Introduction and Review of Literature

1.1 Introduction:

Ralstonia solanacearum [1] is a Gram-negative, soil-borne bacterium which causes one of the most devastating plant diseases known as “bacterial wilt”. Common symptoms of this disease include wilting of foliage, browning of vascular tissue, stunting and yellowing of the plant leading to their death. The disease is known to occur predominantly in the tropical and subtropical regions and in some temperate regions of the world [2] indicating wide geographic distribution of the pathogen. In tropical and sub-tropical regions, bacterial wilt has been reported as the second most devastating disease in potato [3].

R. solanacearum belongs to β-proteobacteria of Burkholderiales order and Ralstoniaceae family and was previously known as *Pseudomonas solanacearum* [4]. The infection caused by this soil and water borne bacterium is not localized; it disseminates from one host to another in soil. It affects more than 200 plant species distributed in 53 families covering many economically important crop plants such as tomato, potato, eggplant, olive, banana, peanut, ginger, etc. [5,6]. Moreover, it can also infects cashew [7], custard apple [8], sunflower [2], eucalyptus [9] etc. Interestingly, host range is continuously increasing covering both herbs as well as trees indicating the significance of the disease and hence the pathogen, understanding of which is still not very clear.

This pathogen exhibits vast genetic diversity in strain level reported from different geographical regions despite causing the common bacterial wilt symptoms in respective hosts and hence is also termed as *Ralstonia solanacearum* species complex (RSSC) [10]. Different approaches have been made to classify and analyze genetic diversity of RSSC. Traditionally, strains of RSSC have been classified into five races and six biovars based on differences in host range and differences in sugar utilization property respectively. Races are loosely defined and taxonomically not that useful. Further in 2005, four monophyletic groups called phylotypes was proposed based on 16S-23S ITS region sequence which has each been connected to initial geographical origin: phylotype I to Asia, phylotypes II to Americas, phylotype III to Africa, and phylotype IV to Indonesia. These phylotypes can also be subdivided into sequevars depending on the endoglucanase (*egl*) gene sequence variation [10]. Based on

multilocus sequence typing (MLST) of 58 strains covering all four phylotypes, in 2007 phylotype II was further subdivided into two subgroups namely IIa and IIb respectively. Recently, in another classification system, division of RSSC into three species, keeping phylotypes I and III together as *R. pseudosolanacearum*, phylotype II as *R. solanacearum* and phylotype IV as *R. syzygii* subspecies indonesiensis has been reported [11].

R. solanacearum exhibits “natural competency” which facilitates the uptake of exogenous DNA via natural transformation [12]. This natural transformation, a form of horizontal gene transfer (HGT); is a major driving force of *R. solanacearum* evolution and a probable reason of emergence of novel pathogenic strains with varying pathogenicity [13]. Phylotype I strains of RSSC is the most recombinogenic due to this nature [14].

R. solanacearum dwells in different ecological niches; lives a saprophytic life in soil without plant hosts and in vascular systems of the hosts as a destructive pathogen [15,16]. As a saprophyte, it can survive for years in moist soil or water and also in decaying plant debris, rhizosphere of non-host plants as well as in asymptomatic weeds. In water microcosms, some strains of this bacterium adapt themselves by maintaining in a nongrowing but culturable or in a viable but non culturable (VBNC) state. They increase their number to favour dispersion, reduce their sizes changing from bacilli to cocci for better absorption of scarce nutrients and aggregate for protection [15]. However, when the pathogen encounters a susceptible host, it switches from saprophytic to parasitic mood. In a suitable condition, the pathogen first enters the root through wounds or natural openings and colonizes the intercellular spaces of the inner cortex. Further, it crosses the endodermis, penetrates into the vascular parenchyma to finally invade protoxylem vessels [17] where it suppresses plant defense mechanisms via the type III secretion system. Subsequently, it spreads up into the whole plant, where the pathogen cell density reaches upto 10^9 CFU/g of host tissue. In the xylem vessels, they multiply extensively and produce large amounts of exopolysaccharide that leads to the collapse of water flow resulting in the wilting symptoms and eventual plant death [6]. Several hydrolytic enzymes are predicted to promote this intercellular progression as well as colonization of the bacterium within the xylem vessels. After the plant death, large amount of bacterial

cells are shed from roots, return to the soil and wait for new hosts. Milky white bacterial oozing has been observed in infected stem which is the characteristic feature of bacterial wilt disease. The pathogen is very peculiar as it causes disease symptoms only after the systemic spreading throughout the hosts unlike many narrow host range pathogens.

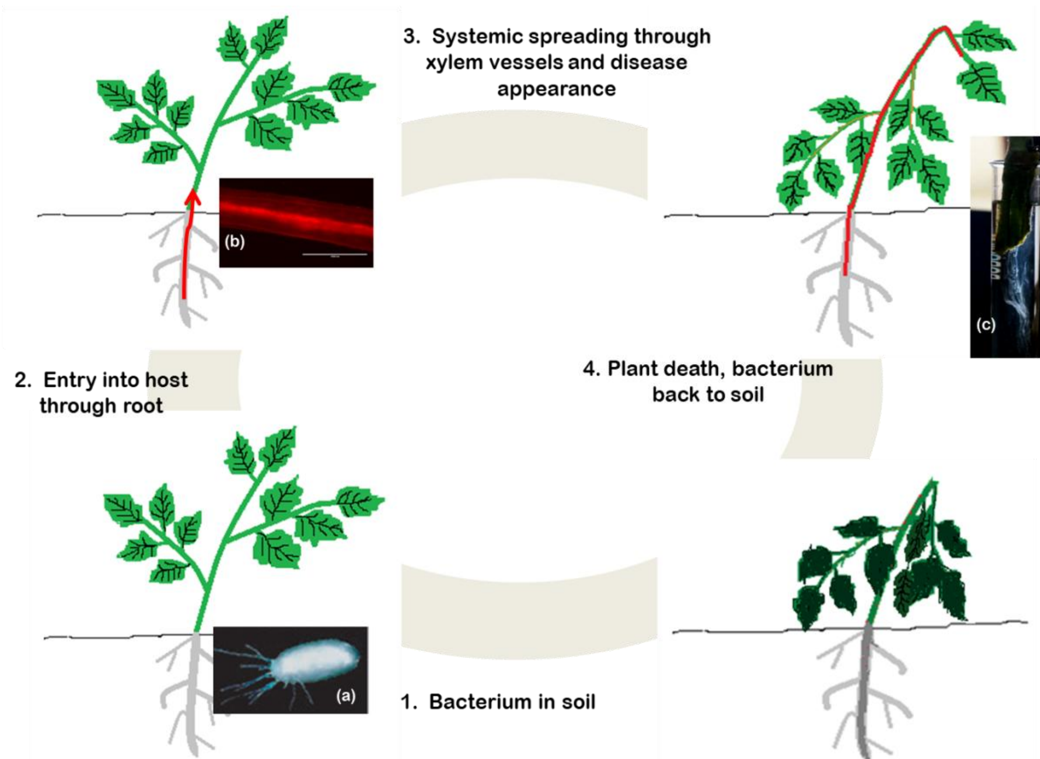

Fig.1.1: Schematic representation of *Ralstonia solanacearum* infection cycle. The bacterium dwells in soil, enters into the plant through root, colonizes xylem vessels, spreads in the entire plant and cause wilting. After the death of the plant, bacterium comes back into the soil (a) Bacterium (GMI1000) under Transmission Electron Microscopy (b) mCherry tagged bacteria (F1C1) observed in root of infected tomato (c) bacterial ooze coming out from the cut end of infected stem [Picture is redrawn after Genin (2010)].

The pathogen expresses different genes or virulence factors for its adaptation to conditions encountered during stay in soil and subsequent host entry, colonization and exit back to the soil. These gene products or factors favour pathogen to use limited nutrients, escape plant recognition, fight defence responses, and manipulate the host to alter its environment better suited for the bacterium. Till date many such virulence factors has been unraveled by functional analysis studies although many potential virulence factors are yet to be characterized. The bacterium produces several extracellular hydrolytic enzymes including pectinases, polygalacturonases and

endoglucanases which facilitate its initial attachment and colonization into the host cells. During pathogenesis, it produces large amount of exopolysaccharides (EPS), one of the most important virulence factors of *R. solanacearum,* to block the vascular system and promote rapid systemic colonization. EPS I-deficient mutants of *R. solanacearum* are nearly avirulent and exhibits less stem colonization compared to wild type. Moreover, it has been shown that it uses EPS I to prevent recognition of pili and or lipopolysaccharide by plant defense mechanisms [18]. *R. solanacearum* exhibits amazing ability to secrete more than 100 proteins into the extracellular milieu [19]. Genome sequencing reveals the presence of all types of protein secretion systems in this pathogen [20]. Out of all six systems, type two secretion systems (T2SS) and type three secretion systems (T3SS) have been shown to play vital role in pathogenic interaction with the hosts [19]. Through T2SS; a form of general secretion pathway, it secretes six major exoproteins including cell wall degrading enzymes and is important for attachment/ colonization inside host cells. In GMI1000, twelve genes (*gspC-N*) were predicted for this pathway [21,22]. *R. solanacearum* employs T3SS encoded by *hrp* (hypersensitive response and pathogenicity) gene cluster to secret large repertoire of effectors into the host cells to modulate the physiological functions. Like other Gram- negative plant pathogens, the *R. solanacearum* mutants defective in T3SS are nonpathogenic in host plants and unable to elicit defensive hypersensitive response in resistant hosts [22] suggesting the collective importance of all effectors, however functional role of each effector protein is still not completely characterized. *R. solanacearum* possesses type IV pili (Tfp) which promotes the attachment to the host surfaces as well as natural transformation [23]. Apart from these, involvement of quorum sensing molecules [24,25], extracellular DNAses [26] as well as biofilm formations [27] essential for virulence functions in this bacterium have been uncovered. Moreover, Tfp dependent twitching motility [28], flagellar dependent swimming motility [29] and chemotaxis [30] have proved to be important virulence functions. Swimming motility plays role during the early stages of host cell invasion while the type IV pili and twitching motility are important for different stages of disease development.

R. solanacearum possesses tightly regulated sensory and regulatory gene networks which facilitates its dynamic life cycle in soil and in host environment. A

LysR family transcription regulator PhcA, which is involved in the Phc cell density sensing system (PhcA-QS, is the master regulator of this network. PhcA positively regulates the expression of endoglucanase gene (*egl)* and the EPS regulator gene (*xpsR*) [31]. Moreover, PhcA indirectly regulates different traits via other regulatory cascades such as *pehSR*, *vsrAD*, *vsrBC*, *flhDC*, and *solIR* [32]. However, these in vitro evidences are not sufficient or completely reliable to understand *R. solanacearum* regulation during pathogenesis in the host microenvironment. For instance, two important virulence factors namely swimming motility and T3SS regulated by PhcA expresses differently in culture and *in planta* condition [16]. In culture, number of motile cells increases after attaining 10^7 CFU/ml bacterial concentration [33], while inside the plant, bacteria become non-motile even at 10^9 CFU/ml population [34]. Similarly, in case of T3SS, in culture PhcA represses its expression at high cell densities [35] while in another studies T3SS expression was found to be high during infection [36]. Very recently, researchers have showed exciting behavior of *R. solanacearum* in flowing xylem sap of host plant with emphasis on its metabolism and pathogenesis. Xylem flow affects many pathogenicity traits of this pathogen; most importantly the quorum sensing system that mediates the major switch from early fast growing colonization phase to late slow growing infection phase where it consumes limited nutrient source but produces large virulence factors [37].

In 2002, first whole genome sequencing of *R. solanacearum* strain GMI1000 has been completed which was isolated from a wilted tomato plant in Guyana [20]. This phylotype I strain has been found to have a bipartite genome structure (5.8 MB) comprising of 3.7 MB chromosome and 2.1 MB megaplasmid that have coevolved. It is a GC rich genome with a G+C content of 67% and encoding for approximately 5120 proteins [22]. Both the chromosome and megaplasmid carry essential and pathogenicity related genes [32]. Till now, 16 strains of RSSC have been sequenced, isolated from different hosts and geographic origin [38]. From India, two strains Rs-09-161 and Rs-10-244 isolated from eggplant and chilli respectively were sequenced very recently [38]. Comparative genomic analysis of all the sequenced genomes covering main phylogenetic groups will accelerate the studies of evolution, pathogenomics as well as adaptation of the pathogen.

It is pertinent to note that, *R. solanacearum* genome carry genes encoding polyphosphate metabolism, an enigmatic molecule whose diverse vital roles has been reported in many pathogenic organisms including pathogenesis.

In relation to its pathogenicity, *R. solanacearum* exhibits several interesting features such as systemic infection in host plant [6], the lethal nature of the disease, a wide host range [5], wide geographical distribution [2], latent infections in distant hosts [6,39], occurrence of all the six types of the protein secretion systems [19,20], the largest repertoire of type III effectors among the known plant pathogenic bacteria [40], a high number of hemagglutinin homologues [20], a complex regulatory network to control its virulence and pathogenicity functions [21,25,32], and long term surviving ability in soil and water [6,15]. Because of its lethality owing to these features, *R. solanacearum* is now one of the most extensively studied phytopathogenic bacteria and is a suitable model for investigating mechanisms of pathogenesis. Notably, it ranks second in the list of most devastating phytopathogenic bacteria [41].

In late 1880's, bacterial wilt disease prevalence was reported from Asia and South America. In 1886, Smith first described the causal agent of this disease as *Bacillus solanacearum*. Since then, several scientists from different parts of the world have been working on this pathogen to exploit the mechanisms of gene expression, to identify molecular determinants implicated in wide host range and evolution as well as the adaptation of *R. solanacearum* to its hosts and pathogenesis. Though, a great amount of knowledge has been accumulated by scientists throughout many years of research on this bacterium, there are still several unanswered questions that needs to be addressed about its pathogenicity. Some of the examples are (i) a comparative pathogenicity of *R. solanacearum* in different susceptible hosts; (ii) pathogen adaptation in two different host plants; (iii) susceptible plants escaping the wilting symptom after inoculation with the pathogen; (iv) the role of polyphosphate molecules in the virulence etc. In this study, we were most interested in these aspects.

In general, *R. solanacearum* pathogenicity in host plants is studied by soil drenching or stem inoculation method. In these methods, soil grown plants are inoculated with the pathogen. Bacteria from the soil inhabit the host plants and there is possible influence of this microbiota on *R. solanacearum* pathogenicity. In Tezpur

University, we are working on *R. solanacearum* F1C1 strain isolated from wilted chilli, Tezpur [42]. A simple and efficient methodology has been standardized in tomato seedlings either by leaf inoculation [43] or by root inoculation method [44]. In this method, very early stages of tomato seedlings can be recruited in microfuge tubes to carry out the pathogenicity study on the seedlings and the pathogenicity assay gets completed by ten days from inoculation. Here, soil is avoided hence the influence of other soil microbiota. One of the difficulties associated with *R. solanacearum* pathogenicity study in host plants is to develop a stable and consistent virulence assay in host plants. This might be a reason for the use of only limited plants such as tomato, *Arabidopsis* as model hosts to study *R. solanacearum* pathogenicity. Apart from tomato, several important crop plants such as potato, eggplant, chilli, etc. are severely get infected by this pathogen. Bacterial wilt disease in eggplant is frequently reported in India. There is less number of studies with regard to *R. solanacearum* pathogenicity mechanisms in eggplant. In this study we have successfully demonstrated that the leaf clip inoculation is an effective method to study pathogenicity of *R. solanacearum* in two cotyledon stage seedlings of eggplant. We established eggplant seedling as a model host by this leaf clip method. It has helped us to compare F1C1 pathogenicity behavior in eggplant and tomato, two phylogenetically closely related hosts.

1.2 Objectives

Here in this study, we have tried to understand the following three main aspects of *R. solanacearum* F1C1pathogenicity.

1. Comparative pathogenicity study of *R. solanacearum* F1C1 between eggplant and tomato seedlings by the leaf clip inoculation method

- *R. solanacearum* pathogenicity study in eggplant seedlings by the leaf clip inoculation method
- To study *R. solanacearum* wild type and mutant colonization in eggplant seedlings
- *R. solanacearum* pathogenicity comparison between tomato and eggplant seedlings

2. A comparative study of the pathogen load in wilted and healthy looking tomato seedlings inoculated by the leaf clip method

   - Pathogenicity study in tomato seedlings by one leaf vs. two leaves inoculations
   - Tracking bacterial colonization in healthy looking and infected tomato leaves
   - Counting bacterial load in healthy looking and infected tomato seedlings

3. Characterization of polyphosphate metabolism homologues of *R. solanacearum* F1C1 with regard to their role in virulence

   - Creation of insertion mutations in *ppk1, ppk2*, *ppx* and *ppnk* genes of *R. solanacearum* F1C1
   - Expression study of *ppk1, ppk2*, *ppx* and *ppnk* genes in BG medium (nutrient rich) and Minimal Medium
   - Characterization of the above mutants for different features such as, motility, hypersensitive response, cellulose activity, oxidative stress and virulence

1.3 Review of literature:

1.3.1 *R. solanacearum* pathogenicity study in host plants

Under natural conditions, *R. solanacearum* infects the host plant through roots, proceeds further to colonize throughout the whole plant before wilting it [6]. Therefore, soil drenching method has been widely used by the researchers in the field to study its pathogenicity by the root inoculation in grown up tomato plants. However, there are also evidences that *R. solanacearum* is pathogenic in host plants artificially inoculated in stem, petiole and leaves [45]. In recent years, different modes of inoculations have been used to study its pathogenicity in early stages of the plant grown either under *in vitro* tissue culture medium or in the laboratory. Recently, our group developed a leaf clip mode of inoculation under gnotobiotic condition to assess the pathogenicity of *R. solanacearum* F1C1 wild type strain and several virulent deficient mutants such as *hrpB*, *hrpG*, *phcA* and *gspD* employing 6-7 days old tomato

seedlings [43]. The method suggested that the bacterium can cause disease upon inoculation in cotyledon leaves at an early stage and is efficient in screening of virulence functions like in grown up plants. Further, we also demonstrated that F1C1 is aggressive in the early stages of tomato seedlings inoculated through a root inoculation method [44]. Collectively, research from our group, as well as from many other groups have demonstrated that plants such as tomato, *A. thaliana*, *M. truncatula* are susceptible to *R. solanacearum* when infected at early developmental stages [17,43,44,46,47].

There are only few plants such as tomato [17], *Arabidopsis thaliana* [48], *Medicago truncatula* [46] and Petunia [49] in which in vitro inoculation assays have been successfully established for studying *R. solanacearum* pathogenicity. Though the pathogen can infects a wide range of host plants, detailed pathogenicity studies uncovering its disease causing mechanisms has not been enlighten. It is pertinent to note that *R. solanacearum* adaptability and aggressiveness in different hosts is not the same [32,39]. Research from the experimental evolution has revealed the role of transcription regulators in host adaptation and colonization [39,50,51]. Considering above information, the question regarding the pathogen adaptation to different hosts is yet to be explored.

Eggplant (*Solanum melongena L.*) is an economically important crop in tropical and subtropical regions belonging to solanaceae family. Reports suggest that eggplant was originated from Africa and thereafter spreaded to Asia [52] which is now the largest producer of eggplant in the world. However, *R. solanacearum* is a great threat to the production of this crop yielding heavy losses every year [53,54,55]. In India, the disease prevalence in eggplant is evidenced in different states [55,56,57] including Assam (Fig1.2). Eggplant cultivation in Goa, India is severely affected due to bacterial wilt and the incidence is around 30-100% [55].

Fig.1.2: Photographs of wilted eggplants from field, near Tezpur University, Assam, India.

In spite of this, *R. solanacearum* pathogenicity as well as pathogenicity determinants in eggplant has not been adequately addressed. Studies regarding *R. solanacearum* pathogenicity in eggplants have mainly been addressed to find out resistant eggplant cultivars against the bacterial wilt disease and to understand resistant mechanisms of eggplant against the pathogen [45,58,59,60]. Recently, approaches has been made to study the genetic diversity of strains isolated from infected eggplants from different regions and studied their virulence in eggplant by soil drenching and petiole pricking method [61] in grown up plants. In none of these studies with eggplant, *R. solanacearum* pathogenicity functions have been characterized. Eggplant being a different host, as well as from economic point of view, understanding eggplant and *R. solanacearum* interaction will be of significant importance and interest. Further work with respect to cause behind variations in the aggressiveness of the bacterium might be of great use in management of *R. solanacearum.*

1.3.2 Escapees: susceptible plants evading the bacterial wilt symptom after *R. solanacearum* inoculation

Unlike narrow host range bacterial pathogen such as *Xanthomonas, Pseudomonas* etc. which causes disease soon after its contact with the hosts, *R. solanacearum* develops a systemic spreading throughout its host plant before causing the disease. In tomato model host of *R. solanacearum*, stem pricking and soil drenching methods are widely used to study its pathogenicity. It is very interesting to notice that among all the tomato plants inoculated with the pathogen, through any

inoculation mood, about 10-20 % plants don't exhibit disease symptoms or escapes the disease. This observation is also common in case of seedlings stages of that host inoculated through leaf clipping. Although several exciting insights into disease causing mechanisms have been made [24,25,26,27,62], which attribute significantly to make this bacterium a successful pathogen, this enigmatic behaviour in its pathogenicity has not yet been explained. In this, it is interesting to notice that there are different strains that have been reported to develop latent infections that are maintained at high concentrations in asymptomatic or distant hosts [5,39,63,64]. It has been shown that the pathogen cannot invade the xylem in these hosts, and restricted to the root cortex or the root surface [15] which can be considered as reservoirs of the bacteria and these include numerous weed species [5,65].

1.3.3 Polyphosphate metabolism

Inorganic polyphosphate (PolyP) is a vital molecule, always been a rich source of energy, abundantly found in every living organisms likely from prebiotic times [66]. Initially, PolyP was observed as metachromatic granules in *Spirillum volutans*, hence was also referred to as volutin granules which was later renamed as PolyP granules [67]. In cells, accumulation of PolyP can be influenced by environmental factors, genetic background as well as culture conditions. It is a polymer of hundreds of phosphate residues linked by high-energy phosphoanhydride bonds as found in ATP [Fig.1.3(A)]. In microbial cells, various functions for polyphosphate have been proposed such as an alternative source of storing energy [68], reservoir for phosphate [69], a chelator of metal ions [70], a buffer against alkali ions [71], a channel for DNA entry [72] and a component involved in cell envelope formation and function. In *E. coli,* PolyP features have been extensively studied where it is found as complexes with RNA polymerase and degradosomes involved in RNA processing and degradation.

This molecule plays significant role during unfavorable environmental conditions as a regulator of stress and survival [66] and as a regulatory component in gene expression [73]. It serves the regulatory role by inducing *rpoS*, the RNA polymerase sigma factor gene that regulates more than 50 stress response genes during stationary phase [74]. Poly P is stable over a wide range of pH, temperatures and oxidants. Recently U. Jacob (2014) has described chaperone like activities of

these molecules *in vitro* [75]. Moreover, studies have suggested that variety of bacterial movements are dependent on PolyP for example swimming, swarming, and twitching as observed in *P. aeruginosa* [76].

Even though the mechanism underlying PolyP accumulation is not clearly understood, the principal enzymes involved in its metabolism have been identified such as polyphosphate kinase (PPK), polyphosphate: glucose-6-phosphotransferase, exopolyphosphatase (PPX), polyphosphate: adenosine monophosphate phosphotransferase (PAP), polyphosphate: NAD-phosphotransferase, 1,3-diphosphoglycerate: polyphosphate phosphotransferase, tripolyphosphatase, polyphosphate glucokinase and endopolyphosphatase [67,77]. In bacteria, among the enzymes that make and hydrolyze Poly P, PPK1 is the most widely conserved that reversibly synthesizes the Poly-P chain using terminal phosphate of ATP as a donor [78]. It is highly conserved across 100 bacterial species, including 20 or more of the major pathogens [79]. The PPK1 structure was first crystallized in *E. coli* which forms a dimer from 80-kDa monomers [80]. Apart from *E. coli,* the genes encoding PPK have been characterized from *Klebsiella aerogenes, N. meningitides, Pseudomonas aeruginosa*, *Acinetobacter* spp. etc. [77]. In *P. aeruginosa*, PPK1 functions have been extensively studied; showing that deletion of *ppk1* affects growth, motility, quorum sensing, biofilm formation, and virulence [81]. Moreover, *ppk1* mutant of strain PAOM5, showed cell envelope distortion, nucleoid compaction, exopolymer production defects and susceptibility to carbenicillin [82]. Proteomic studies have shown that some of the enzymes related to TCA cycle, protein folding, fatty acid catabolism and amino acid biosynthesis were upregulated while motility and transport proteins were down regulated during PolyP deficiency in cells [83].

In *P. aeruginosa,* another form of PolyP named PPK2 (41 kDa protein) have been characterized. The PPK2 is approximately half the length of PPK1 and the primary amino acid sequences do not share any homology. It prefers GTP over ATP as the phosphate donor unlike the PPK1 and the reversible reaction i.e. synthesis of GTP at the expense of PolyP is more favorable [79]. During the stationary phage of bacterial growth, *ppk2* expression goes high where GTP is needed to synthesize alginate, the exopolysaccharide to support the growth. Many pathogenic bacteria have been reported having only PPK1 or only PPK2, or both types of PPKs [79] that can be potential targets for the control of pathogenesis.

In PolyP metabolism, exopolyphosphatase (PPX) plays important role which hydrolyses terminal phosphate from the end of linear PolyP chain having 3 or more phosphoenhydride bonds [84]. *E. coli* possesses two exopolyphosphatases PPX1 and PPX2, predicted to have a role in stress response during starvation. In the opportunistic pathogen *Bacillus cereus*, the deletion mutant of *ppx* was shown to be impaired in sporulation [85].

For many cellular processes and metabolism, synthesis of NADP is important and NAD kinase does this function. It utilizes either PolyP or ATP as a donor in *M. tuberculosis*, *Micrococcus flavus and Bacillus subtilis* [78] while in *E. coli* and *S. cerevisiae* it uses ATP as the only donor.

R. solanacearum genome sequence has revealed the presence of all these four genes encoding PPK1, PPK2, PPX and PPNK enzymes involved in polyphosphate metabolism and is conserved across different strains [Fig.1.3(B); Table1.1]. It is worth mentioning that the role of polyphosphate metabolism genes in plant pathogen is very scares, reported only in *Pseudomonas syringae* pv. *tabaci* 6605, the causal agent of wildfire disease. The deletion mutant of *ppk* showed increased sensitivity heat shock and oxidative stress, reduced exopolysaccharide formation and virulence in tobacco [86]. However, there is no report of characterization of these functions in *R. solanacearum* till date.

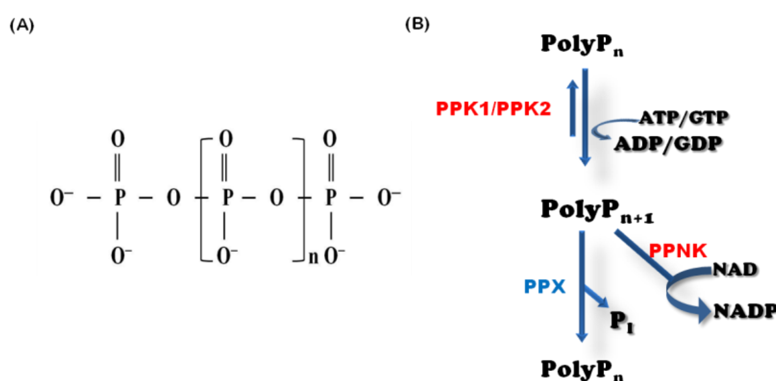

Fig.1.3: Schematic representation of polyphosphate molecule and its metabolism. (A) Structure of linear PolyP molecule and (B) PolyP synthesis and utilization by the enzymes PPK1 and PPK2 (polyphosphate kinases), PPX (exopolyphophatase) and PPNK (PolyP dependent NAD kinase).

Table 1.1: Sequence homology/ conservation of polyphosphate metabolism genes across different strains of *Ralstonia solanacearum* (BlastN)

Sl. No	*R. solanacearum* strains	*ppk1*	*ppk2*	*ppx*	*ppnk*
		% identity	% identity	% identity	% identity
1	GMI1000	100	100	100	100
2	FQY_4	99	99	99	99
3	YC45	99	99	99	99
4	Po82	95	93	98	95
5	CFBP2957	94	84	94	95
6	CMR15	98	97	98	99
7	PSI07	96	95	95	97
8	UY031	95	92	94	95
9	YC40M	99	99	99	99

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