Protocol for assessing bacterial wilt resistance in greenhouse and field conditions

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Layout

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I. INTRODUCTION

Bacterial wilt, caused by *Ralstonia solanacearum* (Yabuuchi *et al.*, 1995) is the second most important potato disease in tropical and sub-tropical regions of the world after late blight (Champoiseau *et al*., 2010). Globally, the disease has been estimated to affect about 1.7 million hectares of potatoes in approximately 80 countries, with global damage estimates of over USD 950 million per annum (Champoiseau *et al.*, 2009a). In addition to potatoes, the disease also affects over 200 plant species from more than 50 families (Hayward, 1991). The bacterium, which is often endemic in the soil, penetrates the plant through the root system and eventually causes irreversible wilting and death (Muthoni *et al.*, 2012). The disease is also referred to as brown rot in potato.

This protocol is an updated version of "Assessing potato clone field resistance to bacterial wilt" issued in The International Cooperators' Guide (CIP 2007). The first edition of the protocol presents a standard procedure for field assessment of resistance to bacterial wilt for documenting levels of resistance of advanced potato germplasm. The protocol aims at promoting uniform data sharing between institutions dedicated to potato breeding and selection.

This second edition has included a standardized procedure for greenhouse screening of potato seedlings for bacterial wilt resistance useful for perform genetic studies, parental selection or identification of new sources of resistance in accessions of wild species propagated or maintained as true seed. In addition, it was updated with procedures for phylotype identification based on DNA sequence so far considered the best method for classifying strains.

***Epidemiology*:** Direct yield losses caused by bacterial wilt vary widely according to host, cultivar, climate, soil type, cropping practices and pathogen strain. The disease affects central Himalayan countries in South Asia such as Nepal and Bangladesh, having more than 30% of potato crops affected by *R. solanacearum* with over 14% reduction in yield to up 100% due to poor cultural practices, such as keeping seed from infected crop (Elphinstone, 2005). In African countries as Uganda and Kenya, yield losses from 30 to 100% have been estimated and increased incidences reported due to the spread and build-up of the disease in the majority of the potato growing areas (Ateka *et al.*, 2001; Kinyua *et al*., 2005).

Transmission and spread: *R. solanacearum* survives in infected plants, plant debris, soil, water, seeds, vegetative propagation material and in the rooting system and rhizosphere of many other host crops and weed. Infected potato seed is the main cause of pathogen's dissemination to long distances. After infestation, *R. solanacearum* can survive in the soil for many years, period that depends on the pathogen strain and the crop rotation program. The pathogen spreads through irrigation with contaminated water, utilization of infected vegetative planting material, and infested soil adhered to tools, hooves and farmers' shoes.

Figure 1. Potato plant showing wilted leaves

Symptoms: *R. solanacearum* primarily enters plants through natural openings or from wounds, particularly in the roots. Natural openings are commonly formed by lateral root emergence, while wounds are a result of root damage caused by soil borne organisms (e.g. root-knot nematode), transplanting, cultivation, or insects. Once the bacterium enters the plant, it spreads upward via the xylem and colonizes in the vascular bundles. This leads to a condition in which infected plants start wilting irreversible

The initial symptom in mature plants is wilting of upper leaves usually first visible at the warmest time of day followed by recovery throughout the evening and early hours of the morning. The wilted leaves maintain their green color as the disease progresses (Fig 1.). Epinasty of the petioles may occur.

Figure 2. Streak discoloration of the stem

Figure. 3. Stem being cut

As the disease develops, massive invasion of the cortex might result in the appearance of water-soaked lesions on the external surface of infected stems and streaky brown discolorations of the stem may be observed on stems above the soil line (Fig. 2) and the leaves may have a bronze tint (Gota, 1992). If an infected stem is cut crosswise (Fig. 3), tiny drops of dirty white or yellowish viscous ooze exude from several vascular bundles (Fig. 4) (Champoiseau et al., 2009b). This test denoted as “the vascular flow test” allows us to confirm the presence of the *R. solanaceraum* a in wilted plants. Under hot and humid conditions, complete wilting occurs, the plant becomes yellow and brown necrotic and eventually the plant dies. Wilting by *R. solanacearum* can be confused with wilting caused by other pathogens such as *Pectobacterium, Dickeya, Fusarium or Verticillium* spp, as well

as insect or mechanical damage at the base of a stem or when there is lack of water.

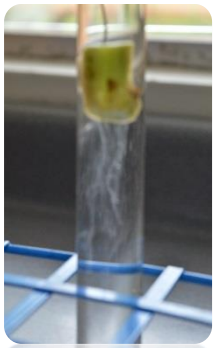

Figure. 4. Stem suspended in a transparent tube to observe the cloudy white streaming of the bacteria from the vascular bundles

On tubers, external symptoms may or may not be visible, depending on the state of development of the disease. Tubers exhibit browning of the vascular ring and/or a bacterial ooze that often emerges from the eyes and stolon-end attachment of infected tubers (Fig. 5.). This ooze is a creamy fluid exudate clearly seen in tubers cut transversely (Fig. 6.). Soil may adhere to the tubers at the eyes as a result of the soil particles sticking on the bacterial ooze. Plants with foliar symptoms caused by *R. solanacearum* may bear healthy and diseased tubers, while plants that show no signs of the disease may sometimes produce diseased tubers. Soil may also be seen to adhere to the eyes of such tubers as a result of the soil particles sticking on the bacterial ooze.

Figure.5. Oozing due to bacterial wilt

Figure.6. Creamy fluid exudate seen in tubers cut transversely

A plant infected with *R. solanacearum* may express all or none of the symptoms outlined above, even under environmental conditions that are ideal for the pathogen. If symptoms are not evident on an infected susceptible host, the condition is known as latency. This occurs usually in cool conditions, such as those found in the tropics at altitudes above 2500 MASL or in cool temperate regions. Warm climates favor multiplication of the bacteria and therefore the development of symptoms. However, symptomless plants may harbor the bacterium and transmit it to progeny tubers. This may lead to severe disease outbreaks or spread to bacterial wilt free areas (Hayward, 1991; French, 1994)

Pathogen description: *R. solanacearum* is gram-negative, rod-shaped bacterium that grows well at 28 to 32°C strictly in aerobic conditions (Hayward, 1991; Schaad *et al.*, 2001). Individual colonies of normal or virulent isolates are usually visible after 36 to 48 hours. These colonies are characterized by irregular shape, fluidal and entirely white or with a pink center on modified Kelman's medium (MKM) with 2,3,5 triphenyl tetrazolium chloride (TZC) (Fig.7) (French et al.,1995).

Figure 7. Colonies of *R. solanacearum* in MKM with TZC

Strains of *R. solanacearum* have previously been grouped into five pathogenic races based on susceptible host plants, and five biovars determined by utilization of a panel of five to eight carbohydrate substrates (Buddenhagen *et al.*, 1962; Hayward, 1964). Most important in potatoes are race 1 and 3. The former occurs in the lowland tropics and warm temperate lands (French, 1994). It has a high temperature optimum (35-37°C). On the other hand, race 3 occurs at higher altitudes (in the tropics) and higher latitudes than race 1 (EPPO, 2004). This race

has a long association with potatoes and has an optimum temperature of 27 - 28°C and average temperatures of 20-22°C (French, 1994).

As for biovars, Biovar 2 (race 3) is known as the potato low temperature race and is found in high latitudes, and high altitudes (from 1,500 to 2,500 MASL) in the tropics (Seal *et al.*, 1999; Hayward, 2000). Race 3/biovar 2A (R3bv2A) causes bacterial wilt of potato in over 90% of cases worldwide because potato is a cool season crop (French, 1994; EPPO, 2004). R3bv2A occurs in the cool tropical highlands and it is widespread in the higher latitudes as far as southern Sweden and southern Argentina (Champoiseau *et al.*, 2009a). Although R3bv2A principally occurs in cool climates, it also occurs in potato plants grown in warmer locations from seed tubers harvested from cool climates (French, 1994). In warmer subtropical and tropical lands, race 1/biovars 1, 3 and 4 occur and bacterial wilt symptoms are severe. This race and their corresponding biovars are expected to spread and cause more severe symptoms under global warming (EPPO 2004).

Aforementioned classification has not resulted very useful as host ranges overlap and tests to define races are cumbersome. In addition, race determination is not possible, because *R. solanacearum* strains do not have race-cultivar specificity on plant hosts and with the exception of R3bv2A, the old “races” do not have phylogenetic unity (Fegan and Prior, 2005; Champoiseau *et al.*, 2010). Molecular characterization focusing on the variation of selected marker genes resulting in the identification of Phylotypes and sequevars has been proposed as a classification scheme that adds more valuable information about the geographical origin and in some cases the pathogenicity of strains (Fegan and Prior, 2005; Prior and Fegan, 2005). Four Phylotypes and tens of different sequevars have been identified. Phylotype I includes all strains belonging to biovars 3, 4, and 5 and strains are isolated primarily from Asia. Phylotype II includes strains belonging to biovars 1, 2A and 2T isolated primarily from America. The strain most commonly infecting potato R3bv2A is Phylotype II and has sequevars 1 and 2, race 2 is also a member of Phylotype II. Phylotype III contains strains primarily isolated from Africa and surrounding islands. Strains in this group belong to biovars 1 and 2T. Phylotype IV

contains strains isolated primarily from Indonesia belonging to biovars 1, 2 and 2T (Table 1).

Table 1. Equivalences among phylotypes, biovars, and races of *R. solanacearum* (Source: Prior & Fegan, 2005).

Species	***Ralstonia solanacearum***			
Phylotype	I	II	III	IV
Origin	Asia	America	Africa	Indonesia
Biovars	3 4 5	2T 1 2A	2T 1	2T 1 2* R B
Races	1 4 5	1 2 3		

R=*Ralstonia syzygii*; B= *Pseudomonas celebense*
*This could be 2A (Lilian Gutarra, personal communication)

Resistance to bacterial wilt: No high level of resistance to bacterial wilt exists in potato cultivars, although some cultivars are less susceptible than others and can give high yields in the presence of the disease. Because race 3 strain belongs to a genetically homogeneous group, the resistance to this race is expected to be more stable than resistance to lowland strains (race 1) of *R. solanacearum* (French *et al*. 1998). Breeding for resistance at CIP has resulted in moderate to high levels of resistance to bacterial wilt; however, the high frequency of latent infection in tubers is still a problem (Priou *et al*. 2001, 2005). Latent infection is responsible for spread of the disease and overcoming of resistance (French *et al*., 1998). The possibility of latent infections demands seed production in areas free of *R. solanacearum*, independently on the level of resistance of the genotype to bacterial wilt. The advantage of the resistant cultivar would be their lower losses upon cultivation in infested commercial fields.

II. GREENHOUSE SCREENING

Screening for bacterial wilt resistance at greenhouse can be performed for several purposes, such as 1) to prove if resistance found in new sources is genetically inherited; 2) to identify parents with ability to transmit resistance in a breeding program; 3) to test the resistance of clones against races and/or biovars others than those present in the crop geographical area where the resistance study is conducted; 4) to search for extremely or highly resistant individuals in true seed accessions of wild species.

It is recommended to develop tuber families from true seed segregating progenies to keep a healthy stock from each individual (see: “Protocol Procedures for Generation of Potato Tuber Families from true (botanical) seed” by Mihovilovich *et al*. 2015). In this case a stem cutting can be taken from each plant 60 days after planting, i.e. 40 days after transplanting to pots. However, when the objective is any of the first two aforementioned, plants derived from seedlings of TS segregating progenies can be directly inoculated.

III. PROCEDURE:

Maintaining and culturing of R. solanacearum strains:

Pure cultures of *R. solanacearum* resulting from isolation procedures can be stored for many years at room temperature (15-20 C) in sterilized tap, distilled or deionized water, or at -80°C in liquid culture broth amended to 40% glycerol.

To obtain fresh cultures, stock suspensions should be streaked on MKM with TZC (French *et al*. 1995) and incubated for 48 h at 30 °C or at room temperature for 2-6 days. Plates should be incubated in an inverted position because water condensation may cause colonies to flow into each other, thereby limiting separation. Separately growing colonies can then be picked and sub-cultured onto fresh media to obtain pure cultures. Remember that *R. solanacearum* easily loses virulence if repeatedly transferred on agar plates and loses viability if plates are stored at 4°C. Cultures can become non-culturable, although viable, if exposed to very low temperatures (van Elsas *et al*., 2001).

Inoculum preparation:

Bacterial suspensions are prepared by culturing strains on MKM without TZC at 30°C for 48 h. The cells are harvested in sterile distilled water and the bacterial concentration evaluated by measuring the optical density (OD) at 600 nm of a diluted aliquot.

***Screening true seed segregating progenies**:*

True seed representing a progeny of individuals are sown after GA treatment (1500 ppm) directly in a small tray (Fig. 8) or plastic crates (Fig. 9) containing a soil substrate composed of soil, sand and peat moss in 2:1:1 proportions. Make rows or wholes of 0.4 inch deep spaced 2 inches apart. Best results are obtained using the Promix BX® substrate (Premiers Brands, INC, Stamford, Canada) or the commercial substrate Plantmax® produced with composite pinus peel, vermiculite and basic fertilization.

Figure 8. Seedlings from true seed three weeks after planting

Figure 9. Emerging seedlings from true seed sown in plastic crates

A complete fertilizer (N-P-K 20-20- 20, diluted at 0.5% in water) can be applied at the time of planting if no commercial substrate was used. Twenty days later, seedlings are transplanted into 250 mL plastic cups containing the same substrate mixed (soil, sand and peat moss in 2:1:1 proportions, or Promix). In case Plantmax® is used, add sterilized soil in equal proportions (Fig.10). When stem cuttings of segregating progenies are used, these can be planted directly into 250 mL plastic cups containing the soil, sand and peat moss in 4:3:1 proportions. The seedlings and stem cuttings are kept in a greenhouse protected with anti-aphid screen, well ventilated and with good luminosity to avoid dampness. Plants are irrigated daily, except a day before inoculation. Ten days after transplanting in the case of seedlings or once stem cutting have rooted (20 days after planting), these are transferred (if required) to a greenhouse with conditions that favor disease

development (28±4ºC and 85-90% R.H.) and inoculated by pouring 10 ml of a suspension into the base of the plant (approximately 10^7 to 10^8 cfu / ml) (Fig. 11).

Figure 10. Seedlings representing replication of a family of 120 seedlings transplanted into 250 ml. plastic cups.

Figure 11. Inoculation of seedling with a suspension of the bacteria.

Heating devices may be required during the nights to ensure infection and reduce the frequency of escapes. Wilting seedlings appeared from seven to 10 days after inoculation and plants will be evaluated in a 1 and 0 score every 5 days, as follows:

1 = apparently healthy plant
0 = wilted or dead

If the objective is to search for extreme or highly resistant individuals in true seed accessions of wild species, wilting and dead seedlings are removed weekly. Seedlings that survive 15 days after inoculation are transplanted individually to 6 inch diameter pots containing a sterile substrate of soil, sand and peat moss in the same proportions aforementioned. Evaluation should continue on surviving plants 15 to 20 days more after transplanting and then moved to a cooler greenhouse (18- 22 C) for tuber production. Plants which wilted in this environment are also discarded. On the other hand, if the screening is for any of the other three objectives aforementioned, wilted or dead seedlings should be counted and the evaluation will end up when more than 80% plants obtained from 10 to 30 stem cuttings of a susceptible control is dead. Plants scored 1 are maintained till harvest whatever the objective is to recover tubers for evaluation of visible oozing at tuber eyes and latent infection analysis. At harvest tubers are evaluated for visible oozing

at the eyes or vascular oozing visible upon slicing rotten tubers. The number of symptomatic tubers is recorded for each plant scored as 1.

The frequency of wilted plants/ progeny is calculated as follows:

$$\text{Percentage of wilted plants/progeny} = \frac{\text{Total number of wilted plants}}{\text{Total number of evaluated plants}}$$

Average for each segregating progeny can then be computed over replications if replicates of the progeny comprising 80 to 120 seedlings were grown at the beginning and randomized in the greenhouse.

Latent infection analysis: *(*Taken from Gutarra *et al.*, 2014)

Healthy looking or asymptomatic tubers harvested from apparently healthy plants (score=1) are analyzed for latent infection. Tubers are washed in tap water and analyzed 2 weeks after harvest. Once in the laboratory, healthy looking tubers are disinfected with 70 % alcohol, rinsed with sterile water and checked again. At this stage, tubers exhibiting BW symptoms (visible oozing at tuber eye) are removed and the number recorded for that plant (individual genotype). Samples may consist of single or composite samples of two or three tubers depending on the total number of asymptomatic or healthy looking tubers/plant. Strips along the vascular ring are cut with a flame-sterilized cuticle remover. The samples are placed in a plastic bag, weighed and crushed and homogenized using two ml of sterile sterile citrate extraction buffer per gram of tuber tissue (0.1 M citric acid, 0.1 M sodium citrate, pH 5.6). Bacteria in the tuber extracts are enriched by incubating a mixture of 500 µl with same volume of modified SMSA broth (Elphinstone et al. 1996, Appendix) in sterile 1.5 ml Eppendorf tubes for 48 h at 30 C. The enriched tuber extracts are analyzed by NCM-ELISA, as described by Priou *et al*. (1999). The CIP kit to perform the post-enrichment enzyme-linked immunosorbent assay on nitrocellulose membrane (NCM-ELISA) is available under request and used

according to the protocol indicated in the kit manual (Priou *et al.* 1999). The presence of *R. solanacearum* in the tuber extract leads to the development of a purple coloration. All races and biovars of *R. solanacearum* can be detected with the polyclonal antibodies. The intensity of the coloration is proportional to the bacterial concentration. After enrichment (e.g., multiplication of the bacterial population in the extract), as few as 10 bacteria per ml of tuber extract (cells/ml) can be detected, whereas 10^7 cells/ml or more are necessary if enrichment is not practiced before conducting the immunoassay. Thus, enrichment increases sensitivity of the serological test by a million-fold, allowing the detection of *R. solanacearum* in potato tubers (or stems) that are latently infected, e.g., with very low infection levels that produce no visible symptoms. Positive results in NCM-ELISA can be confirmed by isolating *R. solanacearum* on MKM with TZC and plates incubated at 30 ° C for 48 h or by PCR with primers 759/760 (Opina et al., 1997).

Calculating the percentage of total infected tubers of an individual plant (genotype)

$$Percent\ of\ symptomatic\ tubers = \frac{Number\ of\ symptomatic\ tubers}{\left(\begin{matrix}Number\ of\ healthy\ looking\ tubers+\\ Number\ of\ symptomatic\ tubers\end{matrix}\right)} * 100$$

$$Percent\ of\ latently\ infected\ tubers = \frac{Number\ of\ samples\ positive\ to\ NCM\ ELISA}{Total\ samples\ tested} * 100$$

$$Percent\ of\ total\ infected\ tubers\ of\ an\ individual\ plant\ (genotype) = \%\ symptomatic\ tubers + \frac{healthy\ looking\ tubers\ * \%\ latently\ infected\ tubers}{100}$$

Calculate the mean percentage of total infected tubers of all plants (genotypes) scored as 1

$$\text{Mean percentage of total infected tubers} = \frac{\Sigma\ \%\ of\ infected\ tubers\ of\ each\ plant}{Total\ number\ of\ genotypes}$$

Greenhouse screening can also be used for testing clones through stem cuttings or tuber sprouts. Both can be planted directly into 250 mL plastic cups for inoculation once rooted (see above).

Decisions:

Parental donor of a progeny with 20% or more of their individuals scored as 1, and 15% or less of infected tubers can be selected and used in a breeding program.

Likewise, parental donor of a progeny with 20% or more individuals scored as 1, and 30% or less of infected tubers can also be selected is breeder considers this can be a novel or complementary source of moderate levels of resistance

Selection of resistant genotypes within progenies:

Genotypes scored as 1 (apparently healthy plants) with 15% or less infected tubers can be considered as resistant while those that achieve 30% are considered moderately resistant.

Resistant genotypes from segregating progenies can be propagated for field trial evaluation from tuber stocks or from two asymptomatic tubers separated at harvest before samples for latent infection analysis were taken. Note that asymptomatic tubers unless rotted under storage due to latent infection can be used upon sprouting for propagation under greenhouse conditions, otherwise the genotype should be discarded.

IV. FIELD EVALUATION

Biotic conditions:

Screening potato for resistance to BW requires a field with a reasonably uniform and moderate level of infestation (between 30% to 50% wilt incidence in the previous potato crop) (Priou *et al.* 1999). Phylotype of the strain present in the field should be identified. Note that cultivars known to be resistant to strains of one phylotype of the bacteria may become susceptible to strains of other phylotype. In addition to the pathogenicity of the strain, phylotyping scheme adds information of the geographical origin.

Abiotic conditions:

Optimum evaluation conditions depend on the environment under which the strain survive or persist and is more pathogenic to potato. Race 3 biovar 2 that belongs to phylotype II and is primarily pathogenic to potato occurs in the highland tropics and thus persist under cool humid conditions, whereas race 1 biovars 1, 3, 4 that belongs to Phylotype I and occurs in warm subtropical and tropical lowlands.

***Isolation of R. solanacerum from soil samples in semi-selective medium (SMSA*)**:

(Taken from The International Plant Diagnostic Network (IPDN), 2014). Soil samples are weighed into 10 g quantities. Each sample is suspended in 100 ml of water or phosphate buffer (4.26 g Na2HPO4 and 2.72g KH2PO4 l-1; pH 7.2; PB) in a conical flask or a heavy gauge polythene bag. The sample is shaken vigorously for 2 minutes and the heavy soil particles were allowed to settle for about 2 minutes. Subsequently, the following steps are undertaken:

− Draw out a 1.5 ml aliquot from the suspension using a sterile pipette tip and a micropipette, being careful to avoid soil or debris. Transfer the suspension to a sterile Eppendorf tube; this forms the stock suspension (10^0).

− Draw out a 100 µl aliquot from the stock suspension and put it in 900 µl of sterile distilled water in a sterile Eppendorf tube; mix thoroughly by pipetting in and out, while stirring simultaneously with the micro-pipette. This forms the first dilution of the stock suspension (10^{-1}).

− Dilute the 10^{-1} suspension by adding a 100 µl aliquot to 900 µl of sterile distilled water in a flat-bottomed tube to get a 10^{-2} suspension.

− Continue the serial dilution procedure to get a 10^{-4} suspension (or higher dilutions if the samples are suspected to have high populations of *R. solanacerum*.

− Mix the 10^{-4} suspension (or the highest dilution made) thoroughly using a pipette tip and draw out a 100 µl (0.1 ml) aliquot.

− Place this suspension on a well-set agar plate containing SMSA (Appendix) and spread with a sterile bent glass rod to cover most of the surface of the medium (this is called ‘lawn-plating’). Be careful not to splash the suspension to the edge of the medium because counting the colonies at the edges can be difficult or impossible. Note that spreading should be done as soon as the suspension is placed on the medium to avoid ‘clumps’ of colonies.

Note: Changing the pipette tip is not necessary between plating o of any two dilutions of the same sample, PROVIDED THAT YOU START WITH THE LEAST CONCENTRATED SUSPENSION per sample; however, a fresh sterile tip must be used for each separate sample.

Note: Whenever possible, it is advisable to prepare at least two plates for each dilution per sample, to cater for experimental error by using the mean of the counts in those plates

− Incubate the plates at 30°C for 48-72 hrs.

Positive control: Ensure that you dose a soil suspension with a confirmed isolate of *R. solanacearum* and carry out serial dilutions from 10^0 to 10^{-5}. Plate the 10^{-1}, 10^{-3} and 10^{-5} suspensions (one plate each is adequate). Also prepare another positive control of *R. solanacearum* in sterile distilled water and plate out the 10^{-1}, 10^{-3} and 10^{-5} suspensions (one plate each is adequate).

− Typical *R. solanacerum* characteristics. The colonies should be fluidal and irregular in shape with a characteristic red or pinkish red centers and whitish periphery. These are best seen when observed against light. Opaque, dark pink/red colonies are likely to be seen but should be ignored.

− Typical bacterial colonies are streaked onto fresh MKM with TZC.

− Well separated typical wild type *R. solanacearum* are further transferred to MKM by exclusion of TZC for multiplication of inoculum.

Six to ten colonies of the bacteria must be transferred in 10 ml of double distilled sterile water and stored at 20 ±2°C.

Procedure to determine the biovar and phylotype:

Classical analysis for biovar determination:

Determination of the five biovars of *R. solanacearum* is done on the basis of carbon utilization in disaccharides and hexose alcohols (Hayward, 1964, Denny and Hayward, 2001). The disaccharides used are cellobiose, lactose and maltose, while the hexose alcohols are dulcitol, mannitol and sorbitol. Sterilized solutions (10% W/V) of these carbon sources prepared separately in distilled water are added to a basal medium (Appendix) before introduction of pure *R. solanacearum* isolates. The hexose alcohols are relatively heat-stable and can be therefore sterilized by autoclaving them at 121°C for 15 minutes. The disaccharides are heat labile and are therefore sterilized by filtration into pre-sterilized universal bottles or small flasks using 0.22 micron millipore membrane and syringe. However,

alternatively all solutions can be sterilized by boiling them in water bath for 20 minutes for three successive days (IPDN, 2014).

After autoclaving the basal medium cool it to about 65 C and mix the 10 ml prepared sugar or alcohol solutions each with 90 ml basal medium. For the control, add 10 ml sterile water (without sugar/alcohol) to 90 ml basal medium. Dispensed 150 µl of the media into the wells of a microtitration plate and seal the plate with sterile (UV-sterilized) plastic tape. The test is performed by adding 40 µl of bacterial suspension in each well of a column of the microtitration plate previously filled with a different alcohol or sugar to the wells, seal the plate with sterile (UV-sterilized) plastic tape and incubate it at 28 C. Results can be observed in 4-7 days. If an isolate utilizes a sugar or alcohol, the color of the medium changes to yellow, otherwise it remains blue-green. In order to differentiate between phenotypes 2A and 2T in biovar 2 prepare test tubes with the same basal medium (2 ml) without agar containing each 1% of D (+) trehalose, D (-) ribose, and L(-) tryptophan or L (+) tartrate. Phenotype 2T gives a positive result with all these carbon sources. The color of the medium turns from green to yellow except for sodium L(+) tartrate and L(-) tryptophan for which the medium changes to blue.

Table 2. Differentiation of *Ralstonia solanacearum* biovars based on utilization of various carbon sources

Test	Biovars					
	1	2A	2T	3	4	5
Mannitol	-	-	-	+	+	+
Sorbitol	-	-	-	+	+	
Dulcitol	-	-	-	+	+	
Lactose	-	+	+	+	-	+
Maltose	-	+	+	+	-	+
Cellobiose	-	+	+	+	-	+
Trehalose and D-ribose / Tryptophan, sodium tartrate		-	+			

Phylotype determination:

For DNA extraction, the bacteria are streaked on MKM without TZC and the plates incubated at 30 C for two days. One colony is suspended in 100 µl of sterile water free of ribonucleases (NFW), boiled for 10 min and kept at –20 C prior to use.

Phylotype specific multiplex PCR (Pmx-PCR) based on primers that amplify specific reference band that recognizes *R. solanacearum* plus specific amplicons from each phylotype have been developed previously (Fegan & Prior, 2005; Prior and Fegan, 2005). The Pmx-PCR amplifies the 280 bp universal *R. solanacearum* specific reference band with primers 759/760 (Opina et al., 1997) plus phylotype specific PCR products as follows: a 144 bp amplicon from phylotype I strains; a 372 bp amplicon from phylotype II strains; a 91 bp amplicon from phylotype III strains; and a 213 bp amplicon from phylotype IV strains (Table 3).

Phylotype Pmx-PCR is carried out in 15 µl final volume of reaction mixture, containing 1 X of the buffer supplied by the manufacturer (Promega), 2.5 mM MgCl2, 0.2 mM of each dNTP, 0.2 µM of each primer (Table 3), 0.3 U of GoTaq G2 Flexi DNA polymerase (PROMEGA) and 1µl DNA template. Amplifications are performed. in an Applied Biosystem Veriti thermocycler as follows: an initial denaturation step at 94ºC for 2 min, followed by 30 cycles of denaturation at 94ºC for 30 sec, annealing at 59ºC for 30 sec, extension at 72ºC for 23 sec, and a final extension step at 72ºC for 5 min. PCR products (10 µl) are subject to electrophoresis on 2% (w/v) agarose gels and visualized with 0.01 µl /ml GelRedTM 10,000X nucleic acid gel staining (Biotium) and photographed under UV light in The Chemidoc ™ MP Photodocumentation System (BIO-RAD). The size of the amplified fragments is estimated by comparison with a 1 Kb Plus marker ladder.

Table 3. Primers used for multiplex PCR to determine phylotypes of the *Ralstonia solanacearum* species complex.

Primer name	Primer sequence	Expected band size	Remark
Nmult:21:1F	5'-CGTTGATGAGGCGCGCAATTT-3'	144	Phylotype I (Asiaticum)
Nmult:21:2F	5'-AAGTTATGGACGGTGGAAGTC-3'	372	Phylotype II (Americanum)
Nmult:22:InF	5'-ATTGCCAAGACGAGAGAAGTA-3'	213	Phylotype IV (Tropical)
Nmult:23:AF	5'-ATTACGAGAGCAATCGAAAGATT-3'	91	Phylotype III (African)
Nmult:22:RR	5'-TCGCTTGACCCTATAACGAGTA-3'		Amorce reverse unique
759R	5'-GTCGCCGTCAACTCACTTTCC-3'	280	Universal R solanacearum primers
760F	5'-GTCGCCGTCAGCAATGCGGAATCG-3'		

***Field experimental trial**:*

Assessment trials can be carried out in naturally infested or inoculated fields. Screening potatoes in naturally infested field requires a reasonable uniform infested field with moderate levels of infestation (between 30 to 50% wilt incidence in the previous potato crop).

Inoculum:

Naturally infested fields with heterogeneous field infestation must be planted with a susceptible potato variety during the previous cropping season. At harvest, rotten tubers are uniformly spread on the field and buried to homogenize and enhance soil inoculum levels.

Field inoculation:

One month after potato plant emergence, all the individual plants of the trial, controls included, are inoculated by burying a piece of agar culture that contains approximately 3 x 10^9 bacteria at the root level (20 cm deep) using a medium size spade. Special care should be taken not to harm the roots of the young potato

plants. A 9 cm diameter plate with a 48 h- culture of R. solanacearum (local isolate) on MKM without TZC will allow the inoculation of about 8 plants (French et al. 1995). Alternatively, the field can be inoculated before establishing the potato evaluation trial. Three or months before planting, rooted stem cuttings of a susceptible potato cultivar are planted into the fields and inoculated 8 weeks later by spraying or dropping a bacterial suspension (3×10^9 bacteria) at the base of the stem. Approximately 6 weeks after inoculation, when at least 80% of plants are wilted, the potato plants are buried in the soil.

Controls:

Resistant controls:

- Cruza 148 (CIP 720118): Also known as “Ndinamagara” in Africa, recommended as moderately resistant control. The variety can present high rates of latently infected tubers
- CIP 394895.7 (BWH87.230R×C90.205): An advanced clone from CIP’s bacterial wilt resistance potato breeding program. This clone was classified as resistant in trials against strains from Phylotypes I and II, and low frequency of latent infected tubers (≤7%) (Gutarra et al. 2014)

Susceptibles:

- Revolución (CIP 720043) (Naranja x (Katahdin x Mantaro). Available in CIP’s genebank
- Monalisa: (Bierma A 1 287 x Colmo) A variety from Netherlands. Not available in CIP’s Genebank

***Experimental design**:*

The plot size or experimental unit should be determined according to the number of tubers available for most of the clones while the experimental design will depend on the number of clones (treatments) to be tested and uniformity of infested field. A random complete block design (RCBD) can bear up to 30 clones per block when experimental units are in plots of one row. Other experimental designs such as lattice or alpha design should be considered when experimental units consists of plots of more than one row or otherwise when there are more than 30 clones for testing including controls when the experimental unit consist of one row/plot. At least 3 replications and a minimum of 3 to 5 plants /plot are acceptable, however it is advisable to use up to 5 replications to obtain more reliable results. If sufficient amounts of seed tubers are available, the number of plants can be increased to 10 or 20 per experimental unit in plots of two rows of 5 or 10 plants each, accordingly.

Blocks and sub-blocks within a block (replication) will be displaced with the soil water movement that follows rainfall or furrow irrigation. Inoculum movement can be minimized by building drainage ditches between blocks. Replications can be placed according to a field infestation map.

If enough healthy seed available, a plot of a susceptible and resistant cultivar should be randomized in every sub-block within a block (replication) of a lattice or alpha design so that the control plots are spread throughout the field and false resistance, due to infection failure resulting from lack of pathogen populations, will be detected.

Field management:

Agronomic practices are identical to those recommended for locally grown commercial potato crops. Additionally, the following sanitary precautions should be taken to avoid the spread of the pathogen:

- Workers' shoes and tools are washed and disinfected with 1% sodium hypochlorite when leaving the field.

- Taking into account the field slopes, a 2 meter-deep well is built in the lowest corner of the field to collect run-off water. The well is regularly disinfected with 1% sodium hypochlorite.
- Rotten leftover tubers are removed from the field after harvest.
- Harvested tubers that are not taken to the laboratory for evaluation are burned or used exclusively for food consumption.

Disease assessment:

Plant wilt severity:

Plant emergence should be recorded 45 days after planting. The field should be observed regularly starting 45 days after planting in naturally infested fields to check for the appearance of the first symptoms in the susceptible control varieties. The first evaluation will take place at 60, and then at 75 and 90 days after planting. In inoculated fields, the first evaluation will take place as soon as the first symptoms are observed in the susceptible control, that can be from 20 to 30 days after inoculation and then every 15 days until the susceptible control achieved 80% of plants with the greatest score or dead.

For each plant, the wilt severity is evaluated using a three-point scale (Figure 12-14):

1 = healthy plant

2 = below or equal to 50% plant wilted

3 = above 50% plant wilted and eventually dead

Data management:

Average wilt severity score for each tested clone is computed over the total number of plants evaluated in each experimental unit and evaluation dates.

. $$Average\ of\ wilt\ severity\ score = \frac{\Sigma \text{ wilt scores of evaluated plants}}{Total\ number\ of\ evaluated\ plants}$$

Figure 12. Grade 1
Healthy plant

Fig. 13. Grade 2
≤ 50% wilted

Fig. 14. Grade 3
> 50% wilted

Average wilt severity scores for each clone/control can then be computed over replications.

Note: In case of plant death due to a cause other than bacterial wilt, the plant data should be registered in the data sheet and not included in further computation.

V. TUBER EVALUATION

***Symptomatic tubers*:**

At harvest, the number and weight of healthy looking tubers and of tubers exhibiting visible bacterial wilt symptoms (e.g., visible oozing at the eyes or vascular oozing visible upon slicing rotten tubers) is recorded separately by plot. This evaluation may also be performed only on marketable tubers.

$$Percent\ of\ symptomatic\ tubers = \frac{Number(N°)/weight(wt)of\ symptomatic\ tubers}{\binom{N°/wt\ of\ healthy\ looking\ tubers+}{N°/wt\ symptomatic\ tubers}} * 100$$

Average percentage of symptomatic tubers for each clone/control can then be computed over replications

***Latent infected tubers*:**

Only asymptomatic tubers of clones with an average wilt score less than or equal to 1.3 and 30% or less mean percentage of symptomatic tubers are selected for latent infection analysis by *R. solanacearum* (see Table 4 below).

For each clone and across all replications, try to sample as many asymptomatic tubers as observed (10-15 per replication is a good number) of any size above 30 mm (Priou *et al.* 2001). Once in the laboratory, sample tubers are washed, disinfected with 70 % alcohol and checked again. At this stage, tubers exhibiting bacterial wilt symptoms are removed and the number recorded. Individual asymptomatic tubers or composite samples of two or three tubers depending on the total number per replication are then analyzed with the NCM-ELISA test and positive samples confirmed by isolating *R. solanacearum* on MKM with TZC. (See latent infection analysis above).

Table 4. Resistance levels to *R. solanacearum* of clones under field conditions

Resistance level	Average wilt severity score	Percent of visible infected and/or latent infected tubers
Highly resistant	1.0	0
Resistant[1]	1.0	≤15
Moderately resistant	1.01-1.30	≤30
Moderately susceptible	1.31-1.60	Not evaluated
Susceptible	1.61-2.20-	Not evaluated
Highly susceptible	≥2.21	Not evaluated

$$Percent\ of\ latently\ infected\ tubers = \frac{Number\ of\ samples\ positive\ to\ NCM\ ELISA}{Total\ samples\ tested} * 100$$

$$Percent\ of\ total\ infected\ tubers = \%\ symptomatic\ tubers + \frac{healthy\ looking\ tubers\ * \%\ latently\ infected\ tubers}{100}$$

Following table 3 only clones with an average wilt score less than or equal to 1.3 are selected and will be evaluated again during the next season to confirm their resistance. Desirable selections are those with average wilt scores equal or smaller than resistant controls.

Sample destruction:

All materials (plant, soil or water) and/or cultures resulting from isolation and/or supplies used in the analyses involving *Ralstonia solanacearum* should be destroyed by autoclaving at a minimum of 121°C (15 psi) for at least 15 minutes before being disposed. All apparatus, equipment and tools should be sterilized appropriately soon after use to prevent any future contamination.

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APPENDIX

Modified Kelman's medium (MKM) with 2,3,5 triphenyl tetrazolium chloride (TZC) (French et al. 1995).

Basal medium:

Dextrose (Merk)	2.5 g
Bacto Peptone (BD)	10 g
Casamino acids (Difco)	1 g
Agar (SIGMA ALDRICH)	15 g
Water (distilled)	1000 ml

The basal medium can be autoclaved and stored. To each liter of the melted medium, add 5 ml of the TZC solution to give a final concentration of 0.005%.

The TZC stock solution is prepared by dissolving 1 g of TZC in 100 ml of distilled water. The solution must be placed in a light-proof capped bottle and autoclaved for only 8 min or otherwise sterilized by filtration. Store refrigerated.

Pour about 20 ml of MKM with TZC in Petri dishes and invert the Petri dish once the medium is set. Keep 1-2 days before using to permit the surface of the medium get dry (longer storage may result in poor bacterial growth).

SMSA medium

Bacto peptone (Difco)	10.0g
Glycerol	5.0 ml
Casamino acids (Difco)	1.0 g
Bacto aga (Difco)	15 g

All per litre of distilled wáter

Once autoclaved add filter sterilized solutions of the following: 25 mg (about 1250 U) per litre Bacitracin (Sigma B-0125). l00mg (about 600000 U) per litre polymyxin B sulphate (Sigma P-1004), 5 mg per litre chloramphenicol (Sigma C-3175), 0.5 mg (about 825 U) per litre penicillin-G (Sigma P-3032), 5 mg per litre crystal violet and 50 mg per litre of 2,3,5-triphenyl tetrazolium chloride (Sigma).

Basal media for Ralstonia biovar testing (taken from IPDN, 2014)

	100ml	700ml*
Ammonium dihydrogen phosphate (NH4H2PO4):	1.0 g	0.70 g
Potassium chloride (KCl):	0.2 g	0.14 g
Magnesium sulphate (MgSO4. 7H2O):	0.2 g	0.14 g
Peptone:	1.0 g	0.70 g
Bromothymol blue:	0.03 g	0.021 g
Agar:	3.0 g	2.10 g
Water:	1.0 L	700 ml

* It is advised to prepare the volume of medium that can easily be divided into seven equal parts, such as: 700 ml or 350 ml.

Bring the medium to boil with constant stirring. Raise the pH of the medium to 7.0 – 7.1 by drop wise addition of 1.0 N sodium or potassium hydroxide. The medium turns green. Divide the medium into seven containers, each container with 90 ml of medium. Autoclave at 121°C, 15 psi for 20 minutes.

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