CHAPTER 7

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Ginger (Zingiber officinale Rosc.), a herbaceous plant of the family Zingiberaceae, is one of the valuable spice crop cultivated in India and outside for the spicy vegetable ginger and dried ginger. Among the cultivated countries, India is the largest producer of ginger and contributes approximately 32.75% of the world production. The major diseases causing economic loss in ginger are soft rot caused by species of Pythium and bacterial wilt caused by Ralstonia solanacearum. Among these bacterial wilt is the most devastating and 50-100% disease incidence has been reported from major ginger fields of Kerala during different years. The pathogen is both seed and soil borne in nature and hence both seed and soil can act as source of inoculum for the perpetuation of disease in the field. The typical topography of ginger fields, heavy rainfall during the monsoon and the conventional agricultural practices would result in the movement of pathogen through water and soil that also predisposes the crop for infection and its further spread.

Lack of suitable detection methods, for checking the presence of pathogen in latently infected rhizomes and soil, before sowing is a lacunae in ginger cultivation, so also proper management strategies. The common strategies employed for bacterial wilt management in ginger include selection of apparently healthy seed rhizomes, selection of fields with no bacterial wilt history, seed treatment using protectant chemicals and strict phytosanitary measures. However, these disease management tactics met with limited success in case of bacterial wilt of ginger and still it remains as a threatening problem in all ginger growing tracts. Based on these lacunae, the present research was focussed mainly on two aspects. 1) To understand the genetic diversity of the pathogen for developing a race specific on farm diagnostic, to avoid the use of latently infected
planting material and conducive land for planting and 2) To explore the diversity of endophytic microflora associated with the apoplast of ginger to exploit them for the biological control of bacterial wilt in ginger.

The salient achievements from the study are

- Forty two *Ralstonia solanacearum* isolates were collected from different bacterial wilt affected ginger and small cardamom from different endemic areas during 2009-2015. The isolates represented geographically separated locations such as Kerala, Karnataka and Sikkim in India.
- The isolates were characterized for biovars and found to be race 4 biovar 3 strains.
- Pathogenicity tests proved that there is variation in the infectivity of *R. solanacearum* isolates on ginger as evidenced by the days taken to express the typical wilt symptoms. The highly virulent isolates caused wilting in 6-13 days of inoculation while the less virulent strains caused wilting in 15-25 days.
- Cross infectivity studies clearly revealed that ginger is infected only by the race 4 biovar 3 strains of *R. solanacearum*; the race 1 biovar 3 strains infecting the solanaceous crops could not infect ginger. However, the race 4 biovar 3 strains of ginger can infect solanaceous crops also.
- Twenty one *R. solanacearum* isolates representing major crops (ginger, small cardamom, tomato, potato, chromolaena (*Chromolaena odorata*), chilli, paprika, and eggplant) and geographical locations in India were selected for comparative genetic diversity analysis using different molecular tools.
- Multiplex-PCR based phylotyping has done to understand the geographical origin of the *R. solanacearum* isolates and the study revealed the predominance of phylotype I among the isolates showing the Asian origin. Only one isolate from potato was found to be in phylotype II indicating its American origin.
• **recN** gene sequencing and phylogenetic analysis was conducted to decipher the genetic diversity within a population of *R.solanacearum* race 4 biovar 3 strains from ginger and small cardamom in comparison with a collection of other strains from various hosts and geographic locations. The rooted Bayesian phylogenetic tree generated using *R.picketti* as an outgroup clearly separated the strains based on their geographic origin in which the 30 strains including the strains from the current study were clustered together in a large group of Phylotype I, which again separated into 10 sub clusters showing the variation in the recN gene sequence within the cluster. Within phylotype I, the highly pathogenic race 4 biovar 3 strains from ginger and small cardamom separately clustered distantly from other strains.

• The high discriminatory power of recN gene for elucidating the intraspecies diversity of *R.solanacearum* strains is also revealed in the study.

• Multilocus sequence typing (MLST) was done with five house-keeping genes (*adk, gapA, gdhA, gyrB, ppsA*) and three virulence related genes (*egl, fliC, hrpB*). Partial sequences of all these eight genes were amplified and sequenced from the 21 isolates of *R.solanacearum*.

• In MLST analysis, specific allelic profiles with the eight genes were obtained for all the 21 isolates based on analysis in PAMDB (www.pamdb.org). The highly pathogenic isolates of *R.solanacearum* from ginger/small cardamom were having same allele numbers for all the eight loci. But the highly pathogenic isolate from Sikkim (GRs-Sik) is having 50% loci same as the pathogenic isolates of Kerala.

• Thirteen sequence types (STs) were found in the 21 isolates of *R.solanacearum* isolates used in this study comprising of race 1 biovar 3, race 4 biovar 3 and one race 4 biovar 4 strains, in which eight STs belonged to the ginger isolates of *R.solanacearum*. The highly virulent strain GRs-Pkd is a single locus variant (SLV)
of the ST1 clonal complex containing the fast wilting ginger isolates. PRs–Pun a race 3 biovar 2 isolate from potato shared all eight allele same with the reported race 3 biovar 2 phylotype IIB strains of potato.

- Sequevar characterization was done for all the 21 isolates using the partial \textit{egl} gene sequences. Based on the sequence similarity and clustering, the most virulent ginger strains and one strain from chilli clustered in sequevar 17. GRs-Per 02, a race 4 biovar 3 strain was clustered with sequevar 31. There are also strains of ginger which were not clustered with any reported sequevars.

- The in-depth gene sequence based analysis opened an entry to the development of specific diagnostic for race 4 biovar 3 strains which is prevalent in all ginger growing tracts.

- From the analysis of nine genes used in the diversity studies, \textit{gyrB} gene sequences displayed high percentage of polymorphic sites and nucleotide diversity for developing the specific LAMP primers for \textit{R.solanacearum} race 4 biovar 3 strains.

- A set of six LAMP primers were designed from the \textit{gyrB} gene of race 4 \textit{R.solanacearum}.

- The LAMP primers were validated using genomic DNA. The primers produced a ladder like amplification only for race 4 biovar 3 \textit{R.solanacearum} strains showing the specificity.

- In order to develop a diagnostic tool for an on-farm detection of \textit{R.solanacearum}, real-time loop-mediated isothermal amplification (Real-time LAMP) was attempted where a portable machine (Geneii II, Optigene UK) is used which can be easily carried to the field for on-farm diagnosis.

- The LAMP primers were used for real-time LAMP where it produced a sigmoid curve with \textit{R.solanacearum} race 4 biovar 3 strains further confirming the specificity.
of primers. Race 1 biovar 3 strains from solanaceous crops or race 3 biovar 2 strain from potato and also bacteria from the genera like *Pseudomonas* and *Bacillus* were not amplified.

- The sensitivity of the real-time LAMP was checked with genomic DNA as well as bacterial cells. Amplification was observed with as little as 5 pg of genomic DNA and at $10^4$ CFU ml$^{-1}$ of *R. solanacearum* cells.

- The real-time LAMP protocol using soil DNA (the extraction of which is cumbersome at field level) was modified by replacing soil supernatant as template. Positive amplification could be obtained with soil supernatant (obtained from soil artificially inoculated with *R. solanacearum* race 4 biovar 3 strain cells). This experiment clearly indicated that even soil supernatant can be used for detection of *R. solanacearum* by real-time LAMP which made the protocol still easier.

- For testing latent infection in rhizomes, homogenized ginger extract can be used as the template for real-time LAMP. This also eases the sample preparation. The detection limit was found to be $10^3$ CFU g$^{-1}$ of soil or rhizomes.

- The diagnostic developed was validated with soil and ginger rhizome samples collected from fields. Amplification was obtained only for soil and rhizomes collected from infected field only. Healthy samples did not yield any amplification.

- Thus real-time LAMP based protocol developed in this study can be used as a quick diagnostics method for testing rhizomes and soil for latent infection by race 4 biovar 3 strains.

- For studying the microbial diversity in the apoplastic fluid of ginger, 150 bacteria were isolated from the apoplastic fluid of ginger pseudostems and leaves collected from different ginger growing tracts and germplasm accessions using vacuum infiltration and centrifugation method.
• No culturable fungi or actinomycetes population could be observed in the apoplastic fluid of ginger.

• The apoplastic bacterial isolates were characterized morphologically and biochemically and assigned to different families. The prominent families from which these bacteria belong to are *Bacillaceae*, *Pseudomonadaceae*, *Staphylococcaceae*, *Enterobacteriaceae*, *Rhizobiaceae*, *Aeromonadaceae*, *Moraxellaceae*, *Staphylococcaceae* and *Micrococcaceae*. Gram staining revealed that around 43.3% apoplastic bacteria are of Gram positive rods and 38% are Gram negative rods.

• Testing of apoplastic bacteria for biocontrol traits like production of siderophore and HCN revealed that 72% of the isolates could produce siderophores, but none of them could produce HCN.

• Based on *in vitro* and *in planta* screening, six bacterial isolates viz, IISRGAB 5, IISRGAB 24, IISRGAB 43, IISRGAB 48, IISRGAB 107 and IISRGAB 146 were shortlisted.

• Evaluation of selected apoplastic bacteria for growth promotion traits revealed that all the six isolates could produce ammonia. IISRGAB 5 and IISRGAB 48 could produce considerable amount of IAA and IISRGAB 5, IISRGAB 24, IISRGAB 107 and IISRGAB 146 could solubilize insoluble phosphate.

• Evaluation of the shortlisted bacteria for antibiosis revealed that IISRGAB 5, IISRGAB 43 and IISRGAB 146 could inhibit *R. solanaceraum in vitro* and acetoin production revealed that isolates IISRGAB 5 and IISRGAB 107 could produce acetoin besides siderophores.

• Evaluation of selected apoplastic bacteria for extracellular enzyme production revealed that IISRGAB 5, IISRGAB 24, IISRGAB 48, IISRGAB 107 and IISRGAB
146 could produce both amylase and cellulase and IISRGAB 5, IISRGAB 48, IISRGAB 107 could produce protease. None of the six isolates could synthesize lipase.

- The shortlisted isolates were identified using 16S rDNA sequencing and identified as *Bacillus subtilis* (IISRGAB 5), *Agrobacterium tumefaciens* (IISRGAB 24), *Bacillus marisflavi* (IISRGAB 43), *Micrococcus luteus* (IISRGAB 48), *Bacillus licheniformis* (IISRGAB 107) and *Staphylococcus haemolyticus* (IISRGAB 146).

- These short listed isolates were evaluated against bacterial wilt under green house conditions using seed priming and soil drenching method.

- *Bacillus licheniformis* (IISRGAB 107) was found to be very promising in inhibiting race 4 biovar 3 *R. solanacearum* by showing a disease reduction up to 67% over pathogen challenged control.

- The increased population of introduced bacteria in the rhizosphere soil, roots, rhizomes and apoplastic fluid revealed the colonization of the targeted bacteria when compared to control showing the efficiency of bacterization of soil and seeds.

- To summarize, the present study leads to the development of a specific diagnostic for a quick, early and on-farm detection of race 4 biovar 3 strain of *R. solanacearum* and also identified one potential biocontrol agent viz. *Bacillus licheniformis* from the apoplastic fluid of ginger which can be used for the biological control of bacterial wilt of ginger. The study also leads to the understanding of the diversity of race 4 biovar 3 strains of *R. solanacearum* and also the diversity of culturable microorganisms in the apoplastic fluid of ginger.