Virulent factors of the phytopathogen *R. solanacearum* infecting eggplant

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I. INTRODUCTION

*R. solanacearum*,a soil-borne bacterium, known to cause bacterial wilt in numerous plant species, is widely accepted for the study of pathogenicity in plants. This pathogen is a very challenging bacterium which has destroyed the fields because of its extreme 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 and contains isolates from all geographical regions. All *R. solanacearum* strains have traditionally 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 was further classified into four phylotypes based on the sequences of selected genes and this correlates the strains with their geographical 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.

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. DISEASE CYCLE OF *R. SOLANACEARUM***

*R. solanacearum* naturally infects roots, penetrates through the cortexand latter disseminates throughout the vascular system *R. solanacearum* has a strong tissue-specific tropism within the host as a result of which it rapidly multiplies in the xylem vessels soon after invading the host. Virulence factors idenified in the pathogenesis are the lytic enzymes (endoglucanases, pectic enzymes) EPS etc. Inside the plant, the bacterium rapidly develops within intercellular spaces of the inner cortex; then, it crosses the natural barrier of the endodermis and penetrates into the vascular cylinder where it multiplies within vascular parenchyma to finally invade protoxylem vessels via cell wall degradation. Extracellular polysaccharide production causes rapid wilting of infected plants as a result of accumulation of pathogenic bacteria. The colonization of approximately 25% of xylem vessels in each vascular bundle above the collar zone is sufficient to induce partial wilting of tomato and ultimately leads to plant death. The functional analysis of pathogenicity genes indicates that several hydrolytic enzymes might be necessary to promote the intercellular progression of the bacterium within the inner cortex and during translation towards the xylem vessels under favorable conditions, symptoms include leaf flaccidity of the youngest leaves, yellowing of foliage, 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 cases grey-white bacterial oozing has been observed on stem surfaces. *R. solanacearum* may invade the susceptible host through microscopic wounds caused by the emergence of lateral roots. Soon after the bacterial colonization, it produces extracellular polysaccharides which help in clogging the vascular tissue, resulting in death of the plant. Under field conditions, the disease first appears in scattered patches 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 occurs 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 augmented by the partial blocking effect of tyloses after collapse. Sudden release of large number of bacteria from disrupted tylosis causes rapid and successful colonization in the xylem vessels and also found that the movement of the bacterium was more rapid in the vessels of the stem than in the root. In certain weed hosts viz. *Solanum dulcamara* infected with the pathogen might display discolouration of vascular tissue but no actual wilting.

**III. PATHOGENICITY TESTING**

Vudhivanich (1997) has used micropipette technique for injection of various concentrations of *R. solanacearum* inoculums directly into the tomato plant by inserting diagonally into the stem at the third leaf axil from the top. Seedling submergence technique for pathogenicity study of *R. solanacearum* was proposed by Marina and El-Nashaar (1993). In that, seedlings of tomato plant were treated with aqueous inoculums suspension of bacteria for 10 seconds and transplanted in field. The root severing and root drenching method were used to inoculate Capsicum plants with *R. solanacearum.* The injury was made to roots of 28 days old seedling and 30 ml bacterial suspension was inoculated into each pot*.* Inoculation of *R. solanacearum* on *Moringa oleifera* has performed by spraying the bacterial suspension onto pin pricked leaf axil of healthy plants and by dipping the cut ends of roots of the healthy plants in bacterial suspension. It was observed that wilting symptoms in plants has been developed after 10 to 20 days of inoculation.

**IV. 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 a variety of extracellular products particularly one with high molecular mass acidic extracellular polysaccharide (EPS I) that contributes to disease symptoms. It is a heterogeneous 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 important 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 negligible.

Egl mutants appear to be reduced in their ability to colonize the stems of infected plants but remain 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*, substantially 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 successive 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) reported that *R. solanacearum* produces Type IV pili (Tfp) required for twitching motility that is composed mainly of a single pilin protein, PilA, assembled to a flexuous polar filament. Tfp is also responsible for its property of attachment to substrates and natural transformation. *R. solanacearum* type IV pili mutants were comparatively 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, migrates to wound sites.

Genin and Boucher (2004) reported the biofilm formation in plants by *R. solanacearum* and assumed that it helps bacterial survival 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 demonstrated that maximum 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 constructed by disrupting the *fliC* (encoding the subunit of the flagellar filament) and *fliM* (encoding the flagellar motor switch protein) genes, showed a reduction in virulence of mutants compared to the wild-type strain, but this difference cannot be observed after wounded petiole inoculations, suggesting that swimming motility is the most crucial virulent factor that is essential during early stages of host plant invasion.

1. **Chemotaxis**

Bacterial chemotaxis is the movement towards regions that contain higher concentrations of beneficial or lower concentrations of toxic chemicals and is required along with the motility for many pathogenic species to colonize and invade a host. *R. solanacearum* uses itschemotaxis system to move towards more favourable conditions. 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 concluded that chemotaxis is an essential 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**

Protein secretion plays an important role in virulence of many bacterial pathogens of plant and animals. *R. solanacearum* displays a remarkable ability for protein secretion since more than 100 proteins can be identified in the cell-free supernatant of wild-type *R. solanacearum* cultured 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 conserved Sec-dependent secretion pathway The significance of the Type II secretion system was proved as the mutants defective in either system are severely impaired in colonization ability and multiplication *in planta*. After its entry in the plant, the bacterium must rapidly find nutrients to multiply and disseminate in the plant leaves.

1. **The Type III Secretion System**

Phytopathogenic bacteria employ type III secretion system (T3SS) inorder to suppress plant defense responses and this secretion system is encoded by the *hrp* (hypersensitive reaction and pathogenicity) gene cluster that translocates effector proteins into plant cells.

The HR basically prevents the spread of pathogen infection by rapid death of cells thereby blocking it within the region adjacent to the surrounding infected area. *R. solanacearum hrp* cluster encode the components of a type III protein secretion pathway (TTSP), which plays a crucial role host pathogenesis. Population of *Hrp* mutant strains remains very low in the infected host plants than the wild type strains due to two factors i. e. low nutrient availability of nutrients and general plant defense responses.

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

It is reported that the recognition between the pathogen *R. solanacearum* and the host involves an interaction between bacterial LPS and plant lectins. *R. solanacearum* LPS is composed of lipid A, the oligosaccharide core (rhamnose, glucose, heptose, and 2-ketodeoxy-octonate) and the O-specific antigen (a chain of repeating rhamnose, N-acetylglucosamine, and xylose in a ratio of 4:1:1). *R. solanacearum* strains that possess Smooth LPS (negative HR inducers) and rough LPS (positive HR inducers) basically indicated the presence or the absence of the O-specific antigen. Kao and Sequeira (1991) have reported that LPS and EPS are co-related as the gene cluster was identified for the biosynthesis of cell surface components.

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

The production of virulence determinants in *R. solanacearum* are controlled by a regulatory network named PhcA, which plays a role in activation of multiple virulence genes involving EPS biosynthesis, Pme and endoglucanase exoproteins, Type IV pili, and repression of genes involving production of polygalacturonases, siderophores, and motility.

During early virulence stage, PhcA remains inactive at low *R. solanacearum* population resulting in inactivtation of polygalacturonases and both twitching and swimming motility. Whereas, during late virulence stage PhcA is actived at high *R. solanacearum* population that leads to production of EPS and essential cell wall degrading enzymes (cellulases and pectin methylesterase). This mechanism of PhcA is regulated by presence of a specific autoinducer molecule 3-hydroxypalmitic acid ester (3-OH PAME).

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

3-OH PAME is synthesized by PhcB, a membrane-associated protein, from S adenosyl methionine. At high cell density in a restricted space, such as the plant vascular system, when extracellular 3-OH PAME accumulates above threshold concentrations (5 nM), it activates a two component regulatory system encoded by PhcS, a histidine kinase sensor, and PhcR, a response regulator. When inactive, this two-component system represses the production of PhcA. Therefore, when bacterial cells are in low density or are dispersed in the soil, levels of 3-OH PAME are low, consequently the two component system is inactive and PhcA levels are low. This results in the lack of expression of late virulence genes (EPS, cellulases) and the induction of expression of siderophore, pili and flagellar movement. 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 taking part in the quorum sensing (QS) system, a well-known mechanism of bacterial cell-cell communication that activates the expression of the virulence genes only when bacteria are in high population levels. In the *R. solanacearum* regulatory network, PhcA positively controls the production of a second QS molecule, an Acyl homoserinelactone (acyl-HSL) dependent autoinduction system consisting of *luxR* and *luxI* homologues, designated *solR* and *solI* respectively.

1. **l-glutamic acid**

According to Brosnan and Brosnan (2013), it is crucial for nutrition metabolism, energy production, immunological response, oxidative stress, and signal modulation. Notably, Wu et al. (2015) showed that glutamate dehydrogenase is essential for pathogenicity and that deletion of glutamate dehydrogenase in *R. solanacearum* lowered EPS generation and bacterial virulence. Xylem arteries in plants transport glutamate from the surrounding tissues to the protein synthesis center. According to Price et al. (2012) and Forde (2014), it can lead to particular alterations in growth, root tip morphology, and root branching. Crucial metabolic processes connected to the plant's defense against pathogens depend on glutamate metabolism as well. It's interesting to note that infections have developed methods for using amino acids from hosts to their own advantage.

1. **Other factors**

Numerous investigations have shown that *R. solanacearum* is capable of modifying substances produced by host plant cells. For instance, extracellular polygalacturonases from plant cell walls can produce galacturonic acid, which can be utilized to feed bacterial pathogen cells and hasten the development of bacterial wilt. In plant hosts that use plant salicylic acid (SA) as a defense signaling molecule, *R. solanacearum* has also been found to degrade SA to reduce host immunity and protect itself. *R. solanacearum* can also utilise a variety of different organic substrates, like tryptophan and methionine, to increase its virulence.

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