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Ralstonia solanacearum Virulence in Two different Susceptible Hosts by the Leaf Clip Inoculation Method

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Abstract

Ralstonia solanacearum causes the lethal bacterial wilt disease in many plant species across different continents. The systemic infection in a host and broad host range are the unique features of this Gram-negative phytopathogenic bacterium. Under laboratory condition, its virulence is usually studied in grown up plants by inoculation in roots using the soil drenching method. Though virulence functions of the pathogen have been reported well in literature, comparative study of its pathogenicity between different susceptible hosts is not common. One of the limitations might be the complex virulence assay. Recently we established an easy and simple method to study its virulence in early stages of tomato and eggplants seedlings by directly inoculating the pathogen *via* clipping a part of the cotyledon leaves using a pair of scissors dipped in bacterial suspension. The pathogen could move downward along the shoot from the leaves to the roots in an infected seedling. Importantly, the leaf clip inoculation method assisted in differentiating *R. solanacearum* virulence functions amidst tomato and eggplant seedlings. This mode of inoculation recruiting young seedlings for virulence assays is instrumental in determining susceptible host range, host specific virulence functions as well as to track pathogenic location corresponding to disease severity.

Keywords: *Ralstonia solanacearum*; Bacterial wilt; Virulence; Cotyledon stage seedlings; Leaf clip inoculation; Tomato; Eggplant

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Introduction

Ralstonia solanacearum is a Gram-negative phytopathogenic bacterium. It causes a lethal wilt disease in more than 450 plant species belonging to 54 botanical families [1,2]. Its broad host range, wide geographical distribution, high genetic diversity amongst sequenced strains, prolong survivability in soil and water, systemic nature of infection and lethality apparent in the plant hosts have encouraged scientists around the world to work on this pathogen [3]. The bacterium lives in soil as a saprophyte until it invades a susceptible host through root. First it colonizes in the xylem of the root and then, systemically spreads to the aerial parts of the host. It is believed that the pathogen colonization and the production of copious amount of exopolysaccharide inside the xylem affect transpiration, which results in wilting and subsequent death of the host. Mutant characterization, genomics and transcriptomics research have revealed that the pathogen uses an elaborate regulatory network to control the expression of its virulence functions inside the host plant [4-7]. Different master regulators such as PhcA, HrpG, HrpB, PehR and various two component regulatory systems such as PhcS/R, PehS/R, VsrA/D and SolR/I have been well characterized in this pathogen [4]. Its virulence functions include the adhesins, type III (Hrp) and type II protein secretion systems and different effectors, cell wall degrading enzymes and toxins secreted by these systems, exopolysaccharides, motility mediated by type IV pili and flagella,

biofilm formation, quorum sensing molecules etc [8-16]. Despite a plethora of knowledge about the pathogenicity determining genes and their regulation in this bacterium, comparative study of its pathogenicity across various susceptible hosts is lacking. It is interesting to note that while wilting in grown up tomato plants is known to occur first in the younger leaves at the top and then gradually occurs in the older leaves towards the bottom, wilting in eggplants is observed to occur first in the older leaves and then gradually occurs in the younger leaves towards the apical meristem (**Figure 1**). There are several plants known as distant hosts where this bacterium is known to colonize without any disease symptoms [17,18]. How does the bacterium adapt to different host environments is yet to be explored. Other questions such as host range of this bacterium and host specific role of virulence functions are needed to be explored in near future.

Figure 1: Wilted eggplant in field. This is a picture of wilted eggplant (60 days old) from inside the Tezpur University campus. It is obvious that the wilting of the older leaves have occurred. The younger leaves are still healthy looking. This is the third wilted eggplant from the field. The other two plants also had similar wilting pattern.

R. solanacearum pathogenicity is studied by the soil drenching method to inoculate them in the root, as well as by direct stem inoculation method, etc., in grown up tomato plants [19]. As the infection is systemic, and the bacterium needs to colonize different host tissues prior to initiation of the disease, the disease penetrance in susceptible host is not always absolute. Several epigenetic factors such as rhizosphere microbes, presence of other microbes inside the host xylem, variation in the host arising during the development etc., are expected to contribute towards disease phenotype variation within a susceptible variety. Therefore, interspecies specific comparison of the host plants with regard to disease phenotypes due to *R. solanacearum* further becomes a more complex phenomenon. To reduce complexity in *R. solanacearum* virulence study, it is essential to study its pathogenicity in gnotobiotic infection condition and also in early growth stage of host plants, where minimal variation among the host plants due to epigenetic factors is anticipated. Recently, we developed a leaf clip inoculation method to study *R. solanacearum* virulence in seedlings of tomato and eggplant [20,21]. The cotyledon stage seedlings are only 6 to 7 days old since the seeds are kept for germination. The seedlings are maintained in a sterile microfuge having only sterile water. This method of inoculation minimizes the above complexities of virulence study to some extent [22]. Therefore, the leaf clip infection method has provided an avenue to compare its virulence between the two different hosts such as tomato and eggplant. In this review we have tried to summarize the efficiency of leaf clip inoculation in studying the virulence of *R. solanacearum* and the differential contribution of certain common virulence functions in the two different hosts. Several laboratories have reported the use of seedlings to study *R. solanacearum* virulence. Recently, seedlings of resistant and sensitive varieties of tomato have been used to study bacterial localization [23]. Seedlings are also recruited as model systems to investigate the biocontrol effect of endophytes against *R. solanacearum* infection [24,25] [Unpublished result from the lab].

Ralstonia solanacearum Virulence in Seedlings of Tomato and Eggplant by Leaf Clip Inoculation

Xanthomonas oryzae pv oryzae (Xoo), the causal agent of bacterial leaf blight in rice, is a vascular pathogen. Leaf clip inoculation is a well-known method to study *Xoo* virulence in rice [25]. Like *Xoo*, *R. solanacearum* is a xylem pathogen, though its natural mode of infection is through root. *R. solanacearum* is also known to cause disease upon stem inoculation under laboratory condition. We were interested to develop an inoculation method for *R. solanacearum* that can be used to its virulence in very early stages of host plant. Inspired by *R. solanacearum* resemblance to *Xoo* at least in this context, and our motivation to study its virulence in early stages of host, we initiated the leaf clip inoculation experiment with *R. solanacearum* in 6-7 days cotyledon stage seedlings of tomato by clipping a part of the cotyledon leaves using a pair of scissors dipped in bacterial suspension. Interestingly, the inoculated seedlings developed the disease symptom within three days of inoculation. Within seven days of inoculation, more than 90% of inoculated seedlings died. The frequency of disease occurrence was consistent and stable using this method. A schematic flowchart of the steps followed in the leaf clip inoculation method is represented in **Figure 2**. The entire infection study starting from seed germination, to inoculation and disease scoring can be completed within maximum three weeks period. It was thus evident that disease can occur in the host plant seedlings in gnotobiotic infection condition since the seeds were germinated under sterile condition and also the seedlings were maintained in sterile water in a microfuge. Different mutants such as hrpB, hrpG, phcA, and gspD were found out to be virulence

deficient in tomato seedlings by the leaf clip inoculation. It eventually emphasized that virulence functions can be studied using leaf clip inoculation in seedlings [20]. Additionally, using the same method, the demonstration that *R. solanacearum* could move from leaf downward to the root within a host was also possible [26]. As the seedlings are maintained in transparent microfuge tubes, it helps to observe the pathogenicity process very closely. Leaf clipping in seedlings is an easy, simple, and economical method to study *R. solanacearum* infection. After the successful study in tomato seedlings, we further studied the successful implementation of the same inoculation method in 6-7 days old eggplant seedlings. It was very distinctly observed that susceptibility of eggplant seedlings is higher than that of tomato seedlings. Like tomato seedlings, virulence functions of *R. solanacearum* could be studied in eggplant seedlings too [21]. Further, we have demonstrated the success of the method in chili seedlings [Unpublished result from the lab]. So the method could aid in studying the virulence of the pathogen in different host seedlings.

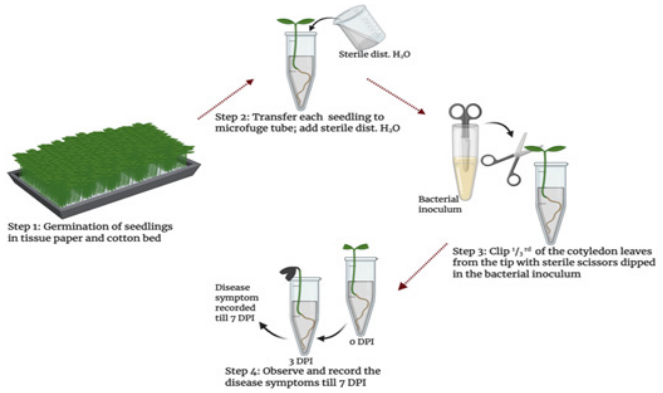

Figure 2: A schematic representation of the leaf clip inoculation method in cotyledon stage seedlings. The seedlings are germinated in sterile tissue paper and cotton bed. Then, the seedlings at the cotyledon stage (around 7 days old since kept for germination) are transferred to 1.5 to 2.0 ml microfuge tube followed by addition of sterile distilled water. A pair of scissors dipped in bacterial suspension is used to clip one-third part of both the cotyledon leaves from the tip to inoculate the pathogen. Wilting symptoms are initially observed in the cotyledon leaves which gradually move downwards from third day onwards. By the end of the seventh days, 80 to 90% wilting is observed in case of tomato seedling while in case of eggplant seedlings, almost 100% wilting is observed.

Virulence Functions of *Ralstonia solanacearum* in Tomato and Eggplant Seedlings

R. solanacearum mutants deficient in several virulence functions such as hrpB, hrpG, gspD, phcA, exopolysaccharide, have been found out to be virulence deficient by leaf clip inoculation in tomato seedlings [20]. Further these virulence deficient mutants have further been recruited in eggplant seedlings to study their virulence. A surprising observation was with regard to the phcA mutant, which was found to be only moderately virulence deficient, in contrast to its severe virulence deficiency in tomato seedlings [21]. It is pertinent to not that phcA regulon is known regulate ~ 1500 genes in *R. solanacearum* [27]. The leaf clip inoculation method can also be recruited to elucidate novel virulence functions in the pathogen by doing large scale screening of mutant population of the pathogen. We have screened ~130 Tn5 induced mutants in tomato seedlings using the leaf clip method and isolated several virulence deficient mutants [26]. Interestingly, the virulence deficient mutants exhibited various magnitude of virulence in eggplant seedlings. One of the mutants is exopolysaccharide (EPS) deficient having insertion in the epsI gene. While this mutant resulted in only 10 to 20% wilting in tomato seedlings, the same strain caused around 50 to 60% wilting in eggplant seedlings [Unpublished result from the lab] (**Table 1**). This observation is in concordance with phcA mutant, because PhcA mutant is deficient for EPS. The observation of moderate virulence deficiency in case of epsI and phcA mutants in eggplant seedlings impels for revisiting the role of *R. solanacearum* EPS function in the young hosts during virulence process. EPS is thought of as a major virulence determinant, which helps in blocking water transport in the xylem. Further EPS has been described as metal chelator in some bacteria [28]. Therefore, the role of EPS in *R. solanacearum* virulence needs further investigation. The comparison of virulence between the two hosts has revealed that a general function affecting virulence significantly in one host may not be that significant in another closely related susceptible host. There may be a significant role of host chemistry attributing to its pathogenicity.

Strains	Virulence in Tomato	Virulence in Eggplant	Reference
F1C1 (Wild Type)	80%-90%	100%	[20, 21]
HrpG mutant	1%-5%	1%-5%	[21]
HrpB mutant	1%-5%	1%-5%	[21]
PhcA mutant	10%-20%	50%-60%	[21]
EpsI mutant	10%-20%	50%-60%	Lab unpublished data
VsrA mutant	10%-15%	10%-15%	Lab unpublished data

Table 1: Virulence of *R. solanacearum* strains in tomato and eggplant seedlings.

R. Solanacearum Localization and Seedling Wilting

Wilting symptoms of seeding starts after two days of inoculation either by the leaf clip inoculation of by the root inoculation [20,29]. Gus staining of the inoculated seedlings at regular intervals after inoculation revealed that the pathogen moves to shoot apical meristem within two days of inoculations, from where the wilting initiates [26]. This indicates that wilting is a result of the direct colonization of the pathogen in the shoot apical meristem and cotyledon leaves and may not be an indirect consequence of pathogen colonization in the shoot xylem and

inhibition of the transpiration. This assumption further support role of EPS in virulence as more direct than indirect i.e. blocking of water transport in the xylem. We also observed very low gus staining in the root of the wilted seedlings in comparison to the staining of the cotyledon leaves as well as shoot apical meristem. *R. solanacearum* mutants deficient to localize efficiently in the shoot apical meristem by root inoculation are found to be virulence deficient. But these mutants can cause wilting by leaf clip inoculation and could localize the shoot apical region in tomato seedlings. This localization study explains why leaf clip inoculation is more efficient in causing disease in seedling in comparison to root inoculation both in tomato and eggplant seedlings.

Conclusion

The leaf clip inoculation mode has indeed helped us to draw out some of the important inferences with respect to the virulence mechanisms of this pathogen such as (i) appearance of conspicuous disease phenotypes under gnotobiotic condition; (ii) downwards migration of the pathogen from leaf to root; (iii) the apical meristem is the favorable niche of the pathogen; (iv) eggplant seedlings are more susceptible than tomato seedlings by leaf clip inoculation; (v) mutants in some common virulence functions exhibit differential virulence in tomato and eggplant seedlings. Recruitment of seedling stages for pathogenicity studies in respective pathogen-host pairs are anticipated to unravel yet unknown facets of plant-pathogen interaction mechanisms as well as responses. There are several questions that have come up from seedlings experiments. Why does the pathogen moves to the shoot apical region? What is multiplication rate of the pathogen with respect to different tissues?

Pathogenicity study, detection efforts in seedling stages of plant species corresponding to particular pathogen types that are generally known to affect respective plant hosts in their adult/grown-up stages have been scarce. Novel insights into pathogen-host seedling interactions involving varying degrees of tolerance/susceptibilities might prove instrumental in long term crop breeding practices to infuse useful resistance mechanisms in important crop plants.

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Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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