*R. solanacearum*, a phytopathogen and its virulence factors infecting eggplant

Gaitonde Sapna S.

Department of Microbiology

Government College of Arts, Science & Commerce

Khandola-Marcela, India

sapna.gaitonde@khandolacollege.edu.in

R. Ramesh

Crop Improvement and Protection (Crop Science)

ICAR - CCARI,

Old Goa, India

 r.ramesh@icar.gov.in

I. INTRODUCTION

*R. solanacearum*,a soil-borne bacterium, known to cause bacterial wilt in many plant species, widely used in horticultural research. This disease is a very difficult disease that destroys fields due to its aggressiveness, geographical distribution and large number of hosts. This pathogen is a very challenging bacterium which has destroyed the fields because due to severe aggressiveness, geographic distribution, and broad host range. *Ralstonia solanacearum* is a soil-borne beta-proteobacterium that causes bacterial wilt in 450 plant species of 54 families across the countries. *R. solanacearum* is a highly heterogeneous species isolated from all regions. All *R. solanacearum* strains have conventionally been classified into races (1-5) based on host range and biovars (six) based on the ability to acidify carbohydrate substrates. Recently, the phenotypic and genotypic variation of the species has been divided into four phylotypes based on the sequences of selected genes linking the species to their geographic origin.

*R. solanacearum* infects plants through roots and spreads rapidly in the xylem vessels and suppresses plant defense mechanisms via the type III secretion system. In the xylem vessels, the bacteria multiply extensively and produce large amounts of exopolysaccharide ensuing collapse of the water flow causing the wilting symptoms and eventually plant death. As previously reported by Araud-Razuo *et al.*, (1998) the pathogen invades plant roots from the soil through wounds or natural openings where secondary roots emerge,inhabits the vascular tissue of its host by colonizing the root cortex and vascular parenchyma by multiplying itself to 109 CFU g-1 of host tissue. In the later stages of infection, it requires highly specialized process of interacting genes and protein products of the pathogen as well as of the plant. This eventually leads to massive amounts of bacterial cells inside the plant vascular tissue. Symptoms include chlorosis, stunting and wilting resulting in the death of the host.

Many factors contribute to this overall infection process. These include Cell-Wall-Degrading Enzymes (CWDEs) viz. polygalacturonase (PG) and endoglucanase (EG), flagella-driven swimming motility and type IV pili-driven twitching motility, extracellular polysaccharide I (EPS I), chemotaxic behavior the type III secretion system (T3SS) (*Hrp* machinery) that allows the secretion and the injection of effector proteins into plant cells and type II secretion sytem (T2SS). Interestingly, all of these virulence factors are controlled by a complex regulatory signal transduction pathway that responds to both environmental signals and quorum sensing. PhcA, a regulatory protein which plays a central role in a complex regulatory cascade is mediated by the specific endogenous signal molecule, 3-hydroxypalmitic acid methyl ester (PAME). Although not much is understood about these virulence factors and their regulation, less is known about how *R. solanacearum* effectively adheres, colonizes and spreads in the host.

In Goa, solanaceous vegetables particularly eggplant are cultivated throughout the year. Eggplant cultivation is severely affected due to bacterial wilt and the disease incidence ranges from 30-100%. *Agassaim* is the popular and high yielding local cultivar of Goa which is highly susceptible to bacterial wilt. Researchers are trying to combat this bacterial wilt pathogen through various physical, cultural, chemical and biological methods. Bacterial wilt could effectively be managed through crop rotation, host resistance and chemical control to a certain extent. In spite of various efforts, it is very difficult to control bacterial wilt using a single strategy as the pathogen is soil borne and has a broad host range in addition to the existence of vast genetic variation. Further, breakdown of host resistance is reported regularly. Therefore studying this pathogen and its virulent mechanisms is very important for making appropriate decision on disease management.

This chapter deals with the virulence of the *R. solanacearum* on eggplant and production of certain important virulence factors differing in aggressiveness on the host.

**II. Host Range**

The host range of the bacterium is unusually broad together with hundreds of plant species (Hayward, 1991). *R. solanacearum* infects 29 natural hosts other than potato and tomato (Pradhanang *et al*., 2000). The major hosts of *R. solanacearum* worldwide are given in Table 1.1.

Table 1.1 List of major hosts of *R. solanacearum*

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| --- | --- |
| **Hosts** | **References** |
| *Lycopersicon esculentum* (tomato),  *Solanum tuberosum* (potato) | Sequeira, 1998, Davis *et al.*, 2000; Lopes *et al.*,2005 |
| *Capsicum annum* (sweet pepper), *Solanum melongena* (eggplant) |
| *Nicotiana tabacum* (tobacco), *Arachis hypogaea* (groundnut) |
| *Pelargonium hortorum (*geranium) | Swanson, 2007 |
| *Arabidopsis thaliana* | Norman *et al.*,2009, Deslandes *et al*., 1998 |
| Bananas and *Heliconia spp* | EPPO, 1999 |
| Sunflower  | Elphinstone, 2005 |
| *Pepper* spp. and *Morus spp* | Aragaki and Quinon, 1965; French *et al.*,1995  |
| *Anacardium occidentale* (cashew) | Shiomi *et al*., 1989 |
| *Annona spp.* (custard apple) | Mayers and Hutton, 1987 |
| *Archontophoenix alexandrae* (Alexandra palm) | Akiew and Hams, 1990 |
| Artichokes  | Aly and El ghafar, 2000 |
| *S. nigrum* (blacknightshade), *Melampodium perfoliatum*, *Datura stramonium*, *Portulaca oleracea* | NPAG, 2001 |
| Strawberry (Japan) | Goto *et al*., 1978 |
| *Cerastium glomeratum*, *Drymaria cordata*, *Polygonum capitatum and Stellaria media* | Pradhanang *et al.*, 2000 |
| *Solanum dulcamara* (bittersweet) | Elphinstone *et al*., 1998 |
| *Brassica* spp., *Chenopodium album* and *Tropaeolum majus* | Janse *et al.*, 2002 |
| *Urtica dioica*  | Wenneker *et al.*, 1999 |
| *Ipomoea batatas* (China) | He *et al*., 1983 |
| *Eucalyptus* (Brazil, China) | Dianese *et al*., 1990 |
| Cassava (Indonesia) | Nishiyama *et al*., 1980 |
| Peanut (China) | Middleton and Hayward, 1990 |

**III. DISEASE CYCLE OF *R. SOLANACEARUM***

*R. solanacearum* normally infects roots, enters the cortex, and then spreads throughout the vascular system. It has a tissue-specific tropism in the host, so it multiplies rapidly in xylem vessels after invading the host. Virulence factors recognized in the pathogenic process are the lyases enzymes (endoglucanases, pectic enzymes) EPS etc. In plants, bacteria grow rapidly in the intercellular space of the endothelial layer; it then crosses the natural barrier of the endothelial layer, enters the vascular colon, proliferates in the vascular parenchyma, and finally invades the proxylem vessels through cell wall destruction. Production of extracellular polysaccharides causes rapid wilting of infected plants due to accumulation of pathogenic bacteria.Colonization of approximately 25% of the xylem vessels in each vascular bundle above the neck region is sufficient to initiate partial wilting and ultimately plant death in tomato.Genetic study shows that several hydrolases are required to facilitate the intercellular growth of bacteria in the endodermis, and during translation into xylem vessels in certain conditions, symptoms include leaf droopiness of the youngest leaves, yellowing of flora, stunted growth of plant, browning of the xylem tissue takes place and the plant collapses within 2-3 days.

In bacterial wilt disease, milky-white exudation of bacterial cells from infected stem tissue is a prominent feature which is absent in fungal wilt diseases. . In case of tomato, sometimes infected plants does not show symptom until fruit ripening stage. Finally, it results in rapid collapse of plant. A longitudinal slice of infected stem and stolon revealed vascular browning with dark brown streaks. And in some instances, grey-white bacterial ooze has been found on stem surfaces. *R. solanacearum* may invade the susceptible host through microscopic wounds caused by the emergence of lateral roots. Almost immediately after the bacteria colonize, it produces extracellular polysaccharides which results in clogging the vascular tissue, leading in death of the plant. Under field conditions, the disease first appears in scattered spots on tomato. Wilting signs are first seen on younger leaves during hot weather. The vascular tissues of the stem show a brown discoloration and when stem is cut displays bacterial streaming that indicate the presence of dense masses of bacterial cells in infected vascular bundles. Browning of vascular system occur in lower parts of the stem. In the tomato plants, the vessels remained occluded by intact tylosis for 48 to 72 h, such structural defense amplified by the partial blocking effect of tyloses after collapse. Abrupt release of large number of bacteria from disrupted tylosis causes rapid and unbeaten colonization in the xylem vessels and also found that the movement of the bacterium was more hurried in the vessels of the stem than in the root. In certain weed hosts viz. *Solanum dulcamara* infected with the pathogen may show display discolouration of vascular tissue but no actual wilting.

**IV. PATHOGENICITY TESTING**

Vudhivanich (1997) used the micropipette technique to inject different concentrations of *R. solanacearum* inoculum directly into a tomato plant by inserting it diagonally into the stem in the axil of the third leaf from above. A seedling immersion technique for studying the pathogenicity of R. solanacearum was proposed by Marina and El-Nashaar (1993). In doing so, tomato plant seedlings were treated with an aqueous bacterial inoculation suspension for 10 seconds and transplanted into the field. The root separation and root watering method was used to inoculate Capsicum *R. solanacearum* plants. Damage was done on the roots of 28-day-old seedlings and 30 ml of bacterial suspension was inoculated into each pot. Inoculation of *R. solanacearum* on *Moringa oleifera* was performed by spraying the bacterial suspension on the pin-pricked leaf axils of healthy plants and immersing the cut root ends of healthy plants in the bacterial suspension. Wilt symptoms were observed in plants to develop after 10 to 20 days of inoculation.

**V. VIRULENCE FACTORS OF *R. SOLANACEARUM***

Over the last three decades several important virulence factors were identified and characterized underlying *R. solanacearum* pathogenicity and virulence.

1. **Exopolysaccharide**

*R. solanacearum* produces many extracellular products predominantly one accompanying extreme microscopic bulk acidic extracellular polysaccharide (EPS I) that contributes to disease symptoms. It is a miscellaneous polymer containing a trimeric repeat unit of N-acetylgalactosamine, 2-N-acetyl-2-deoxy-L-galacturonic acid, and 2- N-acetyl-4-N-(3-hydroxybutanoyl)-2-4-6-trideoxy-D-glucose. EPS I is the most significant virulence factor of *R.solanacearum*, since EPS mutants do not cause wilt symptoms even when introduced directly into stem wounds although they remain slightly pathogenic. EPS I-deficient mutants are known to poorly colonize the stem of infected plants which also reveals that EPS I plays a role to prevent or avoid the recognition of pili and/or lipopolysaccharide by plant defense mechanisms.

1. **Cell wall degrading enzymes (Cellulolytic and Pectinolytic enzymes)**

*R. solanacearum* secretes three polygalacturonases (*PglA*, *PehB* and *PehC*) and an endoglucanase (*egl*), but gene disruption analysis proved that the role played by individual wall degrading enzymes in BW disease is insignificant. Egl mutants emerge to be reduced in their ability to colonize the stems of infected plants but stay pathogenic. Another exoglucanase, a β-1, 4-exocellobiohydrolase, *CbhA*, that releases cellobiose from the non-reducing ends of the chains and it contribute almost as much to disease as *egl*, considerably in the ability of *R. solanacearum* to systemically colonize tomato plants. *R. solanacearum* produces one pectin methylesterase (*Pme*), which helps in removal of methyl groups from pectin enabling the consecutive breakdown of cell wall by the three polygalacturonases (PGs). *R. solanacearum* has two types of PG: anendo-PG, named *PglA* or *PehA*, that cleaves the pectin polymer at random releasing large fragments, and two exo-PG, the exopoly-α-D-galacturonosidase *PehB*, and exopolygalacturonase *PehC*, that release galacturonic acid dimmers and monomers respectively.

1. **Twitching motility (Type IV pili and fimbrial structures)**

Liu *et al*. (2001) mentioned that *R. solanacearum* produces Type IV pili (Tfp) necessary for twitching motility that is composed mainly of a single pilin protein, PilA, assembled to a flexuous polar filament. Tfp is likewise liable for its property of attachment to substrates and natural transformation. *R. solanacearum* type IV pili mutants were reasonably less virulent on host plants. A few factors viz. motility, adherence and/or type IV pili are known to contribute in *R. solanacearum* pathogenesis. Taken together, these results demonstrate the pilus formation promotes the attachment to host cell surfaces, colonises the root surfaces, and migrates to wound sites. Genin and Boucher (2004) reported the biofilm formation in plants by *R. solanacearum* and assumed that it allows bacterial survival at some point during latent infections and saprophytic life.

1. **Swimming motility**

*R. solanacearum* can produce polar flagella (1-4) for swimming motility. This ability is related with cell density and it was confirmed by Clough *et al*. (1997) who established that most number of bacteria exhibited motility in exponential phase as against the stationary phase that was comprised of non-motile bacteria. A soil soak pathogenicity assay on tomato plants with two non-motile mutants designed by disrupting the *fliC* (encoding the subunit of the flagellar filament) and *fliM* (encoding the flagellar motor switch protein) genes, showed a reduced virulence of mutants compared with wild type, however this distinction could not be observed after inoculation of injured petioles, suggesting that swimming ability is the most significant virulence factor required in the course of early degrees of host plant invasion.

1. **Chemotaxis**

Bacterial chemotaxis is the movement to areas with higher concentrations of favorable or lower concentrations of toxic chemicals and is required along with the motility for many pathogens to colonize and host invasion. *R. solanacearum* uses itschemotaxis system to evolve more favourably. Yao and Allen (2006) observed that *R. solanacearum* is more attracted by root exudates from the host plant tomato but it less attracted by rice exudates, hence they accomplished that chemotaxis is an indispensable trait required for virulence in *R. solanacearum*. However, they observed that the non-tactic strains were as virulent as the wild-type strain, when inoculated directly into the stem, indicated that taxis is an important factor in the early stages for successful invasion of host tissues. The wild-type strain out-competed then on tactic mutants by 100 folds when co-inoculated.

1. **The Type II Secretion System**

The main factor in the virulence of many bacterial pathogens of plants and animals is protein secretion. *R. solanacearum* displays a incredible ability for protein secretion since more than 100 proteins can be identified in the cell-free supernatant of wild-type. *R. solanacearum* cultivated in minimal medium. In *R. solanacearum* the plant cell wall degrading enzymes are secreted by the Type II secretion system, (also named as the General Secretory Pathway) a widely preserved Sec-dependent secretion pathway. The importance of the Type II secretion system was demonstrated by the fact the mutants with defects in either system had drastically reduced capacity for plant colonization and multiplication. After its entry in the plant, the bacterium must locate nutrients quickly after entering the plant in order to grow and spread throughout the plant leaves.

1. **The Type III Secretion System**

The hrp (hypersensitive reaction and pathogenicity) gene cluster encodes the type III secretion system (T3SS), which is used by phytopathogenic bacteria to translocate effector proteins into plant cells and suppress plant defensive responses. The HR essentially blocks the spread of pathogen infection in the area next to the nearby contaminated area by hastening the death of cells. The hrp cluster of *R. solanacearum* encodes the parts of the type III protein secretion pathway (TTSP), which is essential for host disease. Due to two factors, namely reduced nutrient availability of nutrients and general plant defensive responses, the population of Hrp mutant strains remains much lower than that of wild type strains in the infected host plants.

1. **Lipopolysaccharide (LPS) and lectins**

According to reports, an interaction between bacterial LPS and plant lectins contributes to the pathogen *R. solanacearum* and host identification. The oligosaccharide core of R. solanacearum LPS is made up of rhamnose, glucose, heptose, and 2-ketodeoxy-octonate, whereas the O-specific antigen is made up of repeating chains of rhamnose, N-acetylglucosamine, and xylose in the ratio 4:1:1. Smooth LPS (negative HR inducers) and rough LPS (positive HR inducers)-containing *R. solanacearum* strains essentially signal the presence or absence of the O-specific antigen. As a result of the discovery of the gene cluster responsible for the manufacture of cell surface components, Kao and Sequeira (1991) showed that LPS and EPS are co-related.

1. **PhcA, a global regulator controlling phenotypic conversion (PC)**

The PhcA regulatory network, which is involved in the activation of several virulence genes involving EPS biosynthesis, Pme and endoglucanase exoproteins, Type IV pili, and the repression of genes encoding polygalacturonases, siderophores, and motility, regulates the production of virulence determinants in *R. solanacearum*. PhcA is inactive during the early stages of virulence in low *R. solanacearum* populations, which inhibits polygalacturonases and both twitching and swimming motility. While PhcA is active at high levels during the late stages of virulence in *R. solanacearum*, which results in the synthesis of EPS and vital cell wall-degrading enzymes (cellulases and pectin methylesterase). A particular autoinducer molecule, 3-hydroxypalmitic acid ester (3-OH PAME), controls this PhcA pathway.

1. **3-OH PAME, an endogenous signal molecule essential to pathogenesis**

S adenosyl methionine is converted to 3-OH PAME by the membrane-associated protein PhcB. Extracellular 3-OH PAME activates a two-part regulatory mechanism encoded by PhcS, a histidine kinase sensor, and PhcR, a response regulator, when it accumulates above threshold concentrations (5 nM) in a region of high cell density, such as the plant vascular system. This two-part mechanism represses PhcA production when it is dormant. Therefore, low levels of 3-OH PAME mean that the two component system is inactive and that PhcA levels are low when bacterial cells are scattered throughout the soil or at low densities. As a result, siderophore, pili, and flagellar motility are induced, but late virulence genes (EPS, cellulases) are not expressed. On the other hand, when *R. solanacearum* cells are in high concentrations 3-OH PAME accumulation takes place which in turn triggers PhcS and PhcR, and accordingly elevates the PhcA levels in all cells. These bacterial cells become highly virulent due to abundant production of EPS I and exoenzymes.

1. **Acyl homoserine lactone: a second Quorum sensing molecule**

Acyl-homoserine lactones are autoinducers that participate in the quorum sensing (QS) system, a well-known bacterial cell-cell communication mechanism that only stimulates the expression of the virulence genes when there are large population levels of bacteria. The generation of a second QS molecule, an acyl-HSL dependent autoinduction system made up of the luxR and luxI homologues solR and solI, respectively, is positively regulated by PhcA in the *R. solanacearum* regulatory network.

1. **l-glutamic acid**

According to Brosnan and Brosnan (2013), it is essential for signal regulation, oxidative stress, immunological response, and energy generation. Wu et al. (2015) demonstrated that glutamate dehydrogenase is necessary for pathogenicity and that deletion of glutamate dehydrogenase reduced EPS production and bacterial virulence in *R. solanacearum*. Plants' xylem arteries move glutamate from surrounding tissues to the site of protein synthesis. It can result in specific modifications in growth, root tip morphology, and root branching, say Price et al. (2012) and Forde (2014). Glutamate metabolism is also required for essential metabolic activities linked to the plant's pathogen defense. It's fascinating to note that infections have figured out ways to benefit from amino acids provided by hosts.

1. **Other factors**

##### The ability of R. solanacearum to alter chemicals produced by host plant cells has been demonstrated in several studies. For instance, plant cell walls' extracellular polygalacturonases can generate galacturonic acid, which can be used to feed bacterial pathogen cells and speed the onset of bacterial wilt. R. solanacearum has also been discovered to degrade plant salicylic acid (SA) in plant hosts that employ SA as a defensive signaling molecule in order to lower host immunity and protect itself. Tryptophan and methionine are only two examples of the numerous organic substrates that R. solanacearum might use to boost its virulence.

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