) (Taghavi et al. 1996). Fegan and Prior (2005) defined a species complex as "a cluster of closely related isolates whose individual members may represent more than one species." The idea of *R. solanacearum* as a species complex was proposed by Gillings and Fahy (1994) and expanded upon by Taghavi et al. (1996). The origin of the *R. solanacearum* species complex is not clear; however, Hayward (1991) suggests that it predates the geological separation of the continents given that the bacterium has been found in virgin jungles in South America and Indonesia. The *R. solanacearum* species complex historically is subdivided into five races, loosely based on host range, and also divided into five biovars, based on the ability to produce acid from a panel of carbohydrates (Denny 2006). There is no general correlation between races and biovars, except that biovar 2 strains are usually race 3 (and vice versa). The five races of *R. solanacearum* have different host ranges and geographic distributions. Race 1 is a poorly defined group with a very wide host range and is endemic to the southern United States, Asia, Africa, and South America. Race 2 principally attacks bananas and is found mainly in Central America and Southeast Asia. Race 3 is distributed worldwide and has primarily been associated with potato. Race 4 affects ginger in much of Asia and Hawaii. Race 5 affects mulberries in China (Denny 2006).

A newer revised phylogenetically based classification system was proposed in 2005, and some modifications have been made since that time. *R. solanacearum* strains were divided into four major genetic groups called phylotypes. These groups were assigned based on phylogenetic analysis of the 16S-23S intergenic linker sequence data and confirmed by the hypersensitive response and sequence analysis of the pathogenicity gene *hrpB* and the endoglucanase (*egl*) gene. The four phylotypes reflect the geographical origin and ancestral relationships between strains (Fegan and Prior 2005): Asia (phylotype I), the Americas (phylotype II), Africa and the surrounding islands (phylotype III), and Indonesia (phylotype IV, which includes *R. syzygii* and the BDB). The phylotype division is now well accepted by researchers working with the *R. solanacearum* species complex.

TABLE 1
Countries that include *Ralstonia solanacearum* on their Harmful Organism List[a]

Pathogen	Country
Ralstonia solanacearum	Azerbaijan, China, Egypt, Eurasian Customs Union, European Union, Georgia, Israel, Jamaica, Jordan, Mauritius, Mexico, Morocco, Nicaragua, Norway, Qatar, Taiwan, Tunisia, Turkey, Ukraine, United Kingdom, and Uruguay
R. solanacearum race 1	Chile, Jordan, Paraguay, and Uruguay
R. solanacearum race 2	Antigua and Barbuda, Brazil, Chile, Colombia, Egypt, Jordan, Mexico, and Paraguay
R. solanacearum race 3	Canada, El Salvador, Jordan, Mexico, Panama, and Taiwan
R. solanacearum race 3 biovar 2	Canada, Chile, Colombia, Egypt, Panama, and United States

[a] Per USDA Phytosanitary Export Database: https://www.aphis.usda.gov/import_export/plants/manuals/domestic/downloads/xpm.pdf (data as of April 5, 2022).

Fegan and Prior (2005) divided the phylotypes into sequevars based on a partial sequence of the *egl* gene. They defined a sequevar as "a group of strains with a highly conserved sequence within the area of the gene sequenced." Currently, over 71 sequevars have been classified within the four phylotypes listed by Lowe-Power et al. (2020) in the database bioRxiv. As new populations are found, sequevar numbers will be added. Although up to now there was no comprehensive list, nor centralized numbering database, Cellier et al. (2023) recently posted a database and a proposed protocol soon to be submitted for publication. Both web pages constitute updated resources for sequevars and their numbering. R3bv2 belongs to phylotype IIB, sequevars 1 and 2 (IIB-1 and 2) and represent a monophyletic group. The classification by Fegan and Prior (2005) is summarized in Table 2. As seen in Table 2, races 1 and 2 are not monophyletic. Race 1 includes members of phylotypes I, IIA, and III, and race 2 includes members of phylotypes IIA and IIB. Race 1 strains that belong to phylotype IIA are established in the southern United States, where they cause bacterial wilt of tomato and other crops, such as tobacco, and, rarely, pepper and potato. This race has not spread throughout the United States, largely due to its inability to cause disease in host plants at temperatures lower than 20°C (Bocsanczy et al. 2012). In contrast, R3bv2 strains are believed to have originated in the Andean highlands. This group can survive in temperate climates including highland tropics, the United Kingdom, and the Netherlands (Elphinstone 2005), and usually in association with plant tissue (Milling et al. 2009). R3bv2 is highly pathogenic on potato and tomato (primary hosts) and can also infect and eventually kill other solanaceous plants (e.g., eggplant and nightshade weeds) as well as geranium. The host range of this group is not as wide as race 1. However, it is of particular concern to the potato industry in the United States and could extend the geographic range of *R. solanacearum* for other crops, such as tomato, within U.S. borders. R3bv2 strains are monophyletic (phylotype IIB, sequevars 1,2), which explains their consistent pattern of host range, so the R3bv2 nomenclature still applies.

Wicker et al. (2012) subdivided the phylotypes into eight clades (Table 3). Clades were based on multilocus sequence analysis (MLSA) of nine single copy genes located either on the chromosome or megaplasmid. Remenant et al. (2011) suggested that *R. solanacearum* should be divided into three species based on sequencing and DNA–DNA hybridization (Table 4). Safni et al. (2014) disputed the names proposed by Remenant et al. (2011). Based on analyses by Safni et al. (2014), the *R. solanacearum* species complex

TABLE 2
Summary of *Ralstonia solanacearum* species complex classification scheme by Fegan and Prior (2005)

Phylotype	Race	Biovar	Geographic origin	Host of origin
Phylotype I[a]	1	3	Asia and Oceania	Many
		4		Many
	4	4		Ginger
	5	5		Mulberry
		2T		Pepper, tomato
Phylotype IIA	1	1	Americas	Many
	2	1		Musaceous
Phylotype IIB	2	1	Americas	Musaceous
	3	2		Potato
		2T		Potato, eggplant
Phylotype III[a]	1	1[b]	Africa	Many
		2T		Potato
Phylotype IV[c]		1[b]	Indonesia and Asia	Clove
		2		Clove, potato
		2T		Potato
		2T & *R. syzygii* subsp. *celebesensis*		Musaceous
		1 & *R. syzygii* subsp. *syzygii*		Clove

[a] Phylotype I and III strains are now named *R. pseudosolanacearum* (from Safni 2014).

[b] Unrelated to biovar 1 in phylotype II (from Denny 2006).

[c] Some phylotype IV strains are *R. syzygii* subsp. *indonesiensis* (from Safni 2014).

was reclassified into three genospecies, which correspond with the phylotype divisions. Only the current phylotype II strains would continue to be *R. solanacearum* (Table 4). Prior et al. (2016) confirmed the reclassification by Safni et al. (2014) using proteomic profiles and genomic sequence comparisons.

It is difficult to make a full correlation between the different classification schemes. The relevant classification for this document is for the monophyletic group R3bv2, which corresponds to the *R. solanacearum* species according to Safni et al. (2014) and the subdivision phylotype IIB sequevars 1 and 2.

III. Signs and Symptoms

1. Symptoms of the disease:

Foliage on potatoes infected with *R. solanacearum* may be stunted, yellowed, and wilted. Early in the infection process, wilting of leaves may be limited to the top portion of plants and to only an individual branch or leaflet. Infected plants may appear to recover at night, probably due to a combination of the stomata closing and lower temperatures, which allows the plant to regain some turgor. As the disease advances, stems of young plants may collapse and/or have narrow dark streaks, wilting becomes irreversible, and death of the plants follows (Fig. 1A).

On tomatoes, symptoms of bacterial wilt caused by the *R. solanacearum* species complex are the same whether the disease is caused by R3bv2 or other groups. Wilt is first seen as a flagging of one or two leaves, usually at the top portions of plants. Under warm, humid conditions, favorable for the pathogen, the disease develops rapidly and may kill plants as quickly as 4 to 7 days after appearance of the first wilt symptoms (Fig. 2).

On geranium, the earliest symptom of bacterial wilt can be very subtle and easily overlooked, developing as a leaf scorch in sectors and abnormal yellowing of lower leaves. A characteristic symptom of bacterial wilt, also called southern wilt, of geranium is the upward curling of leaf margins. The appearance of this symptom indicates high populations in the stem that will lead to the expression of wilt symptoms. Cool weather may slow down the wilt, but eventually symptoms will develop (Fig. 3).

TABLE 3
Clades of *Ralstonia solanacearum* species complex from Wicker et al. (2012)

Phylotype	Clade	Disease	Geographic origin
Phylotype I	Clade 1	Bacterial wilt of numerous hosts including mulberry	Northern and eastern Asia
Phylotype IIA	Clade 2	Moko and bacterial wilt	Northern South America and Central America
	Clade 3	Bacterial wilt	Northern South America, the Caribbean, and southeastern United States
Phylotype IIB	Clade 4	Moko disease and emerging strains isolated from *Anthurium* and *Heliconia*	Northern South America and the Caribbean
	Clade 5	Potato brown rot and Moko disease	South America
Phylotype III	Clade 6	Bacterial wilt	Southern and East Africa, and Indian Ocean
Phylotype IV	Clade 7	Blood disease of banana and bacterial wilt	Indonesia and northern Australia
	Clade 8	Sumatra disease of clove (*R. syzygii*)	Indonesia and northern Australia

TABLE 4
***Ralstonia* classification schemes from Remenant et al. (2011) and Safni et al. (2014)**

Phylotype	*Ralstonia* species (Remenant et al. 2011)	*Ralstonia* species (Safni et al. 2014)
I	*R. sequeirae*	*R. pseudosolanacearum*
II	*R. solanacearum*	*R. solanacearum*
III	*R. sequeirae*	*R. pseudosolanacearum*
IV	*R. haywardii* subsp. *celebensis*	*R. syzygii* subsp. *celebesensis*
	R. haywardii subsp. *solanacearum*	*R. syzygii* subsp. *indonesiensis*
	R. haywardii subsp. *syzygii*	*R. syzygii* subsp. *syzygii*

Wilting symptoms in geranium caused by *Ralstonia* species are similar to wilting symptoms caused by other pathogens such as *Xanthomonas hortorum* pv. *pelargonii*, the causal agent of bacterial blight. Unlike bacterial wilt, bacterial blight symptoms include leaf spots, and symptom expression is favored by high temperatures (29 to 35°C or 85 to 95°F) (Champoiseau et al. 2009).

Another symptom is vascular discoloration of the stem, which appears to be gray or brown. This symptom could be caused by phenolic oxidation as a response of the plant to the pathogen, although the browning could also be caused by production of melanin.

2. Signs of the pathogen:

Bacterial streaming is a common diagnostic sign of *Ralstonia* spp. When freshly cut stem sections from infected plants are placed in water, threads of a viscous white slime

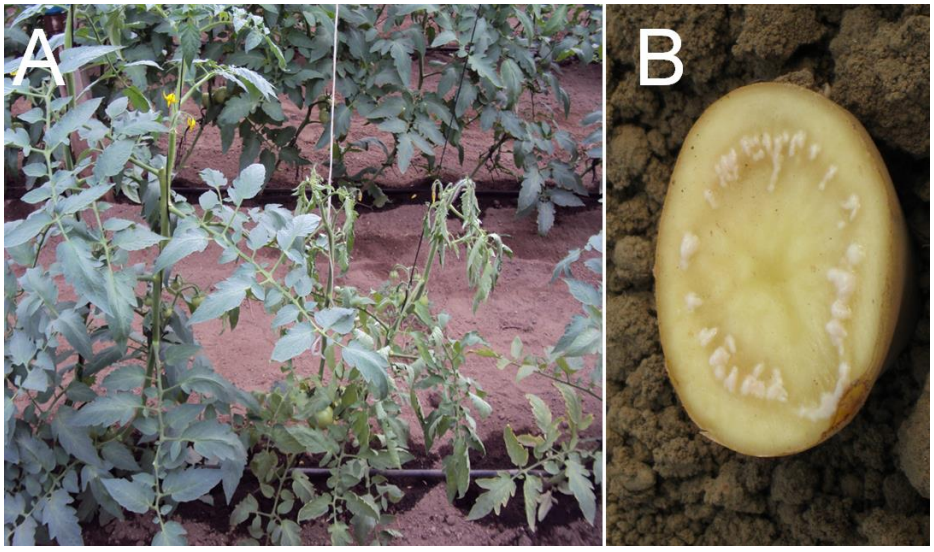

FIGURE 1
Symptoms of brown rot caused by *Ralstonia solanacearum* R3bv2 on **A,** potato plants, and **B,** potato tuber. Photo credits: J. B. Jones.

FIGURE 2
Symptoms of bacterial wilt of tomato caused by *Ralstonia solanacearum* endemic strains in Florida: **A,** flagging of leaves, and **B,** wilted plants. Photo credits: M. Paret.

often can be observed streaming from the cut end of the stem within 15 min. These threads are bacterial ooze exuding from the infected xylem vascular bundles (Fig. 4). This streaming test is a valuable diagnostic tool for quick detection of bacterial wilt or brown rot in the field (Allen et al. 2001). Bacterial ooze may also emerge from the vascular ring of cut potato tubers. Ooze from intact tubers at the eyes or where the stolon attaches may cause soil to adhere to tubers (Fig. 1B). If the vascular tissue has collapsed, sunken skin lesions will also appear.

FIGURE 3
Wilting of geranium caused by *Ralstonia solanacearum* R3Bv2. Photo credit: D. J. Norman.

FIGURE 4
Bacterial streaming from freshly cut wilted tomato stem when placed in clear water. Photo credit: M. Paret.

Vascular rings in symptomatic potato tubers will become gray-brown in appearance (Fig. 1B). Initially the vascular ring appears yellow to light brown, but as the infection progresses the ring will become a darker brown and ooze bacterial cells (Champoiseau et al. 2009). These signs may not be visible in the early stage of disease development.

3. Latent infections:

Due to the dynamics of plant–host interactions and under favorable conditions, plants infected with *Ralstonia* spp. may not show symptoms. This is termed a latent infection and is common in both potato and geranium (Swanson et al. 2005). Latent infections are of major importance in the spread of the pathogen and in the epidemiology of this disease. It was shown that latently infected geranium cuttings were responsible for introduction of R3bv2 to the United States in 2003 and 2004. Janse et al. (2004) demonstrated that both *Petunia* and *Calibrachoa* can also harbor latent infections. Latent infection can also be present in resistant or tolerant crop varieties (Lebeau et al. 2011). Sharma et al. (2016) reported that latently infected seed potato fields were identified in the Oromia and Southern Nations, Nationalities, and People regions of Ethiopia. Latently infected seed potatoes are the major source of bacterial wilt outbreaks in Ethiopia (Parker et al. 2016).

In their survey of commercial fields in Thailand, Dittapongpitch and Surat (2003) found latent infection in tomatoes and peppers growing in a field that previously had high losses due to the disease. Some varieties of plants once considered to be resistant to *R. solanacearum* species complex were actually tolerant, as they could harbor the bacteria without showing symptoms (Huet 2014).

Of great concern is the latent infection of *R. solanacearum* species complex in weeds, found in previously infested fields and along waterways. *Solanum dulcamara* or bittersweet is a weed that commonly grows along waterways and in other wet areas. Although native to Europe, it is now found in many parts of the world, including the United States (Waggy 2009). Latent infection in *S. dulcamara* has been documented in Norway, the Netherlands, and England (Janse 1996). Latent infections have also been found in a variety of weeds worldwide. Some examples are listed in Table 5.

4. Look-alike symptoms:

Symptoms caused by *R. solanacearum* and *R. pseudosolanacearum* are similar to those caused by other pathogens, such as *Fusarium oxysporum* f. sp. *lycopersici* causing Fusarium wilt on tomato, and *V. albo-atrum* and *V. dahlia* causing Verticillium wilt on potato. Abiotic stresses can produce symptoms that may be confused with bacterial wilt.

TABLE 5
List of selected weeds in which latent infection by *Ralstonia solanacearum* has been documented

Weed	Country	Location	Citation
Amaranthus spp.	Thailand	Potato field	Dittapongpitch and Surat 2003
	Uganda	Potato field	Tusiime et al. 1998
Bidens pilosa	Uganda	Potato field	Tusiime et al. 1998
Dopatrium sp.	Nepal	Rice field	Pradhanang and Momol 2001
Monochoria vaginalis	Nepal	Rice field	Pradhanang and Momol 2001
Physalis minima	Thailand	Potato field	Dittapongpitch and Surat 2003
Polygonum nepalense	Uganda	Potato field	Tusiime et al. 1998
Euphorbia hirta	Thailand	Potato field	Dittapongpitch and Surat 2003
Rumex abyssinicus	Thailand	Potato field	Dittapongpitch and Surat 2003
	Uganda	Potato field	Tusiime et al. 1998
	France	River basin	Janse 1996
Solanum dulcamara	England	River basin	Janse 1996
	Netherlands	River basin	Janse 1996
	Netherlands	River basin	Wenneker et al. 1999
	Norway	River basin	Janse 1996
Spergula arvensis	Thailand	Potato field	Dittapongpitch and Surat 2003
	Uganda	Potato field	Tusiime et al. 1998
Urtica dioica	Netherlands	River basin	Wenneker et al. 1999

These include mechanical root damage, drought, and nutrient deficiency. Therefore, symptoms alone should not be used for diagnosis. For this reason, caution is needed in determining whether a symptomatic plant is actually infected with *Ralstonia* spp. This is especially true for bacterial wilt caused by R3bv2, since similar symptoms in the same or different plants may be caused by race 1 (Phy IIA and some Phy I) of the pathogen, which is endemic in the southern United States, and Phy I present in Hawaii and Guam. Furthermore, the current commercial enzyme-linked immunosorbent assay (ELISA) and diagnostic test strips will react with all races and biovars of *R. solanacearum*, including R3bv2.

IV. Distribution, Spread, and Survival

R. solanacearum is primarily a soilborne and waterborne pathogen. It is disseminated by infested soil, contaminated irrigation water, surface water, equipment, or personnel, as well as infected plant material, including latently infected geranium cuttings and seed potatoes (Janse 1996). In greenhouses, it may also be spread by transplanting infected plants, taking cuttings without disinfecting grafting knives between plants, pinching buds of plants, and especially by irrigating with subirrigation or ebb-and-flow systems. *R. solanacearum* normally enters host plants from soil through wounds in the roots formed by lateral root emergence or caused by agriculture processes or soilborne organisms (e.g., the root-knot nematode). The pathogen can also enter plants by way of stem injuries caused by insects, handling, or tools. It does not readily spread from plant-to-plant through the splashing of water, casual contact, or aerially (Swanson et al. 2005). Pathogen spread can be controlled in greenhouses by application of exclusionary and phytosanitary practices, including avoidance of ebb-and-flow and flooding irrigation systems.

R3bv2 entered the United States several times through the introduction of latently infected geranium cuttings produced in greenhouses outside the country. On several occasions in 1999 and 2000, imported geranium cuttings were found positive for R3bv2 (Kim et al. 2002, 2003; Williamson et al. 2002). During 2001 and 2002, the United States had no reported cases of R3bv2. In February 2003, however, the bacterium was again identified in geranium cuttings imported from a facility in Kenya. The outbreak was caused by a breach in sanitation in Kenya that led to the contamination of seven stock plants. As a result, R3bv2 was detected in 127 individual greenhouses in 27 U.S. states. State and USDA-APHIS personnel worked to contain, destroy, and eradicate any diseased geraniums (Daughtrey, *personal communication*). The organism was detected again in December of 2003 and January of 2004, contained to greenhouses, and eradicated. In addition to state and federal regulatory organizations, the National Plant Diagnostic Network (NPDN) provided early detection, diagnostic, and training support to enhance national surveillance capacities and capabilities. The reintroduction that occurred in December 2003 is not believed to be a result of the contamination from the spring of 2003 because the greenhouse did not receive plants from the Kenya cutting station earlier that year, but from new cuttings that came from Guatemala (NAPPO 2004). In 2020, R3bv2 was discovered in a geranium variety in a Michigan greenhouse on plants shipped from Guatemala. Federal and state agencies in partnership with that greenhouse destroyed the affected plants and disinfected the greenhouses. They conducted a standard operating protocol including inspection of all plants shipped from the same facility to U.S. greenhouses, monitoring and testing of geraniums and other host plants commingled or potentially exposed, working with the exporter towards voluntary destruction of all geraniums, and disinfection of the greenhouse facility (https://www.aphis.usda.gov/aphis/ourfocus/planthealth/ppq-program-overview/plant-protection-today/articles/eradicate-ralstonia-greenhouses).

If R3bv2 were to enter the United States and escape into the environment, it is unlikely that climate factors alone would inhibit its survival. Given the wide range of

temperatures in which R3bv2 can survive and infect host plants, the climate across most of the United States is likely to provide temperatures that are suitable for the pathogen. In 1947, Moraes described temperature constraints of a cold-tolerant *R. solanacearum* strain infecting potatoes at high elevations in Portugal. He found that the cold-tolerant strain can grow in temperatures as low as 8 to 10°C or as high as 37 to 39°C, and it can survive in temperatures up to 47 to 49°C (summarized in Janse 1996). Later work showed that R3bv2 can survive for months in infected geranium tissue at a constant temperature of −20°C (Scherf et al. 2010). This wide range of suitable temperatures suggests that few areas of the United States would be too cold or too hot for R3bv2 to survive. Although temperatures are not likely to exclude R3bv2 from most of the United States, temperatures influence R3bv2 survival and symptom expression. Within host plant tissue, R3bv2 can survive long periods of cold temperatures but exposure to multiple freeze–thaw cycles is lethal to the bacterium (Scherf et al. 2010). The stress of freeze–thaw cycles in temperate areas of the United States might limit the areas where R3bv2 can survive. Further, although latent infections of R3bv2 are common in high-elevation potato fields, predicting disease expression based on temperatures alone is also difficult. Plants kept at cooler temperatures have exhibited wilt symptoms (8°C) (French 1985) and even death (16°C) (Ciampi and Sequeira 1980).

Strain competition (see section VI) might also limit R3bv2 survival in some climates. In the absence of competing strains, R3bv2 can cause wilt and plant death at the same temperatures as other *R. solanacearum* or *R. pseudosolanacearum* strains; however, in dual inoculation experiments, temperature influences which strain can infect a host (Ciampi and Sequeira 1980). In cooler temperatures (15 to 20°C), R3bv2 will outcompete race 1 strains within plants, whereas at warmer temperatures (24 to 30°C) race 1 strains prevail (Girard et al. 1992). These competitive interactions would influence whether R3bv2 could establish in areas of the United States where race 1 is present.

Weed hosts are widespread, so it is unlikely that a lack of host material could prevent R3bv2 from establishing in most areas. Solanaceous weeds, such as *Solanum dulcamara* (bittersweet nightshade) and *S. carolinense* (Carolina horse-nettle) can serve as wild host reservoirs (Hayward 1991), and both weeds are widespread throughout the United States (Kartesz 2015).

Because the survival and spread of *R. solanacearum* depends on interactions among factors such as climate, competition with other microorganisms, and availability of hosts, as well as soil, moisture, and the availability of water sources (Elphinstone 1996), accurately predicting where R3bv2 could survive and thrive in the United States is not possible.

R. solanacearum is widespread globally. Although in the past R3bv2 has been found in the United States from imported geranium cuttings, currently there are no active areas where the pathogen is known to be present. Other strains of *R. solanacearum*, such as the phylotype IIA and some phylotype I strains (race 1), are found in the United States, including Hawaii and Guam. The EPPO Global Database (https://gd.eppo.int/taxon/RALSSL) and bioRxiv (Lowe-Power et al. 2020), maintain online pages for *R. solanacearum* presence and global distribution.

V. Modeling

Several statistical approaches have been applied to predict the prevalence and distribution of brown rot of potato. Breukers et al. (2005) developed an aggregate state-transitions (healthy → latent infected → diseased) model to describe the brown rot incidence by observing the disease incidence in seed lots. The model yielded a low basic reproduction factor, meaning a brown rot outbreak was unlikely in Dutch potato farming under existing conditions. This model did not account for the sudden annual fluctuations in disease incidence. They further reported that the disease incidence was

dependent to a large extent on the proportion of potato seed lots with latent infections, which was not easy to determine precisely, and the reported values often deviated to a high degree from actual frequency. Thus, they reported that the state-transitions modeling strategies may not be useful to predict brown rot presence (Breukers et al. 2005).

Subsequently, Breukers et al. (2006) developed a more complex individual-based model that tracked the characteristics (e.g., location) and environment of each individual seed lot. This model incorporated seed lot characteristics and the interactions between seed lot, farm, and field (Breukers et al. 2006). The model showed that primary infection (through irrigation of contaminated water) was responsible for 60% of the infected seed lots. Vertical transmission (from potato seeds that were used to grow new seed lots) was responsible for approximately 22% of the infection. The rest of the infection was due to horizontal transmission (contamination between seed lots), among which harvesting (10.2%) and grading (6.6%) operations were responsible for most contamination (Breukers et al. 2006). They further postulated that irrigation with contaminated water resulted in latent infection in seed lots, which resulted in a sudden increase in brown rot incidence when those seed lots were grown in the field.

Based on the previous epidemiological model, an economic metamodel was developed by Breukers et al. (2007). This metamodel showed that the most cost-effective way to control brown rot infections is through government policies such as sampling intensity and quarantine measures. Sectoral factors, such as farming decisions like adoption of hygienic production systems (particularly use of clean irrigation water) for potato production and regulatory compliance, were also shown to be important.

Analysis of tomato bacterial wilt may also be useful in understanding the risk of *Ralstonia* spp. prevalence in the field. Flavia et al. (2017) developed a theoretical framework for bacterial wilt infection and identified risk factors using Markov chain Monte Carlo (MCMC) simulations. They identified three factors that were most likely to change the basic reproduction number and thus lead to bacterial wilt epidemics. The first parameter, the recruitment rate of susceptible plants, represents the fraction of susceptible tomato varieties for tomato cultivation. The second parameter, the rate of enhancement of bacteria into the soil, indicates the increase of *R. solanacearum* populations in soil due to the presence of infected tomato plants. The third parameter, the exposure rate of tomato plants with the contaminated soil, represents the degree of contact of tomato plants with the infested soil. It contributes to the rate of infection of the healthy tomato plants. Exposure of susceptible tomato to *R. solanacearum*-contaminated soil and enhancement of its population in soil due to infected tomato create a positive feedback loop leading to epidemic levels of bacterial wilt (Flavia et al. 2017).

Among the three risk factors, the exposure rate of tomato plants to contaminated soil was considered most important for preventing epidemics (Flavia et al. 2017). Cropping of resistant varieties is not sufficient for controlling the disease in the long term as the bacteria can slowly overcome plant resistance. Also, the bacteria cannot be completely eliminated from the soil, even after the removal of diseased plants. Thus, the authors recommended (i) soil disinfection by soil solarization, and (ii) sensitization of farmers on using clean and disease-free cropping systems as effective strategies for preventing bacterial wilt epidemics (Flavia et al. 2017).

VI. Climate Change

Luck et al. (2011) observed that the significance of climate to plant diseases has been known for over 2,000 years, dating back to ancient Greece. However, the increase in temperature and carbon dioxide concentrations has accelerated during the past 100 years (Gautan et al. 2013). It has been predicted that worldwide temperatures will continue to increase due to an increasing concentration of greenhouse gases in the

atmosphere. The atmospheric concentrations of carbon dioxide, methane, and nitrous oxide, the so-called greenhouse gases, have risen significantly since 2000 (Yáñez-López et al. 2012). Climate change-driven phenomena such as warmer winter temperatures, elevated humidity, cyclones, hurricanes, drought, and increased carbon dioxide are likely to influence plant diseases (Luck et al. 2011).

Schaad (2008) observed that in recent years several bacteria, including *R. solanacearum*, have become significant worldwide pathogens. This study suggests that one explanation could be global warming. These bacteria (*Acidovorax*, *Burkholderia*, and *Ralstonia*) grow well at higher temperatures than do many other plant pathogenic bacteria. The projected weather conditions associated with climate change, such as heat waves, long periods of rain, and severe storms and hurricanes will favor the occurrence of diseases caused by these bacteria. Temperature is viewed as a key factor affecting the distribution of many plant pathogenic bacteria. If temperatures continue to rise worldwide due to climate change, then the number of disease outbreaks caused by heat-loving plant pathogenic bacteria should be expected to increase (Kudela 2009). Schaad (2008) noted that 1995 was the warmest year in Europe since record keeping began in 1856. In the year 2020 a new high record was achieved in Europe (https://www.ncdc.noaa.gov/cag/global/time-series/europe/land/ytd/12/1880-2020), though no reports were available documenting the severity of bacterial wilt in Europe for that year.

Haverkort and Verhagen (2008) stated that weather conditions associated with climate change, such as irregular rainfall, higher intensity rainfall, flooding, and higher temperatures, will increase the risks of bacterial diseases in potatoes, including bacterial wilt. Castillo and Plata (2016) looked at the spread of brown rot in Bolivia and found that *R. solanacearum* was found at higher altitudes than previously recorded. They suggest that this could be attributed to climate change since minimum temperatures in the Andes are increasing. Climate simulation models show an increase in winter precipitation at higher latitudes (Chakraborty et al. 2000). Projected increases in precipitation and more extreme events such as floods, heavy downpours, and storms could also facilitate the survival of R3bv2 in the Bolivian highlands. They further projected that these factors could increase the spread of the pathogen into areas where previously it did not survive. They also noted that this spread might also be attributed to the increase of latently infected tubers in areas that were previously pathogen free (Castillo and Plata 2016).

Huerta et al. (2015) studied the interaction between tropical strains of *R. solanacearum* and a R3bv2 strain at high (28°C) and low (24/19°C) temperatures. They noted that although R3bv2 can be found in contaminated surface water and imported plant material in the tropical lowlands, it has not been isolated from wilting plants. Individually, all strains grew well at both temperatures. When strain competition was tested in vitro and in planta using R3bv2 and two tropical lowland isolates, both tropical strains outcompeted R3bv2 at warmer temperatures. R3bv2 outcompeted the other two strains at cooler temperatures. Further tests demonstrated that the two tropical strains produced a bacteriocin-like compound that inhibited the growth of R3bv2. However, R3bv2 was able to outcompete the tropical lowland strains at cooler temperatures without the benefit of bacteriocins.

The geographical distribution of plant pathogens and their hosts will likely be altered due to climate change (Kudela 2009). There has been limited research on climate change's effects on pathogens of field crops. Results cannot predict changes in severity of the disease. The change in disease severity will depend on the region and/or weather variables used in the model (Luck et al. 2011).

Although climate change may act as a driving force in altering the geographic range of *Ralstonia* species and plant pathogens in general, it will clearly not be the only factor at play. Research by Huerta et al. (2015) demonstrates that other nonclimate factors could also be important. Extrapolating from their study, factors such as the microbial

community, microflora, microfauna, nematodes, and soil type may also play a role in the redistribution of plant diseases.

VII. Detection and Identification

USDA-APHIS has been testing randomly selected imported *Pelargonium* (geranium) plant material for *R. solanacearum* at Plant Protection and Quarantine's Plant Inspection Stations located at major ports of entry since September 27, 2021 (USDA-APHIS 2021). Plants and cuttings infected with *R. solanacearum* or *R. pseudosolanacearum* can remain asymptomatic for some time where visual inspections could miss infected plants. Therefore, a new testing protocol sensitive enough to detect *R. solanacearum* R3bv2 in symptomatic and asymptomatic plants has been implemented.

For all domestic detections of *R. solanacearum* species complex, confirmatory diagnosis of bacterial wilt caused by R3bv2 must be made by advanced microbiological and molecular tests. The ultimate authority for confirming a diagnosis of the disease rests with the USDA-APHIS-Plant Protection and Quarantine (PPQ)-Plant Pathogen Confirmatory Diagnostic Laboratory (PPCDL) in Laurel, Maryland. Detailed instructions for submitting samples are available at: https://www.aphis.usda.gov/plant_health/plant_pest_info/ralstonia/downloads/nprg-ralstonia.pdf.

1. Preliminary diagnostic screening tests:

a. Bacterial streaming

Clouds of bacterial cells usually appear within 20 minutes when severed petioles or stems of the suspected plants are placed in tubes with sterile water as shown in Figure 4. Note that bacterial streaming may not occur when viewing plant material in the initial stages of infection. This procedure only indicates that wilt may be caused by a bacterial pathogen. The streaming test is a valuable diagnostic tool for quick detection of bacterial wilt or brown rot in the field. Although this test demonstrates the presence of a bacterial plant pathogen, it is not specific to R3bv2 or even to the genus *Ralstonia*.

b. Serological test kits

Symptomatic plants in the field or in the greenhouse can be tested for the presence of *R. solanacearum* and *R. pseudosolanacearum* with immunodiagnostic assays using species-specific antibodies. The USDA-APHIS-PPQ has tested and recommends the use of commercially available diagnostic test strips for rapid detection of *R. solanacearum* and *R. pseudosolanacearum* in the field or lab. Currently there are several available test kits worldwide (Table 6).

Serological screening tests are generally quick, inexpensive, reliable, and require minimum equipment. The minimum detection level (sensitivity) of these tests is 10^4 to 10^6 colony forming units (CFUs). However, they cannot be used to identify the race or biovar and can only detect high populations of the pathogen. Furthermore, the current commercial ELISA tests or diagnostic test strips (Table 6) will react with both R3bv2 and endemic race 1. It is also important to understand that serological methods do not differentiate between living and dead cells. This becomes an issue regarding decisions relating to quarantine issues and destroying contaminated shipments (Denny 2006).

TABLE 6
Serological test kits for *Ralstonia solanacearum* species complex

Product name	Part number	Lower detection limit	Manufacturer
Rs ImmunoStrip Test	ISK 33900	2×10^4 CFU/ml	Agdia, Elkhart, IN, U.S.A.
Rs PathoScreen Test	PSP 33900	2×10^6 CFU/ml	Agdia, Elkhart, IN, U.S.A.
POCKET DIAGNOSTIC *Ralstonia solanacearum*	PD51119	$1–3 \times 10^5$ CFU/ml	Pocket Diagnostics, Abingdon Health, York, U.K.

c. Cellular analysis and notification of antigen risk and yield (CANARY) technology

The CANARY system is a modified cell-based system using B lymphocytes. Lymphocytes are modified to express a single antibody specific to the organism of interest. Aequorin, a luminescent protein obtained from jellyfish, is also expressed in the cell and becomes activated to emit light when the specified antigen is detected. The advantages of CANARY are that it has a relatively low detection threshold and a low rate of false positives (Committee on Materials and Manufacturing Processes for Advanced Sensors 2004; see Web Resources). Systems are commercially available and manufactured by Smiths Detection (https://www.smithsdetection.com).

The assay is simple and quick. Lymphocytes and the sample are added to a microfuge tube and centrifuged to mix. The reaction is read in a luminometer. The entire test can be completed in a few minutes. Tests have shown the minimum detection limit for *R. solanacearum* using the CANARY system is 10^3 CFUs, which is comparable to quantitative polymerase chain reaction (qPCR). This system is also able to detect *Ralstonia* spp. from plant tissue that is in poor condition and that would typically yield a false negative reaction using other diagnostic tests (Levy et al. 2013).

d. Automated methods of identification

Although these methods currently are not as popular as molecular diagnostic methods, they are still available.

i. The Sherlock Microbial Identification System and fatty acid analysis

The Sherlock Microbial Identification System (MIDI, Newark, DE) can identify bacteria to species based on their fatty acid profiles. The RTSBA6 library version 6.21 contains three species of *Ralstonia*, *R. eutropha*, *R. pickettii*, and *R. solanacearum*. When grown under their standard conditions (Sasser 2006), the fatty acid profile for *R. solanacearum* is as shown in Table 7.

There have been several papers demonstrating the use of fatty acid profiles for identification of *R. solanacearum*. Salete de Melo et al. (1999), Khakvar et al. (2009), and Behiry et al. (2015) all conducted research on using fatty acid profiles to distinguish among *R. solanacearum* strains. Although there was some preliminary evidence suggesting that fatty acid profiles might be able to separate out strains by host or by geographic origin, there was no evidence that the profiles could be used to distinguish biovars or sequevars. Janse (1991) was able to distinguish between races and between virulent and avirulent strains using fatty acid analysis. It should be noted that in each study strains were grown under different conditions and different extraction methods were used. Therefore, the results are not comparable. More work under standard conditions using a larger number and greater diversity of strains is needed to determine the usefulness of fatty acid profiling to distinguish subgroups of *Ralstonia* sp.

ii. The Biolog System and substrate utilization

The GEN III system (version 2.8) from Biolog (Hayward, CA) identifies bacteria based on a 96-test profile of carbon source utilization and chemical sensitivity. The database contains four species of *Ralstonia*, *R. insidiosa*, *R. mannitolilytica*, *R. pickettii*,

TABLE 7
Fatty acid profile for *Ralstonia solanacearum* from Microbial Identification System (MIS) database

Fatty acid name	Percent of profile
Summed feature 3 (16:1 w6/7c)	26.46
16:00	26.34
Summed feature 8 (18:1 w6/7c)	18.77
Summed feature 2 (14:0 3OH and its breakdown products)	7.45
17:0 cycle	5.16
18:1 2OH	4.87
16:1 2OH	4.57
14:00	4.39
16:0 2OH	0.89

and *R. solanacearum*. The Biolog plate contains seven of the eight carbohydrates used in the *R. solanacearum* biovar test. The seven carbohydrates and well locations are: glucose (C1), maltose (A3), trehalose (A4), cellobiose (A5), lactose (B2), sorbitol (D1), and mannitol (D2). The plate does not contain dulcitol. Although this system has not been validated for definitive identification of biovars, it can be useful as a preliminary screen to rule out biovar 2.

e. Bacteriophages

Kutin et al. (2009) developed a protocol using bacteriophages combined with qPCR to detect *R. solanacearum* in plant tissue. The assay was very sensitive in detecting both active and latent infections at concentrations as low as 2.7×10^2 CFUs/g of plant tissue. This method is also useful because it eliminates the problem of PCR inhibitors in plant tissue and only detects live cells.

In his review of *R. solanacearum* bacteriophages, Yamada (2012) discusses the potential use of bacteriophages for detection of *R. solanacearum* in plants and soil. The filamentous phages RSS1 and RSM1 were identified as possibly being useful for bacterial and strain identification. However, there are currently no validated methods for using bacteriophages to identify *R. solanacearum* to the biovar level.

2. Isolation, culture, and identification:

a. Water, plant, and soil sampling

With experience, *R. solanacearum* can be isolated and cultured from diseased tissue and infested soil and water samples. Optimized sampling protocols from the European Union (EU Commission implementing regulation 2022/1193 2022) should be used for isolation of the bacterium. Sampling from natural bodies of water is more reliable when the water temperature is above 15°C. To increase the likelihood of detection, each site should be sampled at several time points to reduce the effects of environmental variation (Stevens and van Elsas 2010; Wenneker et al. 1999).

b. Use of semiselective medium (modified SMSA)

The bacterium may be cultured from diseased tissue, soil, and water samples in a diagnostic laboratory by streaking from soil suspensions, diseased tissue suspensions, or water samples onto modified SMSA medium (Elphinstone et al. 1996). The modified SMSA medium contains antibiotics and fungicides that inhibit or reduce growth of competing saprophytic bacteria and fungi. The plates should be incubated at 28°C for 3 to 5 days. Virulent colonies of *R. solanacearum* appear as small mucoid, irregular, whitish colonies with a pink center after 48 to 60 hours. The colonies develop blood-red whorls in the center after further incubation. Other fast-growing bacterial species may form similar mucoid colonies; however, most appear red throughout. This still could create confusion, resulting in false positives if only the semiselective medium is relied upon for detection Therefore, other complementary tests such as twitching motility are used to observe colony growth for identifying presumptive *Ralstonia* colonies. This test is not diagnostic for race or biovar.

c. Other culture media

Other media can be used to retrieve *R. solanacearum* from liquid or frozen stock suspensions or for routine culture of the bacterium (Denny and Hayward 2001). In tetrazolium chloride (TZC) medium, colonies also are mucoid whitish with pink centers as in modified SMSA medium (Fig. 5). On rich solid media, virulent colonies can be differentiated from nonvirulent ones by their morphology (Fig. 6). While virulent colonies are white or cream-colored, irregularly shaped, highly fluidal, and opaque, nonvirulent types appear uniformly round, smaller, and butyrous (or dry). It is important to note that *R. solanacearum* is a slow-growing microbe even on this rich medium. Colonies that are visible in less than 36 h at 28°C are not *R. solanacearum*. Nonvirulent colonies are favored by long-term storage. Often there is brown discoloration of the medium around the colonies. It is best to incubate at 28°C to increase pigment produc-

tion. This test is not diagnostic for race or biovar. Information on the use and preparation of media for working with *R. solanacearum* can be found in French et al. (1995).

d. Twitching motility

It is helpful to observe the microscopic colonies at 18 to 24 h on solidified rich growth media (i.e., modified SMSA, TZC) for twitching motility (Fig. 7). Twitching motility is a form of bacterial translocation over solid surfaces involving the Type IV pili system. Relatively few bacterial species exhibit this distinctive colony morphology, and those that do can be distinguished from *R. solanacearum* once visible colonies develop (Liu et al. 2001). Twitching motility at cool temperatures has been associated with cool-virulent *R. solanacearum* and may have direct or indirect effects on the cool-virulence phenotype (Bocsanczy et al. 2012; Stulberg et al. 2018).

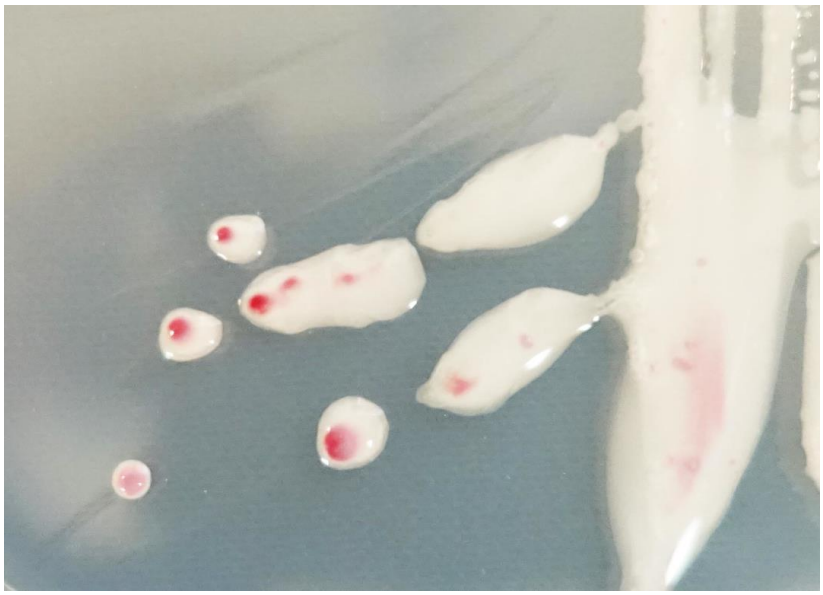

FIGURE 5
Virulent colonies of *Ralstonia solanacearum* on tetrazolium chloride (TZC) agar growth medium. Photo credit: D. J. Norman.

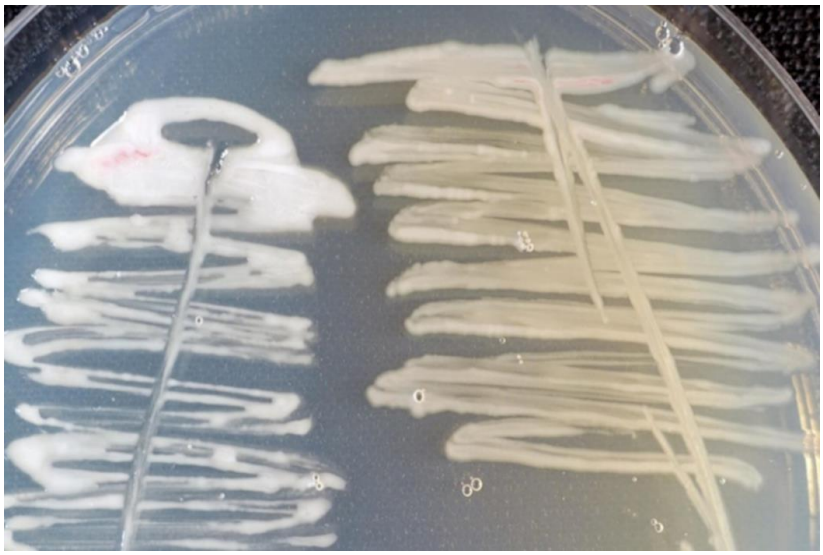

FIGURE 6
Virulent (left) and nonvirulent (right) colonies of *Ralstonia solanacearum* on tetrazolium chloride (TZC) agar growth medium. Photo credit: D. J. Norman.

3. Phylotype and biovar identification:

a. DNA-based methods

i. DNA extraction

There are many DNA extraction kits commercially available. Stulberg et al. (2015) and Remenant et al. (2011) successfully utilized Qiagen's DNeasy Blood and Tissue Kit (part 69504 or 69506) to extract genomic DNA from *R. solanacearum*. Albuquerque et al. (2014) used the Axygen AxyPrep Bacterial Genomic DNA MiniPrepKit (AP-MN-BT-GDNA-250). Baharuddin et al. (2014) and Guidot et al. (2007) each used different versions of a phenol–chloroform extraction protocol. Stulberg and Huang (2015) developed an extraction buffer for a quick, inexpensive, and easy DNA extraction from infected plants including geranium for detection of *R. solanacearum* by qPCR.

Other commercial kits for bacterial DNA extraction used successfully to extract *R. solanacearum* genomic DNA include the Wizard Genomic DNA Purification Kit from Promega, UltraClean Microbial DNA Isolation Kit from Qiagen, and the PureLink Microbiome DNA Purification Kit from Invitrogen. Extraction methods that use phenol and chloroform, as well as other extraction protocols, can be found in publications on molecular biology techniques such as those by Green and Sambrook (2012) and Ausubel et al. (2003). The choice of a kit and/or method should be based on whether DNA is being extracted from cultures, infected plant material, or infested soil and the intended use of the extracted DNA.

ii. Flinders Technology Associates (FTA) cards

FTA cards are manufactured by Whatman Technology. They contain a proprietary matrix that lyses cells while preserving and protecting the DNA. Grund et al. (2010) first documented that DNA from *R. solanacearum* could be stored on FTA cards and later amplified by PCR. Tran et al. (2016) observed that cells were killed within 60 min of being applied to a Whatman FTA (R) card. When PCR was run using extracts from

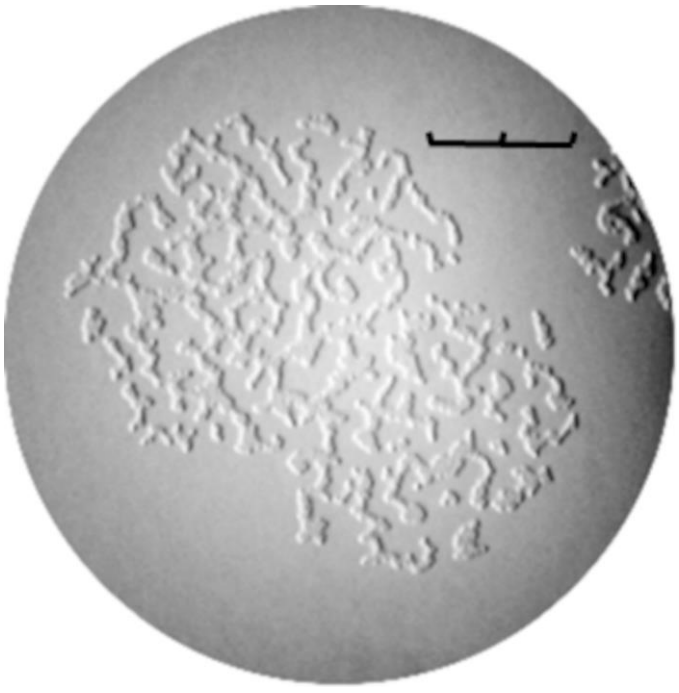

FIGURE 7

Twitching colonies of *Ralstonia solanacearum* on nutrient agar medium at 18 hours after plating photographs were taken at 80× magnification. Bar length represents 0.25 mm. Photo from Bocsanczy et al. (2012). © The American Phytopathological Society.

an FTA card, the sensitivity level was approximately 10^4 CFUs – a level similar to that found in latently infected plants.

Chandrashekara et al. (2012) collected, stored, and amplified DNA from *R. solanacearum* infected solanaceous plants. Their results using the FTA cards compared favorably to results from DNA extracted directly from plant sap. FTA cards also bind PCR inhibitors present in plant tissue, such as in geraniums. However, inhibitors can be eliminated prior to running PCR with additional treatment steps in the processing (Tran 2016). Another benefit is that samples on FTA cards can be stored at room temperature and no refrigeration is needed. Chandrashekara et al. (2012) found that when stored at room temperature the DNA was stable for at least 6 months.

Jenkins et al. (2014) demonstrated that FTA cards may provide a secure method for collecting *R. solanacearum* DNA from suspect plants in the field, particularly in remote locations, and transferring them to a lab for testing. With the increasing occurrence of bacterial wilt, FTA cards could become a valuable aid in the process of sampling and diagnosis.

iii. Polymerase chain reaction (PCR) and qPCR

Race determination using PCR is not possible because *R. solanacearum* species complex strains do not have race-cultivar specificity on plant hosts. The term "race" does not equate with phylogenetic unity except for R3bv2, which represents the monophyletic group phylotype IIB-seq 1 and 2. However, PCR and qPCR assays that use phylotype/sequevar or biovar-specific primers can be used for identification of IIB-1 and 2 or biovar 2 strains of *R. solanacearum* (Fegan and Prior 2005; Paudel et al. 2022; Stulberg and Huang 2015; Stulberg et al. 2015, 2016; Weller et al. 2000). Generally, qPCR analysis uses a variation on a protocol that amplifies both a DNA fragment present in all *R. solanacearum* species complex strains and one that is largely R3bv2-specific (Weller et al. 2000).

Conventional PCR can amplify both a 280-bp "universal" *R. solanacearum* species complex amplicon (primer pair 759/760) (Opina et al. 1997) and a 357-bp fragment that is found only in R3bv2 strains (primer pair 630/631) (Fegan et al. 1998) (Table 8). Strains of the *R. solanacearum* species complex can be subclassified into phylotypes with a single multiplex PCR reaction. Sequevar designation requires PCR amplification, sequencing, and phylogenetic analysis of the conserved endoglucanase (*egl*) gene (Fegan and Prior 2005).

Stulberg et al. (2015) developed a multiplex PCR assay targeting nonphage genome sequences that identifies *R. solanacearum* species complex strains, differentiates the select agent R3bv2 (phy IIB-1 and 2) strains from those that are not, and eliminates false negatives due to PCR inhibition or unsuccessful DNA extractions in one reaction (Table 8). The *R. solanacearum* species complex-specific primers target the predicted glycosyl transferase domain and the IIB-1 and 2-specific primers target the predicted ferric siderophore receptor. The lower detection limit is 200 CFUs/PCR reaction.

TABLE 8
PCR primers used for the detection and identification of *Ralstonia* species

Primer	Primer sequence (5′–3′)	Size (bp)	Target	Specific
759-F 760-R	GTCGCCGTCAACTCACTTTCC GTCGCCGTCAGCAATGCGGAATCG Opina et al. (1997)	282	NA	*R. solanacearum*, *R. pseudosolanacearum*, and *R. syzygii*
630-F 631-R	ATACAGAATTCGACCGGCACG AATCACATGCAATTCGCCTACG Fegan et al. (1998)	357	NA	IIB-1&2 (R3bv2)
RsSC-F RsSC-R	CCGAGCGCATATCGTTCACAC TTTGGCGTTCCGGTCGGAG Stulberg et al. (2015)	296	Predicted glycosyl transferase	*R. solanacearum*, *R. pseudosolanacearum*, and *R. syzygii*
RsSA-F RsSA-R	CAACGATGCCTGGAAACTGACC TGGTCCGGGTTCAGGTAAATGTCAC Stulberg et al. (2015)	132	Predicted ferric siderophore receptor	IIB-1&2 (R3bv2)

Stulberg and Huang (2016) identified specific single nucleotide polymorphisms (SNPs) for *Ralstonia* genospecies and for sequevars 1 and 2 by comparing their *egl* genes. They also designed primers (Table 9) to amplify a 526-bp *egl* fragment from *R. solanacearum* for easy sequencing of the amplicon and to facilitate direct and specific amplification of *egl* from *R. solanacearum* species-infected plants. This eliminates the need for bacterial isolation from infected plant material to obtain pure cultures. They then wrote a user-friendly *R. solanacearum* typing program that places a partial *egl* sequence into one of the three newly proposed *Ralstonia* genospecies and determines if it corresponds to the highly regulated sequevars 1 or 2.

TaqMan-based real-time qPCR is commonly used in federal and state diagnostic laboratories due to its speed and sensitivity. Stulberg and Huang (2015) developed a multiplex qPCR assay that identifies and confirms R3bv2 (phyIIB-1 and 2) strains, and controls for false negatives. One of their IIB-1 and 2-specific qPCR primers and probe sets has been applied in a portable POCKIT system (https://www.genereach.com/index.php?func=product&action=view&product_no=1), which has the potential to facilitate R3bv2 detection at ports of entry and in field settings (Di et al. 2016). Stulberg et al. (2016) also developed new primers and probe sets, and systematically validated and compared them with the previously published ones for reliable detection of *R. solanacearum* at both the species complex and R3bv2 levels by qPCR.

Recently, Stulberg et al. (2018) identified a DNA region associated with cool virulence of *R. solanacearum* R3bv2 strains and developed the first PCR assay targeting the region for specific detection of this important group. A gene encoding the type III secretion system effector protein RipS1 was recently reported to play a role in the cool virulence phenotype of the R3bv2 strains (Schachterle and Huang 2021). By using a 96-bp DNA fragment from the ripS1 gene for melting temperature analysis, Schachterle and Huang (2023) developed a melting curve assay to differentiate the cool virulent R3bv2 and phy IIB sequevar 4 strains from other *R. solanacearum* species complex strains.

iv. Loop mediated amplification (LAMP)

LAMP was developed by Notomi et al. (2000). The protocol uses a *bst* DNA polymerase large fragment, and the reaction mixture is incubated at approximately 60 to 65°C for 1 h. This technique has been successfully used for detection of R3bv2 and offers some advantages over conventional PCR. Using this method, DNA can be amplified under isothermal conditions. Therefore, an expensive thermocycler is not required.

Kubota et al. (2011a) developed a noninstrumental system for running LAMP reactions under low-tech conditions. By combining calcium oxide powder with water, a temperature of 63°C can be obtained and maintained in an insulated container for 1 h. A precipitate is formed by pyrophosphate, which is a byproduct of DNA strand synthesis. A positive reaction is indicated by the white pyrophosphate precipitate. This provides a visual confirmation that the DNA amplification was successful, without running a gel.

Lenarcic et al. (2014) also worked with LAMP and found that it worked best when the *egl* gene was targeted. However, Tran et al. (2016) obtained 5 to 25% false negative

TABLE 9
PCR primers for amplification and sequencing of a partial endoglucanase gene for determination of genospecies and sequevars 1 and 2 of *Ralstonia* (Stulberg and Huang 2016)

Primer designation	Primers (5′–3′)	Number of base pairs
Amplification primers		
MJS-amp-egl-F	CGGACACGGACACCACGACTCTGAA	526
MJS-amp-egl-R	TAGCGCGCATAGTTGTGCGGATCGAGC	
Sequencing primers		
MJS-seq-egl-F	ACACGGACACCACGACTCTG	413
MJS-seq-egl-R	GCATAGTTGTGCGGATCGAGC	

results when testing infected geranium plants using LAMP. Kubota et al. (2011b) and Tran et al. (2016) obtained false positives due to cross contamination. As LAMP evolves, this technique has the potential to become a powerful low-tech DNA-based tool for the detection of R3bv2, particularly in remote areas lacking infrastructure.

v. Recombinase polymerase amplification (RPA)

RPA has generated a great deal of interest as a diagnostic tool because of its high sensitivity and selectivity, rapid turnaround time, and low cost (Rani et al. 2019). RPA is done with three enzymes that operate at isothermal ranges between 25°C and 40°C. The three enzymes are a single-strand binding protein, a recombinase, and a strand-displacing DNA polymerase (Ghosh et al. 2018).

RPA is a good tool for use in field diagnostics because it produces rapid results, operates at a single reaction temperature, and is portable. It is also more tolerant than conventional and real time PCR to the inhibitors present in crude sample extracts (Strayer-Scherer et al. 2019). However, false negatives can still occur due to errors in DNA preparation and inhibitors. Therefore, an internal or host control is needed to enhance test reliability (Ahmed et al. 2018). Various methods can be used to assess RPA products including gel electrophoresis, fluorescent probes, and lateral flow dipsticks (Li et al. 2021).

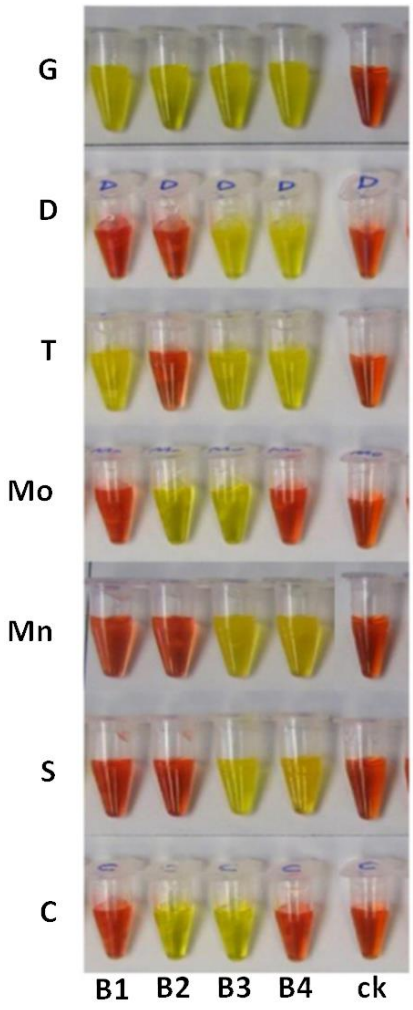

FIGURE 8
Indicator changes in improved biovar tests 4 days after inoculation. G, glucose; D, dulcitol; T, trehalose; Mo, maltose; Mn, mannitol; S, sorbitol; C, D (+) cellobiose. B1, biovar 1 strain Rs124; B2, biovar 2 strain UW552; B3, biovar 3 strain Pss106; B4, biovar 4 strain Pss262; and ck, CPG broth. Reproduced, by permission, from Huang et al. (2012). © Elsevier.

Dobhal et al. (2020) found a unique gene region in R3bv2 genomes for an RPA. The assay was tested for stringency and sensitivity. There were no false positives or false negatives and the detection limit for nonspiked samples was 1 pg.

Agdia (www.agdia.com/AmplifyRPAXCS) has developed an AmplifyRP pathogen-specific kit for R3bv2 (part XCS 51300). Currently, the kit does not include an internal control. It was found that without the internal control the sensitivity of PCR and qPCR was retained in those assays. However, one may be added in the future (Bryant Davenport, Agdia, *personal communication*). Reagents are available for those wanting to develop their own tests, as well as instruments and other detection tools from both Agdia and TwistDX (https://www.twistdx.co.uk).

b. Carbohydrate utilization

Although DNA-based approaches are more accurate with a faster response to identify RSSC species and strains, many labs rely on biochemical methods. Biovars of *R. solanacearum* can be differentiated based on the differential ability of strains to acidify culture media containing a panel of carbohydrate substrates (Denny 2006) (Fig. 8). This is a simple, reliable assay that can be used routinely in combination with other methods in diagnostic labs.

Identification of R3bv2 should be based on at least two independent methods, including the biovar test and one of the DNA-based methods (i.e., PCR or qPCR). In addition to tomato inoculations to determine pathogenicity, DNA sequencing analysis may be necessary for confirmation. There is a great need to replace the SMSA plating method and serological tests with economical, easy-to-use, robust, rapid, highly sensitive, and specific methods that will be effective for monitoring this pathogen at both the species and subspecies levels. See

Appendix B of USDA guidelines for USDA-approved R3bv2 identification procedures (USDA-APHIS 2020).

VIII. USDA Pathogen Permits and Regulations

USDA-APHIS-PPQ permit and registration requirements for plant diseases and laboratories fall under two authorities, the Plant Protection Act (7 CFR Part 330) and the Agricultural Bioterrorism Protection Act of 2002 (7 CFR Part 331). Laboratories and diagnosticians must follow the requirements of the USDA Select Agent Program: https://selectagents.gov/regulations/index.htm.

The Plant Protection Act permit requirements apply to all plant pests and infected plant material, including diagnostic samples, regardless of their quarantine status, that when shipped interstate require the receiving laboratory to have a permit. For further guidance on permitting of plant pest material, consult the USDA-APHIS-PPQ permit website at https://www.aphis.usda.gov/aphis/ourfocus/planthealth/import-information/permits/regulated-organism-and-soil-permits, call 301-851-2357 or toll free 866-524-5421, or email Pest.Permits@usda.gov.

The Agricultural Bioterrorism Protection Act of 2002 (7 CFR Part 331) specifies the requirements for possession, use, and transfer of organisms listed as select agents, such as R3bv2. Based on the Federal Select Agent Services regulations, all races and biovars are select agents. Once a sample is determined to be positive for *R. solanacearum*, the sample must be handled according to the Select Agent Program regulations. The USDA-APHIS-DASAT forms are necessary depending on your course of action to (i) destroy the sample or (ii) send it for further testing. The appropriate form(s) needs to be completed and approved by the USDA-APHIS-DASAT. Guidelines for submitting and handling samples can be found at the following website, Appendix B: https://www.aphis.usda.gov/plant_health/plant_pest_info/ralstonia/downloads/nprg-ralstonia.pdf.

IX. Response

While this plan is focused primarily on recovery, certain aspects of the response to a new detection involve a continuum of activities from response to recovery. The response is under USDA-APHIS-PPQ's authority delegated from the Secretary of Agriculture under the Plant Protection Act of 2000. After a detection of R3bv2 has been confirmed by USDA-APHIS-PPQ-PPCDL, USDA-APHIS, in cooperation with impacted states, is responsible for the response. The response may be immediate with advance assessment teams and survey personnel sent to the site of initial detection to place holds, conduct investigations, and initiate delimiting surveys. Further actions may include regulatory measures to quarantine infested or potentially infested production areas and to stop the movement of infested or potentially infested articles in commerce, and control measures, such as host removal and destruction, or other required phytosanitary practices. USDA-APHIS imposes quarantines and regulatory requirements to control and prevent the interstate movement of quarantine-significant diseases or regulated articles and works in conjunction with states to impose these actions parallel to state regulatory actions that restrict intrastate movement.

For a confirmed detection of R3bv2 in geraniums, the current new pest response guidelines (USDA-APHIS-PPQ 2020) prescribe eradication and control options. It may require holds at production facilities, traceback and trace forward investigations, and required destruction of infested and potentially infested geranium lots. All host material associated with these lots by shared water irrigation systems or unsanitary greenhouse practices are also candidates for destruction. All production areas, greenhouse articles, soil, and water systems must be disinfested according to the guidelines prior to the regulatory release of a facility or growing area.

Confirmed infestations by R3bv2 of potato or other solanaceous crops will require quarantine of fields, seed tubers, seedlings, or other plant material associated with infested lots, including processing facilities, storage bins, means of conveyance, soil, and irrigation water. Host destruction is required along with disinfection, water decontamination, and several years of nonhost production in infected fields or associated growing areas before quarantines can be removed. More details on response procedures to confirmed identification of R3bv2 in the United States can be found in the USDA-APHIS-PPQ's New Pest Response Guidelines (USDA-APHIS 2020).

X. Economic Impact and Compensation

R. solanacearum R3bv2 is one of the most damaging pathogens on potato worldwide (Hayward 1991; Janse 1996; Savary et al. 2019). The pathogen is a USDA select agent because it has the potential to pose a severe threat to plant health (USDA-APHIS 2020). The bacterium is adapted to cooler temperatures and could be particularly damaging to potato production regions of the northern United States. While race 1 (*R. solanacearum* and *R. pseudosolanacearum*) causes losses to tomato and other crops in Florida and the southeastern United States, the potential economic impact of potato brown rot caused by R3bv2 is unknown in temperate climates where much of U.S. potato production occurs. The actual potential of this race to cause losses in temperate climates is uncertain because data on yield reductions are limited due to quarantine and eradication efforts in potato fields in the United Kingdom, the Netherlands, and Sweden. Despite widespread detection of the pathogen in northern Europe, direct losses of potato to the disease have been limited to a few outbreaks during unusually hot summers (Priou et al. 2006). Nonetheless, the economic impact of the additional quarantine-related testing has been significant.

As mentioned previously, R3bv2 is listed as a Select Agent in the Agricultural Bioterrorism Protection Act of 2002. Current authority under the Plant Protection Act only allows for compensation under an Extraordinary Emergency declaration by the Secretary of Agriculture. The current policy does not allow the Risk Management Agency of USDA to pay for undamaged plants. Therefore, any commingled and/or uninfected plants could be destroyed without compensation. This aspect has caused significant economic losses to geranium producers in the past.

Corral et al. (2013) report that crop insurance will cover losses from plant diseases and give an excellent discussion on the subject. Additional information can be obtained on the USDA Risk Management Agency's website (https://www.rma.usda.gov/).

XI. Mitigation and Disease Management

Any disease mitigation strategy that is used should be coordinated with federal, state, and local regulatory officials.

1. Control on potato and tomato

Because *R. solanacearum* and *R. pseudosolanacearum* are soilborne pathogens and host resistance is limited, bacterial wilt is very difficult to control in field production on crops such as potato and tomato (Hayward 1991; Saddler 2005). Moreover, they are very widely distributed and have an unusually broad host range (Denny 2006). Thus, no single strategy is 100% effective to control the disease. However, in locations where the pathogen is established, some level of bacterial wilt control is possible by using a combination of diverse control methods. These methods should be used as part of an integrated management strategy, and include:

a. Host resistance

The best that conventional breeding has achieved for most solanaceous crops is a moderate level of host resistance to bacterial wilt on a regional level when conditions are not excessively hot or wet. Some potato cultivars are less susceptible to bacterial wilt, at least in some regions. There are active potato resistance breeding programs, some of which are focusing on resistance to latent infection.

Lebeau et al. (2011) tested multiple strains of *R. solanacearum* against 30 resistant accessions of tomato, eggplant, and pepper. Only eggplant and pepper showed resistance to R3bv2. However even in the accessions with a low percentage of wilt, stem colonization of *R. solanacearum* ranged from 10 to 93%. Three cultivars of eggplant resistant to bacterial wilt have been used in India (Gopalakrishnan et al. 2005).

Another study evaluated resistance to bacterial wilt in 82 accessions of *Solanum* (formerly *Lycopersicon*) species. Only two accessions showed partial resistance. These were the tomato cultivar Hawaii 7996 and *Solanum pimpinellifolium* accession 63280 with 52 and 63% wilt, respectively (Carmeille et al. 2006; Huet 2014).

Seven genotypes from two wild Andean potato species were found to have high levels of resistance to wilt and tuber infection. These genotypes provide resistance to breeding for resistance in commercial potato cultivars (CIP 2004). *Solanum phureja*, a relative of the cultivated potato, has shown resistance to bacterial wilt. It has been used in breeding programs for several decades. However, the resistance is only effective at cool temperatures or high altitudes (Patil et al. 2012). When Aliye et al. (2014) screened 55 potato cultivars for resistance to bacterial wilt, 28 were resistant in greenhouse trials. However, when these 28 resistant cultivars were tested in the field, only 2 showed any resistance. This study highlights the significance of cultural and environmental conditions on the expression of resistance.

The difficulty in developing resistant plants in Brazil in an interdisciplinary and interinstitutional 30-year program is described by Lopez et al. (2016). Approximately 1% of the plants will be selected for disease resistance when screening plants from true seed for disease resistance using greenhouse inoculations. Accessions must then be tested in the field. Through this program, several clones were selected for stable resistance to bacterial wilt under field conditions. They are currently being used in a breeding program to also select for good quality tubers.

Kunwar et al. (2019) evaluated 67 tomato accessions with reports of bacterial wilt resistance to phylotype I and IIB-1(R3bv2) strains. Plants with QTL *Bwr-6* contributed to resistance to both phylotypes, and QTL *Bwr-12* contributed to resistance to phylotype 1 strains. One of the accessions tested in the study that lacked both *Bwr*-6 and *Bwr-12* had high resistance to the R3bv2 strain, indicating potential for other QTLs involved in bacterial wilt resistance.

Transgenic resistance has also been demonstrated to provide effective field level management of a R3bv2 strain affecting potato and a phylotype II strain affecting tomato (Boschi et al. 2017; Kunwar et al. 2018). A pattern recognition receptor EFR from *Arabidopsis thaliana* was inserted in the susceptible tomato variety FL 8000 and was shown to reduce bacterial wilt incidence and severity under field conditions. EFR expressed in the susceptible commercial potato variety INIA Iporá and the breeding line 09509.6 showed similar trends against a R3bv2 strain in a controlled greenhouse experiment.

Grafting of susceptible tomato varieties to resistant rootstock has been a successful method for management of bacterial wilt in fields against phylotype II strains in Florida and North Carolina (McAvoy et al. 2012; Rivard et al. 2012).

Extensive international research has produced some highly resistant tomato breeding lines, such as Hawaii 7996, but the resistance is usually linked with undesirable traits like small fruit size. Some large-fruited resistant tomato cultivars (e.g., FL7514 and BHN 466) have become commercially available in recent years and provide moderate resistance against bacterial wilt. Grafting susceptible tomato cultivars

onto resistant tomato or other solanaceous rootstocks is used on a commercial scale in different locations worldwide (Rivard et al. 2012) to control bacterial wilt. However, information on the field-level effectiveness of grafting for use against R3bv2 strains is not available. Transgenic resistance offers possibilities in management, but consumer acceptance and commercial hurdles are limiting factors.

b. Chemical control

Direct control of brown rot or other bacterial wilt diseases caused by *Ralstonia* species in the field is difficult because of the wide host range, strain variation, and lack of adequate chemical treatments. Chemicals tested for the control of bacterial wilt include:

- Actigard (e.g., acibenzolar-S-methyl, ASM) (Abo-Elyousr et al. 2012; Anith et al. 2004; Pradhanang et al. 2005)
- BABA (DL-3-aminobutyric acid) (Hassan and Abo-Elyousr 2013)
- Phosphorous acid (Ji et al. 2007; Norman et al. 2006)
- SBP (stable bleaching powder, calcium hypochlorite) and a urea-lime mix (Dhital et al. 1997)

In summary, it has been demonstrated that these chemicals have both greenhouse and, to a lesser extent, field efficacy. However, they have not been validated and tested on a larger scale against R3bv2.

Soil fumigation with Vapam, methyl bromide, or chloropicrin is thought to be of limited efficacy and utility (Yamada 2012). However, in field trials Mao et al. (2014) obtained good control of *R. pseudosolanacearum* infection on ginger with methyl bromide and chloropicrin. Although the isolates infecting ginger are race 4 biovar 4, this study is still applicable for control of R3bv2 strains. It should be noted that the use of methyl bromide has been phased out as per the Montreal Amendment in 1997 of The Montreal Protocol on Substances that Deplete the Ozone Layer in 1987. The phaseout was effective in 2005 for developed countries and in 2015 for developing countries (https://www.epa.gov/ozone-layer-protection/international-treaties-and-cooperation-about-protection-stratospheric-ozone).

Beginning in the early 2000s, the plant-derived essential oil thymol has been evaluated as a soil fumigant to control *R. solanacearum* (R1bv1-PhyIIA). It was found to effectively reduce bacterial wilt incidence on tomatoes when used for preplant soil fumigation (Ji et al. 2005). Popoola et al. (2012), Pradhanang et al. (2001), and Hong et al. (2011) observed some disease control when soil was treated with thymol. In addition, several other plant essential oils have been tested against *R. solanacearum/pseudosolanacearum* under greenhouse conditions. Excellent control was demonstrated for bacterial wilt of tomato by palmarosa oil and lemongrass oil (Pradhanang et al. 2003), and bacterial wilt of tomato and geranium by clove oil (Huang and Lakshman 2010). Work by Hosseinzadeh et al. (2013) showed that sub-bactericidal concentrations of oils from *Cinnamomum zeylanicum*, *Thymus vulgaris* (thymol), *Lavandula angustifolia*, and *Eucalyptus camaldulensis* suppressed both pathogenicity and virulence factors in race 3 of *R. solanacearum*.

Van Bueningen et al. (2005) found that 100 ppm of hydrogen peroxide was sufficient to eliminate *R. solanacearum* in irrigation water. It is a common practice for potato processing facilities in the United States to add a disinfectant such as chlorine to their wash water (Steven Sargent, UF, *personal communication*). However, there is no information on testing for its effectiveness against *Ralstonia* species. Yao et al. (2010) obtained close to 100% inhibition of bacterial growth with 1.3 ppm chlorine dioxide. The strain tested was a pathogen of *Ipomoea aquatica* in Taiwan, so it was probably a race 1 biovar 4 isolate. However, it is reasonable to assume that this chemical treatment would also be effective for R3bv2. Additional compounds approved for skin and surface disinfection are also listed in USDA-APHIS (2020) guidelines.

Hayes et al. (2022) compared various chemical treatments to eradicate or inactivate R3bv2 cultured or in planta cells under different conditions. They assessed active

chlorine, hydrogen peroxide, UV radiation, heat, and desiccation for their effects on viability of R3bv2 cells on contaminated laboratory surfaces, infected plant material, infested soil, and/or irrigation water. Bacterial cells suspended in water were completely susceptible to most treatments. UV irradiation and desiccation were mostly effective in killing bacterial cells on biotic and abiotic surfaces. However, bacterial cells inside stems of infected tomato plants showed increased tolerance to heat and UV radiation, but not to oxidative stress (hydrogen peroxide and active chlorine treatments). The authors concluded that these protocols are useful for compliance with select agent regulations on cultured cells but for in planta material they recommend aggressive treatments such as autoclaving or incineration.

c. Seed priming

Seed priming is a method used to enhance germination and growth by presoaking seeds prior to planting. The potential of seed priming with sodium chloride to induce plant resistance to bacterial wilt was demonstrated by Nakaune et al. (2012). Tomato plants from seeds primed with a solution of 300 mM NaCl showed a greater tolerance to bacterial wilt than plants from hydroprimed seeds or nonprimed seeds.

d. Biological control

Research by Aliye et al. (2008) and Lemessa and Zeller (2007) identified bacteria that inhibited bacterial wilt disease in potatoes. Several soil bacteria and plant growth-promoting rhizobacteria (PGPR) are currently being investigated for their role in control of *R. solanacearum* in small-scale experiments. Currently, none are commercially available, and the efficacy of the biological control agents has yet to be determined on a commercial scale. Anaerobic soil disinfestation (ASD), also known as biological soil disinfestation, has proven to reduce the level of *R. solanacearum*, including R3bv2, in the soil (Messiha et al. 2007; Momma et al. 2013). ASD is a biological preplant technique that consists of applying organic amendments, tarping the soil, and then saturating the soil to capacity. Typically, the soil is covered for up to 3 weeks, by which time the environment under the tarp becomes anaerobic (Rosskopf et al. 2015).

Bacteriophages have been used to control bacterial plant pathogens (Jones et al. 2012). Currently there is only one company, OmniLytics (Sandy, Utah, U.S.A.; www.omnilytics.com), that markets bacteriophages for biological control of several plant diseases. Although they developed a phage mixture for biocontrol of *R. solanacearum* (Iriarte et al. 2012), it is not commercially available. Much of the research on use of bacteriophages to control bacterial wilt has been for race 1 strains. Therefore, the specific phages may not be directly applicable for use with *R. solanacearum* R3bv2. However, the information serves as a useful and relevant model for future work using bacteriophages to control R3bv2. Recently, Ahmad et al. (2018) discovered a novel *Ralstonia* phage RsoP1EGY, isolated from soil in Egypt, that specifically infected only phylotype IIB sequevar 1 (R3bv2) strains of *R. solanacearum*. Such phage proved to be effective in controlling bacterial wilt in tomatoes caused by R3bv2 under greenhouse conditions (Elhalag et al. 2018).

A difficulty in using bacteriophages for control of plant diseases is that they do not persist when applied to leaf surfaces due to environmental factors such as sunlight (UV-A and UV-B), high temperatures, and desiccation. Sunlight was shown to be the most problematic of all the environmental factors (Jones et al. 2012). Iriarte et al. (2012) demonstrated that when bacteriophages are applied in the rhizosphere, the problem of sunlight is eliminated.

Tanaka et al. (1990), Tan et al. (2010), Fujiwara et al. (2011), Addy et al. (2012), Iriarte et al. (2012), and Young et al. (2012) all had some success using phages to control *R. solanacearum* and *R. pseudosolanacearum* on plants in pots under growth chamber type or greenhouse conditions. Recently, three lytic phages were isolated from environmental water in Spain and found effective as biocontrol for *R. solanacearum* R3bv2 (phy IIB-1) by applying single or combined phages through irrigation water (Álvarez et al. 2019). Using a phage cocktail containing two lytic phages isolated from

Korea suppressed disease symptoms in agricultural soil (Magar et al. 2022). So far, two *Ralstonia*-infecting phages, RsoM1USA and RsoM2USA, have been isolated in the United States (Addy et al. 2019; Ahmad et al. 2021). Infection of a susceptible *R. solanacearum* strain by phage RsoM1USA resulted in significantly reduced growth of the infected bacterium in vitro, but not virulence in tomato plants (Addy et al. 2019). RsoM2USA is the largest *Ralstonia*-infecting bacteriophage sequenced and reported to date, with a genome size of 343,806 bp (Ahmad et al. 2021). The jumbo phage RsoM2USA has a wide host range, infecting each of the three newly established *Ralstonia* spp.: *R. solanacearum*, *R. pseudosolanacearum*, and *R. syzygii*, and it significantly reduced the virulence of *R. solanacearum* in tomato plants under greenhouse conditions (Ahmad et al. 2021). Although a great deal of work needs to be done before bacteriophages are commercially available to control R3bv2, the potential clearly exists.

e. Phytosanitation and cultural practices

The best strategy for controlling bacterial wilt in the field consists primarily of phytosanitation and cultural practices. In regions where bacterial wilt of potato is endemic or in locations where *R. solanacearum* and/or *R. pseudosolanacearum* are present but not yet established, these methods can be effective under some conditions. Several cultural practices can reduce disease, including planting pathogen-free (tested) seed potatoes, early detection, accurate identification and reporting of the pathogen, and quarantine measures on infected fields and farms.

In areas where the pathogen is already established and widespread, crop rotation provides the best control. A 2- or 3-year rotation is recommended. Using grasses in the rotation has been successful in reducing the incidence of bacterial wilt (Denny 2006). One example of a recommended rotation is fava beans-garlic-maize-soybean or wheat in succession. A highly successful rotation used in a study in India included potato-wheat-lupine-maize-potato (Saddler 2005). Work in East Africa on bacterial wilt in potato demonstrated that different rotation system combinations including pulses, cereals, sweet potatoes, cabbage, and onions were all effective in decreasing wilt incidence and increasing yield (Lemaga et al. 2005) although the cereals were more effective than the pulses.

Irrigation water and wastewater from potato-processing facilities can spread disease if potato plants are infected. Brown rot was found in Sweden in 1972. The infected field was downstream from two potato factories that released untreated wastewater into a river that was used to irrigate potato fields. The factory potatoes originated from the Mediterranean region. A similar situation occurred in Belgium in 1989. In the early to mid-1990s, the Netherlands and other western European countries experienced an outbreak of brown rot. Contaminated surface water was also involved in this occurrence (Janse 2012). Several measures were implemented in Sweden to eradicate *R. solanacearum.* Potatoes were not grown in infested fields for two years. *S. dulcamara*, an alternative host of *R. solanacearum*, was removed from waterways. Only certified seed was used, and waterways were not used for irrigation. These measures, combined with environmental monitoring for the presence of *R. solanacearum*, were successful in eradicating the pathogen from Sweden (Persson 1998).

Recommended strategies for best management of bacterial wilt of tomato caused by *Ralstonia* species should be followed (Momol et al. 2005). These include sufficient crop rotation, use of cover crops and other measures to reduce the impact of weed hosts and volunteer plants (and in some cases of nematodes), and testing and treatment of surface water used for irrigation. Integrated application of these strategies to other crops is also critical for successful management of diseases caused by *R. solanacearum* species.

f. Exclusionary practices

In locations where the pathogen is not present, it is important to prevent introduction and, if inadvertently introduced, to prevent subsequent movement of the bacterium

from infested to noninfested locations or fields. Effective cultural sanitation practices are critical to keep noninfested areas clean. Sanitation efforts include planting only certified disease-free plantlets, disinfesting all equipment before moving it between fields, controlling flood water flow, and never using surface water for irrigation. In the greenhouse, sanitary practices for tomato transplant production may include avoidance of subirrigation; wide separation of greenhouses from field production areas; disinfestation of all frames, trays, and tools; use of pathogen-free soils or potting mix; control of weeds; and limited handling of plants (McCarter 1991).

2. Control on geranium

In offshore geranium production facilities, USDA-APHIS requires use of sanitation protocols to exclude the pathogen (USDA-APHIS 2004). Exclusionary practices, along with regular testing and personnel training, provide good control when carefully followed by the exporting company. Offshore training efforts could be enhanced by providing funding through USDA, FAS, and/or USAID to U.S. scientists specialized in outreach on *R. solanacearum.*

XII. Current Infrastructure, Needs, and Experts

Studies for initial screening of suspect samples can be carried out by diagnostic laboratories including private, state, NPDN or cooperating university diagnostic laboratories, state and federal regulatory officials, and industry. This screening may include serological testing methods or isolation and identification of *R. solanacearum* to genus and species. If these facilities cannot perform these screening steps, samples can be referred to the appropriate NPDN Regional Centers located in California, Florida, Hawaii, Kansas, Michigan, New York, and Oregon. If a positive result is obtained through initial screening, the PPCDL is required to conduct confirmatory testing to determine if the sample is R3bv2.

If the diagnostic test gives a positive result for *R. solanacearum* (including plant material, water, or soil samples, and/or culture) diagnosticians must follow USDA Select Agent regulations. The PPCDL has all necessary authorizations to receive samples submitted for identification. Diagnosticians must fill in a Form 4 Section AB and submit to USDA-APHIS within 7 calendar days of identification. A confirmatory determination of R3bv2 is only performed at the PPCDL and will require a Form 2, initiated by PPCDL, for transfer.

The infrastructure to handle research on R3bv2 is currently very limited due to its Select Agent status. To conduct experiments with this pathogen, registration is required with the USDA-APHIS-DASAT. Registration is approved on a site-specific basis, considering biocontainment measures, research objectives (i.e., if plant inoculation will be conducted), security, and a variety of other factors. Permits and advance permission are required for any movement of cultures or infected plant materials. These regulations allow laboratories to conduct important research on these potentially dangerous materials while making sure it is done as safely and securely as possible. For more information on agricultural select agents please visit the website selectagents.gov.

The destructive nature and quarantine status of this pathogen has significantly elevated its importance and the need for effective detection, management, and better understanding of this disease and its causal agent. Therefore, a team of experts on *R. solanacearum* from the universities of Florida, Georgia, Hawaii, and Wisconsin was granted a 4-year (2007 to 2011) USDA-NRI funded project to conduct research on *R. solanacearum* R3bv2. Objectives of the project were:

i) to develop rapid, robust, and reliable diagnostic assays for *R. solanacearum* R3bv2;

ii) to identify R3bv2 genes involved in cold adaptation and growth in plant hosts, using a microarray-based postgenomic approach; and

iii) to develop a package of optimized education and management training modules that will educate stakeholders to control this pathogen, primarily by exclusion.

More details about this project (summary, contact information, and accomplishments) can be found at: http://plantpath.ifas.ufl.edu/rsol/NRI_Project/Projectsummary.html.

Since 2011 more national and international scientists have joined the list of *R. solanacearum* experts, thanks to their research on various aspects of *Ralstonia*, including genetics of cool virulence, phylogenomics, genome informed diagnostic methods, control, and interactions with the host.

Noncomprehensive list of current *R. solanacearum* experts:

Allen, Caitilyn: Phytobacteriology, specializing in biology of *Ralstonia* interactions with tomato, geranium, and potato, Department of Plant Pathology, University of Wisconsin, Madison, WI, U.S.A. Email: cza@plantpath.wisc.edu

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Bocsanczy, Ana Maria: Genetic determinants of cool virulence, diagnostic tools, host specificity of *Ralstonia solanacearum*. Blueberry resistance to bacterial wilt. Department of Plant Pathology, University of Florida, FL, U.S.A. Email: anamariab@ufl.edu

Cellier, Gilles: Diversity and genetic population of RSSC strains, biological traits and phylogenetics. Anses – Plant Health Laboratory. Tropical Pests and Diseases unit (RAPT). Saint Pierre, Reunion Island. Email: g.cellier@anses.fr

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Stulberg, Michael J.: *Ralstonia solanacearum* diagnostics, particularly R3bv2 diagnostics. Branch Chief. USDA APHIS Biotechnology Regulatory Services. Riverdale, MD, U.S.A. Email: Michael.Stulberg@usda.gov

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Vinatzer, Boris: Genome-based classification of the *R. solanacearum* species complex, lineage-resolved genome-based and metagenome-based identification with a focus on R3Bv2. School of Plant and Environmental Sciences (SPES), Virginia Tech University, Blacksburg, VA, U.S.A. Email: vinatzer@vt.edu
Vlami-Bergsma, Maria: Phytobacteriology, specializing in diagnostics, risk assessment, and management of quarantine plant pathogenic bacteria, including *Ralstonia solanacearum*, species complex in crops, and waterways. Head of Bacteriology Laboratory, Netherlands Institute for Vectors, Invasive Plants, and Plant Health (NIVIP), Netherlands Food and Consumer Product Safety Authority (NVWA), Wageningen, The Netherlands. Email: m.vlami@nvwa.nl

XIII. Research, Education, and Extension Priorities

RESEARCH PRIORITIES – MOST IMPORTANT

1) Develop disease management tactics to control bacterial wilt on potato, tomato, and geraniums (phylotypes I and IIA strains could be used as a model to advance this area), including:
    a) Develop or screen additional chemical and biological control products.
    b) Exclude the pathogen from potato seeds, geranium cuttings, and tomato transplants, and develop vegetative plant material certification schemes.
    c) Study the effects of cover crops, crop rotation, mulches, and ASD on pathogen dynamics and disease incidence.
2) Develop novel, easy-to-use detection methods to reliably distinguish subgroups of *R. solanacearum*, especially R3bv2, from other endemic strains of *Ralstonia*.
3) Develop a better understanding of latent infections by *R. solanacearum* because these are an important source of potential pest introductions on potato and geranium. Research is needed to understand what factors predispose plants to be latently infected, how bacteria move in latently infected plants, and what triggers symptom development in latently infected plants.
4) Determine whether any other subgroups of *R. solanacearum* besides R3bv2 have cold tolerance capability. What gene sequence(s) code for cold tolerance? This knowledge might allow regulations directed against all strains of *R. solanacearum* with the sequence(s).
5) Develop and enhance bacterial wilt resistant potato and tomato cultivars.

RESEARCH PRIORITIES – HIGHLY IMPORTANT

1) Develop disease-resistant tomato rootstock together with efficient, preferably robotic, grafting techniques for seedling production.
2) A broader host range study should be done to determine which commonly grown ornamentals and other plant species act as hosts or carriers of *R. solanacearum* including R3bv2.
3) Develop novel methods (i.e., use of nanotechnologies, isothermal DNA amplifications) for rapid, sensitive, and accurate detection and identification to subgroups of *R. solanacearum* in environmental samples (including host plant, water, soil, and other media).
4) Explore novel diagnostic techniques to detect R3bv2 quickly and at low concentrations, such as electronic nose and highly sensitive antibody-responsive cell culture systems.

5) Continue the work on cold tolerance of R3bv2 strains, including those from Guatemala, Mexico, Costa Rica, Kenya, and China, to evaluate their ability to survive in temperate U.S. locations.
6) Develop rapid and inexpensive gene sequencing methods for identification of sequevars of *R. solanacearum* species complex. Create a database including all partial gene sequences that allow differentiation of the sequevars.
7) Develop impact network analysis (INA) tools to identify key risk factors in production and movement of planting material (tubers, cuttings) from areas with endemic populations of R3bv2.
8) Establish a reference collection repository for R3bv2 material.

RESEARCH PRIORITIES – IMPORTANT

1) Develop methods that can be used to disinfest contaminated soils, including soil fumigation, other chemicals, and biologicals. Testing of other area sanitizers for site cleanup (such as uses of quaternary ammonium and peroxyacetic acid sanitizers with foam carriers, Virkon S, etc.).
2) Determine a practical disposal method for potatoes in the event of detection of the bacterium in potato-growing areas and/or subsequent quarantine.
3) Develop transgenic tomato and potato cultivars that are resistant to the disease.

EDUCATION PRIORITIES

1) Educate new plant pathologists in the epidemiology and management of bacterial diseases.
2) Develop training courses on detection, monitoring, and management of *Ralstonia*-related diseases.

EXTENSION PRIORITIES

1) Educate county extension agents, growers, and crop advisors in sampling, monitoring, and management of related diseases and in the utility of map-based tracking and information systems such as PIPE.
2) Develop contingency plans to test potato propagation material to ensure that it is free of R3bv2. Existing EU-EPPO protocols for such testing should be used as a starting point.
3) Survey tomato, potato, and other host growing areas and greenhouse areas in the United States with previous R3bv2 history, including soil, water, crops, and weed samples.

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XVI. Web Resources

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