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Ralstonia solanacearum: A wide spread and global bacterial plant wilt pathogen

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Abstract
R. *solanacearum* causes serious wilt disease in plants which have a huge impact on yield and quality of many crop plants around the world. The host range of this pathogen is continuously expanding due to intensive agriculture practices at the global level. It is an aerobic non-spore forming, Gram-negative, soil borne, motile, which multiplies in the vascular tissue of plants that are responsible for wilting symptoms on the infected plants. The pathogen overwinters in diseased plant debris, propagative organs such as tubers, rhizomes, suckers, or seeds of some crops. It is presently considered as species-complex consisting of three species, five races, and four phylotypes. It can be detected through the use of vascular flow test and sensitive methods like Enzyme-linked immunosorbent assay (ELISA), PCR, etc. The high incidence of plant mortality and lack of effective control methods make R. solanacearum as one of the world's most destructive plant pathogens. For control and eradication of bacterial wilt, the integrated disease management approach must be adopted to avoid or limit pathogen survival and dissemination.

Keywords: pathogen, bacterial plant, *Ralstonia solanacearum*

1. Introduction
Plant diseases are caused by a diverse group of micro-organisms which include fungi, bacteria, viruses, viroids, and phytoplasma and largest number of plant diseases are caused by fungi (Sajad *et al*., 2017) [36]. Bacterial wilt disease caused by *Ralstonia solanacearum* (Smith 1896, Yabuuchi *et al.*, 1995) [41] is one of the most important constraints in production of vegetables in the tropical and sub-tropical regions (Devendra *et al*., 2017) [9]. *R. solanacearum* is responsible for wilting symptoms on the infected plants and the disease has a worldwide distribution (Holt *et al*., 1994) [20]. The host range is continuously expanding due to intensive agriculture practices at the global level. *Ralstonia solanacearum* (Smith 1896, Yabuuchi *et al.*, 1995) [41, 46] is an aerobic non-spore forming, Gram-negative, soil borne, motile with a polar flagellar tuft, belongs to β-Proteobacteria that is one of the major limiting factors in the production of many crop plants around the world. *R. solanacearum* multiply in vascular elements of plants that are responsible for wilting symptoms on the infected plants (Holt *et al*., 1994) [20]. *R. solanacearum* was earlier named as *Bacillus solanacearum* by Erwin Frank Smith and thereafter named as *Pseudomonas solanacearum* and *Burkholderia solanacearum* before assuming present nomenclature as *R. solanacearum* with similarity in most aspects to *Pseudomonas* group of bacteria, except that it does not produce fluorescent pigment like *Pseudomonas.*

2. Disease cycle and symptoms
The pathogen overwinters in diseased plants or plant debris, in vegetative propagative organs such as potato tubers or ginger rhizomes or banana suckers, or on the seeds of some crops like capsicum and tomato, and also in the rhizosphere of weed hosts e.g. *Solanum dulcamara*, *Solanum carolinense* and *Solanum cinereum* (Hayward, 1991; van Elsas *et al*., 2000) [16, 45]. This result in latent infection as the host is sometimes further cultivated (Denny *et al*., 2001) [7]. It is able to survive in aquatic habitats and contaminated irrigation water and municipal wastewater, used in the processing of diseased plant tissue, have been identified as sources of inoculum (Elphinstone *et al*., 1998; Janse *et al*., 1998) [33, 23]. The pathogen usually enters the plant *via* a natural wound created by excision of flowers, the genesis of lateral roots or unnatural ones like agricultural practices or nematodes and xylem-feeding bugs attack (Kelman and Sequeira, 1965) [25]. The bacteria get access to the wounds partially by flagellar-mediated swimming motility and chemotaxis attraction toward root exudates. Unlike many phytopathogenic bacteria, *R. solanacearum* potentially requires only one entry site to establish a systemic infection that results in bacterial wilt (Denny, 2006) [8].

The bacteria move towards the xylem vessels where they multiply and spread (Salanoubat *et al*., 2002) [37]. After invading a susceptible host, *R. solanacearum* multiplies and moves systemically within the plant before bacterial wilt symptoms occur. Therefore it is suggested that wilting should be considered as the most visible side effect that usually occurs after extensive colonization of the pathogen. When the pathogen gets into the xylems through natural openings or wounds, tyloses may form to block the acropetal migration of bacteria within the plant. In susceptible plants, this sometimes happens slowly and infrequently to prevent pathogen migration, and instead may lead to vascular dysfunction by unspecifically obstructing uncolonized vessels. Wilting occurs at the high level of bacterial population in the xylem and is partially due to vascular dysfunction in which water cannot reach the leaves sufficiently. At this time, extracellular polysaccharide (EPS1) content is about 10 µg/g tissue in the taproot, hypocotyl, and mid-stem; EPS1 concentration is higher later on at more than 100 µg/g tissue in the fully wilted plant (Denny, 2006) [8]. High bacterial densities, byproducts of plant cell wall degradation; tyloses and gums produced by the plant itself are other contributing factors to wilting (Denny, 2006) [8]. Experiments using *R. solanacearum* constitutively expressing the green fluorescent protein (gfp38) have demonstrated the progress of infection and timing of disease symptoms (Kang *et al.,* 2002) [24].

3. Diversity

Ralstonia solanacearum has been classified in numerous genera since it was first described (E.F. Smith, 1896) [42]. Although Thomas J. Burrill was probably the first to isolate the bacterium in 1890, E.F. Smith was the first to publish a scientific description and classify it in the genus *Bacillus* as *B. solanacearum* in 1896 (E.F. Smith, 1896, 1907; Kelman, 1953) [41, 26]. However, afterward it was moved to the genus *Bacterium*, and to the genus, *Pseudomonas* with the name of *P. solanacearum* (Kelman, 1953) [26], temporarily reclassified in the genera *Phytomonas* and *Xanthomonas* and eventually transferred back to the genus *Pseudomonas* in 1948 (Kelman, 1953) [26]. In 1992 it was placed in the genus *Burkholderia* (Yabuuchi *et al.,* 1992) [47]. But, more recent phylogenetic and polyphasic phenotypic analyses pointed out that it would rather be accommodated in the newly established genus of *Ralstonia*, in 1995 (Yabuuchi *et al.,* 1995) [46]. Since then, the bacterium is named *R. solanacearum* and belongs to the family *Ralstoniaceae* included in the β-subdivision of the Proteobacteria (Stackebrandt, 1998) [42]. *R. solanacearum* is currently considered a heterogeneous species or a "species-complex" (Fegan *et al.,* 2005) [12], supporting the concept of "species-group" already proposed in 1964 (Buddenhagen & Kelman, 1964) [3], following the hypothesis that strains of this species "are the product of long evolution occurring independently in various areas on different hosts" (Buddenhagen & Kelman, 1964) [3]. Recently *R. solanacearum* species complex is classified into three species such as *R. pseudosolanacearum, R. solanacearum, R. syzygii* based on significant variations in the whole genome (Prior *et al*., 2016) The species is further divided into 5 different races (race 1-5) based on host range, five biovars (biovar 1-5) based on carbon utilization/oxidation and four phylotypes (phylotype 1-4) based on conserved nucleotide sequences in the intergenic regions of ribosomal DNA (Fegan and Prior 2004). The accepted convention is to employ a two-fold classification system, which is based on the host range of the strains to classify them into races (Buddenhagen *et al*., 1962) [2], and the ability of the strains to oxidize various disaccharides and hexose alcohols to classify them into biovars (Hayward, 1964). RFLPs on the hrp gene region and 16S rRNA sequence analysis have also been used as the basis of a classification system for *R. solanacearum* (Cook *et al*., 1989; Cook *et al*., 1994; Poussier *et al.,* 2000). The phylogenetic analysis revealed that each phylotype broadly originated from the same location. Within each phylotype, there were a number of strains containing highly conserved sequences, which were grouped as sequevars. Some of these sequevars are pathogenic on the same hosts or strains of common geographic origin (Fegan & Prior, 2005) [12]. The robustness of this phylogeny was demonstrated by Guidot *et al*. (2007) who showed, based on *R. solanacearum* microarray genomic data, that the organism phylogenetic relationships of a set of strains chosen as representative of the four phylotypes matched the classification scheme of Fegan & Prior, (2005) [12]. The relationship between races, biovars, and RFLP and phylotype division for the classification of *R. solanacearum* is summarized in Table 1.

The PCR-RFLP approach was adopted by Fouche-Weich *et al*., (2006) in determining the causal agent of bacterial wilt from eucalyptus plantations in the Democratic Republic of Congo (DRC), South Africa, and Uganda and from potato fields in South Africa. The eucalypt isolates were identified as *R. solanacearum* biovar 3 while the potato isolates, except for one, were classified as biovar 2. This study further qualifies the PCR-RFLP approach as a useful tool for classification of *R. solanacearum*.

Table 1: Host range, Geographical distribution, Characteristics of races and their relationship to biovar and phylotype sub-division of *R. solanacearum*

Race	Host Range	Geographical distribution	Biovar	RFLP division	Phylotype	New Proposal for phylotypes
1	Wide	Asia, Australia, America Africa	3,4 1 1	I, II III	I III	*R. pseudosolanacearum* Phylotype I, III
2	Banana and other Musa spp.	Caribbean, Brazil, Philippines Indonesia	1	II	II IV	*R. solanacearum* Phylotype II
3	Potato	Worldwide	2	II	II	*R. syzygii* Phylotype IV
4	Ginger	Asia	3,4	I	I	
5	Mulberry	China	5	I	I	

4. Host range and Epidemiology

It infects over 200 plant species representing more than 53 plant families. Hosts include solanaceous crops such as tobacco (*Nicotiana tabacum*), tomato (*Solanum Lycopersicon*), potato (*S. tuberosum*) and eggplant (*S. melongena)* (Hayward, 1994), leguminous plants such as groundnut and French bean (Genin and Boucher, 2002) [14], and in monocotyledonous plants, such as banana, ginger (*Zingiber officinale*), small cardamom (*Elettaria cardamomum*) and several other ornamental plants in the

family of Zingiberaceae. *R. solanacearum* also causes bacterial wilt disease on several shrub and tree species such as cashew, mulberry, olive (He *et al*., 1983; Shiomi *et al*., 1989) [19, 39] and *Eucalyptus*. *Eucalyptus* was initially reported as a host in Brazil and China but is currently also a host of the pathogen in Australia and Africa i.e. South African and Uganda (Hayward, 1991; Hayward, 1994; Coutinho *et al*., 2000; Roux *et al*., 2001) [16, 35]. The severity of the disease in Africa may be underestimated as a limited number of *Eucalyptus* plantations have been surveyed. There is a discrepancy in the distribution of bacterial wilt on specific hosts i.e. bacterial wilt may pose a problem on a certain host in one geographic location, and be absent from the same host in another location. A typical example is groundnut bacterial wilt that is very serious in China but absent in India. This suggests that a combination of environmental factors conducive to disease incidence is necessary for *R. solanacearum* prevalence on a particular host (Hayward, 1991) [16]. In India, the predominant races responsible for crop loss are race 1 and race 4 with the limited occurrence of race 3.While race 1 affects solanaceous vegetables, the race 4 is known to infect several plants in the family Zingiberaceae. A single virulent lineage of race 4 is recently implicated in bacterial wilt of small cardamom (Kumar *et al*., 2012) [27]. Recently geranium is found to be a new host for *R. solanacearum* (race 3 biovar 2, phylotype IIB, brown-rot ecotype) (Maurício *et al*., 2017).

It has the ability to survive in the soil in the absence of a host for extended periods as well as in the protected niche of a weed's rhizosphere (Hayward, 1991) [16]**.** High soil moisture in well-drained soils is conducive to *R. solanacearum* survival; however, its survival in the soil is temperature dependent. A high day temperature of 40°C maintained for more than four hours has been shown to reduce bacterial populations (van Elsas *et al*., 2000) [44] although an increase in ambient temperature between 30-35°C has been correlated with an increase in disease incidence and rate of onset of bacterial wilt on hosts such as tomato (Hayward, 1991) [16]. Some soil types suppress the pathogen as the soil moisture determines the antagonistic population levels, which compete with *R. solanacearum.* Nematode infestation (*Meloidogyne* species) also contributes to spreading of the disease. This is thought to be primarily a result of the increase in wounding of plants by the nematodes, which promotes bacterial infection, however, the nematode may also injure plant tissue making it suitable for bacterial invasion (Hayward, 1991) [16].

5. Detection and Diagnosis

The disease is quickly diagnosed through observation of rapid wilting of plant leaves and stem during the 'warmest' (Ooshiro *et al.,* 2004) [30] daytime which at the initial stage recovers at night or when it is cold. At an advanced stage, leaves show necrotic areas and the vascular bundles look brownish (Osborn 1995) [31] which extends into the entire system of the plant and then never recovers. Infected plants are distributed in patches in the field, and it's observed even the presence of sufficient moisture. The degree of infection can be detected through the use of vascular flow test and sensitive methods like Enzyme-linked immunosorbent assay **(**ELISA), PCR, etc which are as under following points.

5.1 Vascular flow test

Wilting alone can be mistaken with other pest attacks like termites; the only ways to be sure is by cutting the fresh living infected part transversally like stem and leaf stalk and dip it in a clear or transparent container with clean water. The milky substances ooze out from the specimen (Gildemacher *et al.,* 2007) [15] if the infection is bacterial wilt. This method is popularly called vascular flow test, is fast and convenient in the field or anywhere when fast results are the priority. Its drawback, however, is that it only manages to detect an infection of a certain degree.

5.2 Enzyme-linked immunosorbent assay (ELISA)

Sensitive methods have been developed to detect latent infections of *Ralstonia solanacearum* to prevent trans-boundary movement of the pathogen. Some of these methods are serological techniques like ELISA, as it becoming common in most pathogen detection experiments due to its sensitivity, easiness, quickness, and reliability to produce quantifiable results from a variety of samples (French *et al.,* 1995) [13]. There are two common types of ELISA test namely nitro-cellulose membrane (NCM-ELISA) and double antibody sandwich (DAS-ELISA). The two methods have these four main steps respectively: coating the micro titration plates with soluble antigens from bacterial cells at a pH of 9.6, addition of the sample extract to the plates; if the pathogen is in the extract it will stick to the antigen, the addition of the second conjugated antibody to enzyme which reacts to the bound antigen-antibody complex. The unbound and excess are removed through washing with well-prepared buffer after each step. The fourth step then involves the addition of enzyme substrate of which produces yellow color as an indication of the presence of reaction in third step (French *et al*., 1995; Priou *et al.,* 2006) [13, 34]. The intensity of the color tells the concentration of *Ralstonia* found in the extract which is normally read with a spectrophotometer.

5.3 Polymerase chain reaction

Highly sensitive method and less labor demanding to come up with pathogen-specific DNA (French *et al.,* 1995) [13]. It was developed for the diagnosis of *Ralstonia solanacearum* by various workers, a quantitative, multiplex, real-time, and fluorogenic PCR (TaqMan) has also been developed (Elphinstone *et al*., 1996; Weller *et al*., 2000) [10, 45]. The results from this method can be obtained within 2-5 hours (French *et al*., 1995) [13]. The method uses designed primers which through heating cycles are able to detect even one copy of *Ralstonia* DNA in the sample (Pastrik & Maiss 2000) [32]. In research, the method is used to develop interactions between the pathogen and its hosts. It is more sensitive than ELISA but its drawbacks rely on technical complexity and expensiveness.

6. Management

Total control or eradication of pathogen in specific and *Ralstonia solanacearum* in particular still remains the dream of most researchers. There is no almost 100% method massively practiced by farmers in the world due to imbalances in knowledge, economic stamina, and technology. Eradication of the pathogen is difficult because of the experimental errors, sampling errors, level of efficiency of the eradication method or tool, the natural reintroduction of the pathogen (Janse & Wenneker 2002) [22]. To control and eradicate bacterial wilt, the main components are the use of healthy seed and planting in clean soils. However, many additional factors influence the incidence of the bacterium, such as environmental conditions (temperature and soil moisture), rotation with nonhost plants, the use of less susceptible varieties and cultural practices (crop sanitation

and nematode control). Thus only an integrated control strategy can succeed in reducing bacterial wilt incidence or eradicating it. Moreover, social and economic factors that influence farmers' decision-making should be factored into the management scheme.

6.1 Cultural Practices

Crop sanitation and cultivation measures aim to avoid or limit pathogen survival and dissemination. After harvest of a bacterial wilt-infested crop, the plant must be removed from the field and buried deep, down-slope and far from irrigation canals, alternatively, they can be burned. After harvest, sorted-out and leftover diseased tubers must also be removed from the field and destroyed using the same procedure as for haulms.

Infected seed tubers are the main means of dissemination of *R. solanacearum*. In cool conditions, such as tropical elevations above 2500 m, infected but symptomless plants may harbor the bacterium and transmit it to progeny tubers as latent infection, leading to severe disease outbreaks when grown at warmer locations. In seed certification schemes, no bacterial wilt must be tolerated during the growing season. For seed production, only bacterial wilt-free seed originating from disease-free areas must be used.

Rotation with cereals or gramineous pastures can be implemented to eliminate soil inoculum. The duration of the rotation necessary to eliminate soil inoculum is variable because *R. solanacearum* survivability in soil varies according to environmental conditions (temperature, moisture) and soil characteristics (biotic and abiotic factors).

Ralstonia solanacearum survives in weeds, so weeding must be done before planting and any other crop used in the rotation. Volunteer plants are another means of survival of *R. solanacearum* and must be removed in the subsequent crop soon after their emergence. If the incidence of bacterial wilt is low in a field, wilted plants must be removed as soon as they are observed, avoid contamination of healthy neighboring plants. They must be destroyed carefully as described previously for potato haulms and sorted-out tubers

To prevent movement of soil from an infested to a disease-free field, all tools must be decontaminated by washing with water and calcium hypochlorite (or other available bactericide) or sterilized by flame. Machinery, vehicles, hooves of animals used for traction, and shoes of the personnel coming from an infested field must be washed at least with water before entering another field. The flow of water from an infested field to adjacent fields must be avoided. In infested areas, irrigation by good water is preferred over surface water from rivers or irrigation canals.

6.2 Plant Resistance

Plant resistance is one of the most effective and economical means of controlling bacterial wilt. However, although many potato varieties have been found to have a degree of resistance to bacterial wilt, they still transmit the latent infection to their progeny tubers. The use of moderately resistant varieties must be thus coupled with a seed program that provides bacterial wilt-free seed tubers. Resistance is strain specific and is overcome when the levels of factors that favor bacterial wilt are increased: high temperature, excessive soil moisture, wounding of roots and stolons, etc. An essential step in the development of resistant varieties is local screening.

6.3 Biological control

Biological control measures are becoming common in integrated disease management (IDM). Research has been developed in trying to come up with plants that can be grown with potato to reduce bacterial wilt incidences, coming up with positive beneficial microbes to counter soil pathogens, and incorporation of some parts of the plant to inhibit survival of pathogens. In tomatoes, the bacterial wilt, though in the greenhouse, can be controlled almost 100% by incorporation of about 20% of fresh aerial parts of *Cajanus cajan* (Cardoso *et al*., 2006) [4]. Furthermore, *Cajanus cajan* is easily available since it is grown as cash and soil fertility crop. *Pseudomonas fluorescence*, *Bacillus subtilis* and *B. amyloliquefacience* has been reported as effective biocontrol agent of *R. solanacearum* in tomato and potato (Ciampi-Panno *et al*., 1989; Chen *et al*., 2012; Singh *et al*., 2012) [6, 5, 40].

6.3 Quarantine

Once bacterial wilt is introduced to an area, quarantine regulations have to be applied to avoid spreading of bacterial wilt to non-infested areas. Measures restrict the production of seed potatoes and impede the commercialization of ware potatoes to bacterial wilt-free countries or regions, affecting the economy of the quarantined regions. Avoid transport of ware or seed potatoes from an infected area to a bacterial wilt-free to avoid spreading the disease. While it is often difficult to fully control all potato trading, this is one of the best preventative measures available.

6.4 Chemical control

Chemical control measure is the last option when all other avenues of disease control are exploited and not effective as expected level. Even though there are a few synthetic chemicals which effectively control bacterial wilt. Janse 2002 [22] reported that the use of sodium or calcium chloro-oxide (Na or CaClO), chlorine dioxide (ClO_2), anti-biotic, organic acids like lactic acids and acetic acids had shown some effectiveness in controlling bacterial pathogens.

7. Conclusion

Bacterial wilt disease caused by *Ralstonia* is one of the most important constraints in production of vegetables in the tropical and sub-tropical regions. It is having broad host range in over 50 plant family with more than 400 reported host plants. The high incidence of plant mortality makes *R. solanacearum* as one of the world's most destructive plant pathogens. Hence to manage the disease caused by this pathogen needs to be proper detection and diagnosis and multipronged approach needs to be adopted for its management.

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