# Final Report

## Path. X1. (813): Studies on bacterial wilt of ginger

**Submitted by**

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**Acknowledgements**

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# RPF - III
# (PERFORMA FOR SUBMISSION OF FINAL REPORT OF RESEARCH PROJECTS)

## Part- I: General Information

**800 Project Code** :
8001 Institute Project Code No. : Path XI (813)
8002 ICAR Project Code No.

**801 Name of the Institute and Division**
8011 Name and address of Institute : Indian Institute of Spices Research, Calicut 673 012
8012 Name of Division / Section : Crop Protection/ Pathology
8013 Location of the Project : Plant Pathology Section, Indian Institute of Spices Research Calicut 673 012

**802 Project Title** : **Path. XI (813): Studies on bacterial wilt of ginger**
**803 Priority Area** : 01,03 & 04

8031

| Applied Research | Basic Research | Process/Technology development | Transfer of Technology |
|---|---|---|---|
| 01 | 02 | 03 | 04 |

804 Specific Area : **Spices Research**
**805 Duration of Project** : **12 years**
8051 Date of start : 1992
8052 Date of Completion : 2004

**806 Total cost /Expenditure Incurred: Rs. 2350000**

**807 Executive Summary**

Diseases are one of the major constraints on production of ginger, and of these bacterial wilt or 'Mahali' or 'Ginger blast') is one of the most important. Bacterial wilt of ginger inflicts serious economic losses in many ginger-growing countries on small and marginal farmers who depend on this crop for their livelihood. Bacterial wilt of ginger is caused by a prokaryote, *Ralstonia solanacearum*, with a wide host range including both dicots and monocots. Biovar 3 of *Ralstonia solanacearum* as causal agent of bacterial wilt of ginger was found in Kerala and Karnataka. The incidence of biovar 4 of *R. solanacearum,* which was relatively less virulent in ginger, was less frequent in Kerala. *Ralstonia solanacearum* from other hosts such as tomato, *Chromolaena*, chilli and potato were non-pathogenic on ginger. It is

interesting to note that the *R. solanacearum* isolates from *Chromolaena*, a common weed of ginger fields were not pathogenic on ginger though they belonged to biovar 3. *Kaempferia galanga* and *Zingiber zurumbet,* a close relative of edible ginger is susceptible to bacterial wilt caused by ginger strains of *R. solanacearum*. Turmeric is another member of Zingiberaceae susceptible to the disease. The molecular analysis by rep-PCR and ITS-PCR revealed that the ginger strains isolated from different locations during different years were having 100 % similarity according to Dice's coefficient. The analysis further revealed that the genetic diversity of *Ralstonia* is very low within ginger. The analysis could identify the host, biovar, pathogenic behavior and geographical origin of *Ralstonia solanacearum* strains. Techniques have been standardized to detect the pathogen in soil as well as in rhizomes using post enrichment ELISA. The PCR-based method for detection of the bacterium in soil has been based on universal primers specific for *R.solanacearum*. The post enrichment ELISA could detect as low as 40 cells per g of ginger tissue. *In vitro* and *in vivo* techniques are available for screening germplasm for bacterial wilt tolerance in ginger. Almost all cultivated edible ginger is susceptible to bacterial wilt. Over 600 accessions screened for bacterial wilt tolerance using soil inoculation method were found to be susceptible to the disease.

808 **Key words:** *Ralstonia,* ginger, bacterial wilt, rhizome microwaving, rep-PCR

## Part-II: Investigator Profile

(Please identify clearly changes, if any in Project personnel)

| | | |
|---|---|---|
| **810** | **Principal Investigator** | : |
| 8101 | Name | : A.Kumar |
| 8102 | Designation | : Scientist (Senior Scale) |
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| 8104 | Location | : Calicut |
| 8105 | Institute Address | : Indian Institute of Spices Research, Calicut 673 012 |
| **811** | **Co- Investigator** | : |
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| | | |
|---|---|---|
| **812** | **Co- Investigator** | : |
| 8121 | Name | : R. Suseela Bhai |
| 8122 | Designation | : Senior Scientist |
| 8123 | Division/ Section | : Crop Protection/Pathology |
| 8124 | Location | : Calicut |
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| **813** | **Co- Investigator** | : |
| 8131 | Name | : Y.R.Sarma |
| 8132 | Designation | : Principal Scientist |
| 8133 | Division/ Section | : Crop Protection/Pathology |
| 8134 | Location | : Calicut |
| 8135 | Institute Address | : Indian Institute of Spices Research, Calicut 673 012 |
| **814** | **Co- Investigator** | : |
| 8141 | Name | : G.N.Dake |
| 8142 | Designation | : Senior Scientist |
| 8143 | Division/ Section | : Crop Protection/Pathology |
| 8144 | Location | : Calicut |
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| **815** | **Co- Investigator** | : |
| 8151 | Name | : T.G.Nageshwar Rao |
| 8152 | Designation | : Senior Scientist |
| 8153 | Division/ Section | : Crop Protection/Pathology |
| 8154 | Location | : Calicut |
| 8155 | Institute Address | : Indian Institute of Spices Research, Calicut 673 012 |

## Part-III: Technical Details

### 820 Introduction and objectives

### 8201 Project Objectives

Development of IDM package for the management of bacterial wilt of ginger by understanding the pathogens behavior

### 8202 Background information and importance of the projects

Among the diseases of ginger, bacterial wilt caused by *Ralstonia solanacearum*, is rhizome borne in nature. Bacterial wilt that leads to rhizome rot is a major problem and one of the constraints in the production of ginger owing to their wider host range and genetic variability it exhibit. Besides the pathogens has got multiple modes of survival and fast

lateral transmission within and between fields. During the investigation basic information on i. Etiology of these diseases, ii. Characterization of pathogens, iii. Their survival and detection in soil and rhizome, iv. Their vulnerability to heat and the ultimate disease management using rhizome treatment were attempted. From the host side, the systematic screening programme was carried out to locate resistant line of ginger against bacterial wilt.

## 821 Project Technical Profile

### 8211 Technical programme

(Indicate briefly plan of procedure, techniques, instruments and special materials, organisms, special environments etc.)

i. Etiology and characterization of bacterial wilt pathogen and its diversity in India

ii. The vulnerability of the bacterial wilt pathogen to heat and its exploitation for rhizome disinfection

iii. The survival of the pathogen and method for its detection in soil and rhizome

iv. Development of cultivars resistant to bacterial wilt

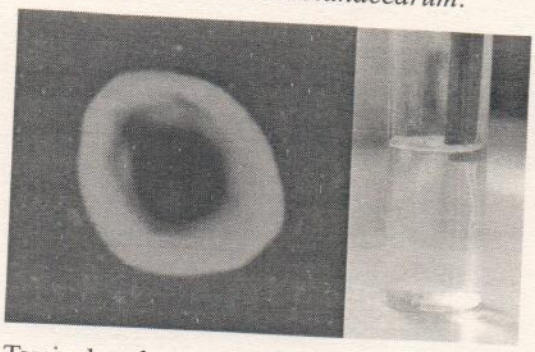
### 8212 Total man month's involvement of component project workers

| | |
|---|---|
| A. Kumar | : 48 (From 1997-2004) |
| G.N.Dake | : 32 (1992-1996) Left IISR and ICAR in 1996 |
| T.G.Nageshwar Rao | : 8 (1992-1996) Left IISR in 1995 |
| Y.R.Sarma | : 8 (1997-2001) Retired from Service in 2002 |
| M.Anandaraj | : 4 (2001-2003) |
| R.Suseela Bhai | : 2 (2003-2004) |
| Total | 102 |

## 822 Final Report on the Project

Detailed report containing all relevant data with a summary of results (not exceeding 2-5 pages)

**1.Etiology of bacterial wilt:** Pathogen causing bacterial wilt of ginger has been isolated from all the major ginger growing regions of the country and identified as Biovar 3 or Biovar 4 of *Ralstonia solanacearum*.



Typical colony of *Ralstonia solanacearum* & Bacterial ooze from cut end of ginger pseudostem: a useful diagnostic tool for confirming bacterial etiology

**2. Pathogen characterization:** Bacterial wilt pathogen *Ralstonia solanacearum* isolated from host such as ginger, tomato, *Chromolaena*, *Capsicum* and potato were characterized for biovar, pathogenicity, infectivity titer, and intrinsic antibiotic resistance. The isolates were also characterized on the basis of their membrane protein pattern and amenability of the isolates for serological detection (NCM-ELISA) using *R. solanacearum* specific antibodies. All the ginger isolates were highly fluidal with characteristic spiral pink center on tetrazolium-amended medium. Among the isolates characterized nine belongs to biovar 3 while one from potato to biovar 2. Among the various isolates tested, only ginger ones except one from Assam induced cent percent wilting in ginger cv. *Himachal* within a week. Plants were wilted even at a concentration of 100 cfu $ml^{-1}$ in stem inoculation while in soil inoculation it was $10^5$-cfu $ml^{-1}$. All the isolates were resistant to antibiotics tetracycline, polymyxin B sulphate and Chloramphenicol and isolates GRSTms and its spontaneous mutant were resistant to Rifamycin. Isolates were detected with NCM-ELISA and biovars on the basis of membrane pattern on SDS-PAGE and biovar specific protein from *R. solanacearum* could be isolated. Phenotypic characterization of bacterial wilt pathogen confirmed the dominance of biovar 3 over biovar 4 in India. An expert system for quick scoring of large number of isolates was developed using server side scripting language PHP and HTML and it was validated using the data generated in the wet lab (published in *Indian Phytopathology* 2004).

**Infectivity titer of *Ralstonia solanacearum* in ginger**

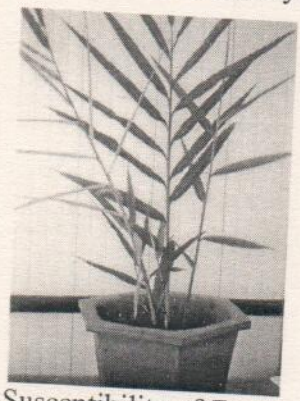
| Inoculum concentration (cfu/ml) | Pseudostem inoculation | | Soil inoculation | |
|---|---|---|---|---|
| | Initiation of disease (Days) | % wilt | Initiation of disease (Days) | % wilt |
| $3.2 \times 10^8$ | 6 | 100(8) | 12 | 100(19) |
| $3.2 \times 10^7$ | 6 | 100(9) | 12 | 30 |
| $3.2 \times 10^6$ | 7 | 100(20) | 13 | 30 |
| $3.2 \times 10^5$ | 7 | 100(15) | 14 | 10 |
| $3.2 \times 10^4$ | 8 | 80 | * | * |
| $3.2 \times 10^3$ | 8 | 80 | * | * |
| $3.2 \times 10^2$ | 9 | 80 | * | * |

Figures in parenthesis indicate number of days to wilt all the inoculated ginger plants.

*- No wilt incidence

**Host range studies**

*Ralstonia solanacearum* from other hosts such as tomato, *Chromolaena*, chilli and potato were non-pathogenic on ginger. It is interesting to note that the *R. solanacearum* isolates from *Chromolaena*, a common weed of ginger fields were not pathogenic on ginger though they belonged to biovar 3. *Zingiber zurumbet*, a close relative of edible ginger is susceptible to bacterial wilt caused by ginger strains of *R. solanacearum*.

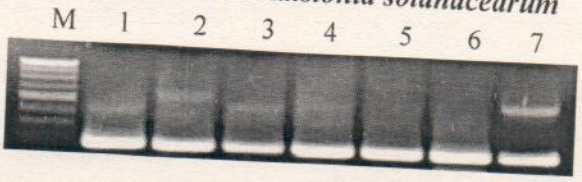


Susceptibility of *Zingiber zurumbet* to bacterial wilt

**3. Identification of *Ralstonia solanacearum***

PCR based identification of *Ralstonia solanacearum* using universal *R. solanacearum* specific primer was standardized and routinely used in the identification of samples received through AICRP centers. Basic data on growth, morphology, generation time, and stability were generated for *Ralstonia solanacearum*

**PCR based identification of *Ralstonia solanacearum***




M Marker 100bp ladder, Lane 1: Ginger, Lane 2: Chilli, Lane 3: Paprika, Lane 4: Potato, Lane 5: Eupatorium, Lane 6: Tomato, Lane 7: Brinjal

**4. Characterization and identification of biovar**

Amplified Ribosomal DNA Restriction Analysis (ARDRA) technique using primers designed from 16s rDNA and 16-23s rDNA intergenic regions was standardized for bacterial strain characterization. RFLP using *Msp I* of 16s rDNA did not produce much polymorphism. However, the technique was found to differentiate the biovars of the bacterium when using restriction enzymes *Msp 1* or *Sau 3A-I* to restrict the amplified hyper variable intergenic (16-23S) sequence

A band with size of 290 bp was found to be specific for Biovar 3 of *R. solanacearum* irrespective of host origin whereas a band with 210 bp was found to be specific for biovar 2 *R. solanacearum* from potato when the amplified intergenic region was restricted with *Sau 3A-I*. The same when restricted with *Msp I* found to produce a band of size 384 bp specific for biovar 2. A band of 928 bp was found when 16s rDNA of biovar 2 was amplified which was absent in biovar 3 isolates of different host including ginger.

**5. Evaluation of genetic diversity of *Ralstonia solanacearum* causing bacterial wilt of ginger using Rep-PCR and ITS-PCR DNA Fingerprinting:** Thirty three strains of *R. solanacearum* isolated from ginger, paprika, chilli, tomato, eupatorium and potato from the states of Kerala, Karnataka, West Bengal and Assam in India were phenotypically and genotypically characterized. Phenotypic characterization for biovar revealed the predominance of Biovar 3 in India. The molecular analysis by REP-, ERIC-, and BOX

PCR (REP-PCR), ITS-PCR and RFLP-PCR classified the strains in to three clusters at 70% similarity where ginger strains are grouped in Cluster I and II. Strain from potato (Biovar 2) clustered in the III cluster. The molecular analysis also revealed that the ginger strains isolated from different locations during different years were having 100 % similarity according to Dice's coefficient. The analysis further revealed that the genetic diversity of *Ralstonia* is very low within ginger confirming that the pathogen population is of clonal lineage and is distributed through 'rhizome transmission' of the inoculum between locations and also between seasons within the locality (published in *Current Science* 2004)

**6. Comparative genetic analysis of *Ralstonia solanacearum* strains causing bacterial wilt of ginger isolated from Sikkim, an Eastern Himalayan state and Southern States of India**: In India bacterial wilt disease has been found in all major ginger growing states and is particularly severe in hot and humid southern states (ambient temperature is 28-36°C) as well as in cold high altitude Eastern Himalayan state of Sikkim (ambient temperature is 7-22°C). These geographical, micro & macro climatic variations and differences in the method of ginger farming in these locations did not deter the severity of bacterial wilt in Indian subcontinent. We compared two populations of strains causing bacterial wilt of ginger from these geographically well-isolated locations. Initially the bacterial wilt pathogen was isolated from wilted ginger plants from these geographical locations. Ten isolates were obtained from wilted ginger plants from North and East Sikkim districts of Eastern Himalayan regions, an altitude of over 5500 feet above sea level. These isolates were phenotypically and genotypically compared with 13 other strains isolated from Kerala and Karnataka, the Southern States of the India. The strains were isolated on CPG agar and identified by PCR based assay using universal Rs specific primers that produced single 280bp amplicon specific for *Ralstonia solanacearum*. Phenotypic characterization revealed the occurrence and dominance of Biovar 3 over 4 among the collections. Interestingly biovar 4 was rarely encountered in both locations compared with biovar 3." The biovar 3 strains were highly aggressive on ginger, causing wilt in 5-7 days of soil inoculation whereas the biovar 4 strains took 3-4 weeks to wilt ginger plants.

Genetic analysis of strains using Rep-, ERIC, and BOX PCR and Amplified Ribosomal DNA Restriction Analysis (ARDRA) revealed that the biovar 3 strains were highly similar and they all belong to single haplotype with 100% similarity according to Dice Coefficient index. Pooled analysis of the data using NTSys (Applier Biostatistics Inc.) clearly indicated their high similarity. High similarity among the isolates from these geographically well-separated locations

indicated that the strains had migrated from one place to another, most likely through the rhizomes, as ginger rhizome exchange among small and marginal farmers in India is an important activity during peak season of planting. This result further confirmed the role of infected rhizomes in the spread and distribution of bacterial wilt in India.

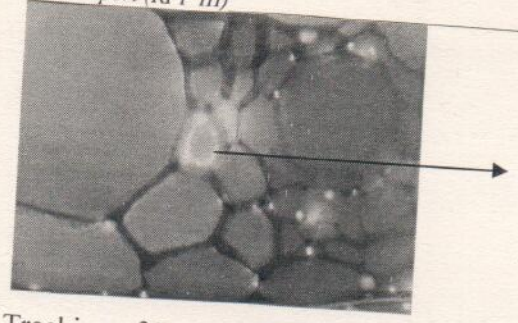
**7. Pathogen detection**

Detection of bacterial pathogen by protein and nucleic acid based methods are widely reported. Exploiting other cellular components of bacteria for specific detection in plant and other complex ecosystem is easy and would supplement the former detection data

**i. Tracking of *Ralstonia solanacearum* in ginger using nileblue staining and PCR**

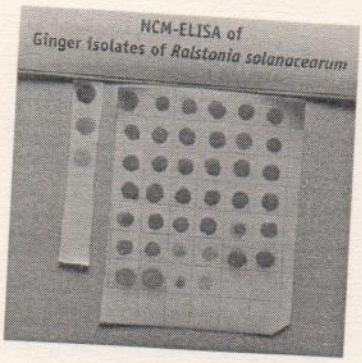
The work reported here is aimed to develop a detection method for bacterial wilt pathogen, *Ralstonia solanacearum* exploiting the poly-beta-hydroxybutyric acid granules located in cytoplasm of cell. Nileblue is reported to selectively stain these granules, which in turn excites at 460nm wavelength in epifluorescence microscope. In order to know the effect of nileblue on multiplication of bacterium, assays were done and the growth was determined spectrophotometrically. The constant absorbance value obtained for 0.001 to 0.006% nileblue amendments clearly indicate the non-toxic nature of nileblue at these concentration. However, at higher concentration cells could not multiply probably the stain is toxic at higher concentration. Incorporation of nileblue in the medium is recommended for detection of *R. solanacearum*. The cell thus multiplied in nileblue added medium was inoculated in plants to know its effect on pathogenicity of the cells. The inoculated plants succumb to the disease after 8 days indicating the retention of virulence after nileblue labeling.

Experiments conducted to detect the movement of bacterial cells in plant; nileblue staining was performed on sections made from the different plant parts. It was found that there was greater accumulation of cells in base and middle of the pseudostem than upper part of the plant. This clearly indicates the effectiveness of this staining procedure in tracking the movement of bacterial cells in plant tissues. This was further confirmed by PCR based tracking of *R. solanacearum* using Rs specific primers. Maximum concentration of bacterial cells was observed in mother rhizome followed by young rhizomes. Among the other parts of the plans pseudostem particularly based pseudostem harboured maximum of bacterial cells. The leaf harboured very less bacterial cells.




Tracking of *Ralstonia solanacearum* in xylem elements of ginger

**ii. Serological detection of pathogen in rhizomes:** The foregoing method would fail to detect when the pathogen population is less. Under field condition the pathogen is carried on to the rhizome surface as contamination or latently infected in the rhizomes. **In order to detect very low level of inoculum the sensitivity of the assay must be very high.** Detection in soil and rhizomes using NCM-ELISA and Double Antibody Sandwich ELISA (DAS-ELISA) was successfully achieved. CIP's DAS-ELISA Kit has been found suitable for the detection of *R. solanacearum* in bacterial wilt affected soil and ginger samples. Bacterial wilt pathogen *R.solanacearum* was detected in apparently healthy rhizomes, which confirms the rhizome borne nature of *R. solanacearum* in ginger. The apparently healthy rhizomes are the protected carrier of bacterial inoculum at low levels. *Ralstonia solanacearum* can survive both in vascular tissue and external surface of naturally infected ginger rhizomes




**iii. Detection of *Ralstonia solanacearum* in naturally infected rhizomes**

Mature ginger rhizomes were collected from six different bacterial wilt affected fields of Wyanad such as Ambalavayal, Poothady, Pulpally, Kenichira, Vadakkanad and Beenachy. Post enrichment DAS-ELISA for the detection of *R solanacearum* in ginger rhizomes. Among the different samples, positive reaction could be noted only from three locations viz., Kenichira, Poothady and Pulpally as their absorbance values are more than three times

of that of negative control value, which clearly indicates the rhizome borne nature of the bacterial wilt pathogen in ginger.

**iv. Serological detection of pathogen in soil:** Sensitivity and selectivity of the antibiotics is key to the success of serological assay. DAS-ELISA kit developed in International Potato Centre, Peru, was evaluated in soil inoculated with *R. solanacearum in vitro*. The sterile soil sample, which was inoculated with *R. solanacearum,* shows high A 405 values when compared to unsterile soil. Assay was also conducted in soil co-inoculated with *P. fluorescens*. It is clear from the results that the kit could detect the *R. solanacearum* released into soil either alone or along with *P. fluorescens*. Positive results are obtained only with enriched sample indicating the need for selective multiplication of *R.solanacearum* to a level, which could be sensitized by the antibodies. The sensitivity of the ELISA has been reported to be $10^4$-$10^5$ cells $g^{-1}$ of soil (soil extract)

**Detection of *R. solanacearum* in ginger rhizomes obtained from bacterial wilt affected plots using -DAS-ELISA**

| Ginger Samples | *A 405 values against *Rs*. Specific antibodies | | | |
|---|---|---|---|---|
| | External Washing | Negative Control | Vascular issue | Negative Control |
| Kenichira -1 | 0.024 | 0.035 | **0.076** | 0.035 |
| Pulpally | 0.015 | 0.035 | 0.007 | 0.035 |
| Poothady -1 | 0.021 | 0.035 | 0.035 | 0.035 |
| Ambalavayal | 0.010 | 0.035 | **0.114** | 0.035 |
| Kenichira | **0.188** | 0.035 | 0.012 | 0.035 |
| Poothady | 0.062 | 0.035 | 0.007 | 0.035 |
| Vadakkanad | 0.022 | 0.035 | 0.026 | 0.035 |
| Beenachy | 0.023 | 0.035 | 0.034 | 0.035 |
| **Pulpally -1 | **0.808** | 0.282 | **0.440** | 0.282 |
| Peruvannamuzhi | 0.264 | 0.333 | **0.560** | 0.333 |

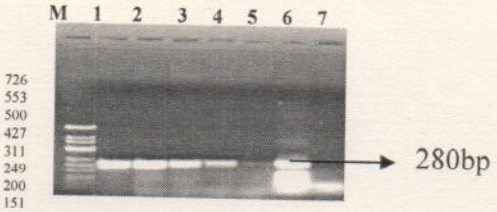
*Mean of four replicates, **Mean of eight replicates

**v. PCR based detection of pathogen soil:** An efficient DNA isolation protocol and PCR based detection of bacterial pathogen in soil is developed The use of this DNA isolation method and PCR based method using universal *Ralstonia solanacearum* specific primer for detection of the bacterium in soil offer a rapid method for unambiguous detection of this pathogen in soil, which can be employed, for monitoring soil samples for this globally important plant pathogen. The PCR based assay could detect the pathogen at a concentration of $10^3$-$10^4$cells per gram of soil (published in *Indian Phytopathology2006*)

**Quantitative and qualitative analysis of DNA isolated from soil seeded with bacteria**

| Concentration of bacteria in soil* (Number of cells gram$^{-1}$) | Yield (µg g$^{-1}$) | A** 260/280 | A** 260/230 |
|---|---|---|---|
| 1. Soil +*Ralstonia solanacearum* (GRS Vy) $10^7$ | 4.10 | 1.56 | 0.53 |
| 2. Soil +*Ralstonia solanacearum* (GRS Vy) $10^6$ | 0.80 | 1.89 | 1.50 |
| 3. Soil +*Ralstonia solanacearum* (GRS Vy) $10^5$ | 0.78 | 2.00 | 1.80 |
| 4. Soil+ *Ralstonia solanacearum* (GRS Vy)$10^4$ | 0.28 | 1.96 | 1.05 |
| 5. Soil +*Ralstonia solanacearum* (GRS Vy) $10^3$ | 0.21 | 1.80 | 0.80 |
| 6. Soil +*Ralstonia solanacearum* (GRS Vy) + *Pseudomonas fluorescens* (IISR 6) | 1.95 | 1.97 | 1.96 |
| 7. Soil +*Ralstonia solanacearum* (GRS Vy) + *Pseudomonas fluorescens* (IISR 51) | 2.00 | 1.98 | 2.04 |
| 8. Soil +*Ralstonia solanacearum* (GRS Vy) + *Pseudomonas fluorescens* (IISR 6)+ *Ralstonia solanacearum* (GRS Vy) + *Pseudomonas fluorescens* (IISR 51) | 2.15 | 2.00 | 2.14 |
| 9. GRS Vy as pure culture ($10^9$ cells per ml)*** | 80.0 | 1.99 | 2.24 |

*Soil consist of 2:1:1 mixture of forest soil, farmyard manure and river sand; **Ratio of absorbance of DNA at two wave lengths, *** Concentration of bacteria per ml of pure culture of *R. solanacearum* GRS Vy




PCR based assay for detection of *R. solanacearum* using Rs specific primers

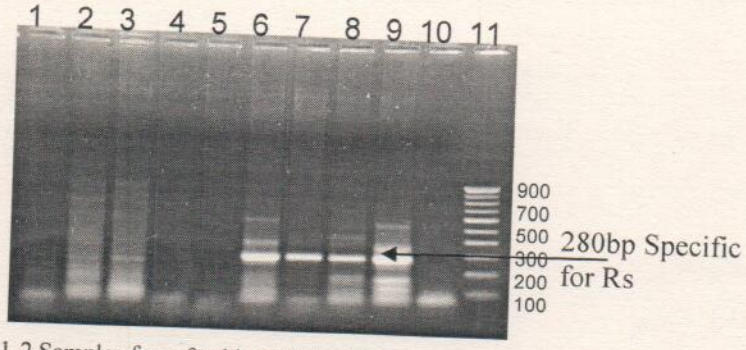
### vi. Survival of *R. solanacearum* in soil

Serological assay was conducted to detect *R solanacearum* on the debris of the rhizomes collected from bacterial wilt affected ginger plants. Only two of 26 samples tested positive for *R. solanacearum* after enrichment. Healthy rhizomes were planted in the pots where bacterial wilt was previously recorded. Disease could be recorded only after five months in two of the 10 pots. The survival data generated using serological and bioassay method further confirms that the pathogen fail to survive in soil and also in ginger periderm collected from bacterial wilt affected soil

### vii. Investigations on spread of the bacterial wilt in soil

Plant to plant spread of bacterial wilt disease was studied in simulated plastic trays. The wilt disease was artificially created in the tray by planting infected rhizome among the lot

of healthy rhizomes. Then the disease spread was studied by PCR based technique. The plants were carefully monitored and the soil from the rhizosphere region around the plant was collected. Soil was collected from different stages of diseases viz., very early stage of the disease when only wilting was noticed, late stage when the plants expressed golden yellowing and lodging on the ground. Thus collected soil was subjected to PCR assay for *Ralstonia* detection using Rs specific primers. The data revealed the absence of the bacterial wilt pathogen in the soil collected from wilted plants during very early stage indicating the containment of the pathogen with the vascular elements whereas the bacterium could be detected in the soil around the completely lodged plants indicating its abundance and spread due to release of bacterial cells in soil around the such a yellowed plants. It served as a source of inoculum for further spread in the soil as indicated by the wilt incidence in the nearby plants emerged from the healthy plants.

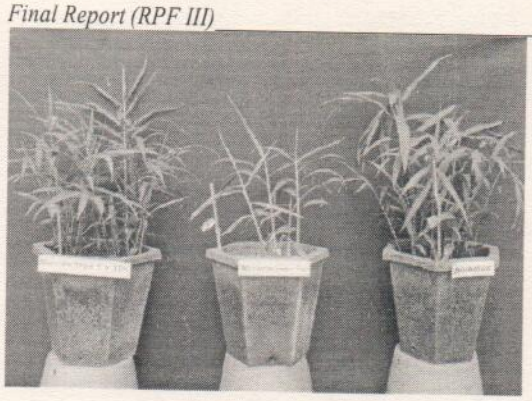



Lane: 1-2 Samples from freshly wilted plants, Lane: 3-4 Samples from Rotten rhizomes, Lane 5-8 Samples from completely collapsed plants, 9. Positive Control, 10. Negative control, 11. 100bp DNA ladder

**Spread of bacterial wilt pathogen in soil**
(Evidence for the role of rhizome borne inoculum and importance of early diagnosis of disease)

**Seed rhizome disinfection by heat induction aided by microwaves for management of bacterial wilt of ginger**

Rhizomes subjected to pulse microwaving involving 4-5 cycles of 10s, with the pause time of 5s between cycles, also confirmed the effectiveness of microwave disinfection. This incidentally is the first report of disinfection of ginger from bacterial wilt pathogen using microwaves. This technology can be adopted in plant quarantine station or material meant for small scale planting.



Effect of microwave treatment on bacterial wilt incidence

Extreme right: The plants emerged from microwave treated (infected) rhizomes

**Effect of microwave treatment on germination of ginger sprouts**

| Exposure in seconds | Temperature (°C) | Sprouts | Height (cm) | *Plant stand | *Wilt (%) | **Plant stand | **Wilt (%) |
|---|---|---|---|---|---|---|---|
| UT | - | $6.00^{ab}$ | $11.5^{abcd}$ | 8.0 | 0.0 | 10.0 | 0.0 |
| 0 | 26 | $5.7^{ab}$ | $15.3^{abc}$ | 8.3 | 8.3 | 7.0 | 26.7 |
| 10 | 40 | $3.0^{bcd}$ | $17.7^{a}$ | 5.3 | 0.0 | 5.0 | 0.0 |
| 20 | 40 | $4.3^{abc}$ | $17.2^{ab}$ | 7.3 | 0.0 | 8.7 | 0.0 |
| 30 | 45 | $6.3^{a}$ | $9.1^{bcde}$ | 6.3 | 0.0 | 7.3 | 0.0 |
| 40 | 59 | $2.3^{cd}$ | $7.3^{cdef}$ | 3.0 | 0.0 | 3.0 | 0.0 |
| 50 | 74 | $0.7^{d}$ | $2.3^{ef}$ | 0.7 | 0.0 | 1.0 | 0.0 |
| 60 | 75 | $0.0^{d}$ | $0.0^{f}$ | 0.0 | 0.0 | 0.0 | 0.0 |
| 70 | 88 | $1.0^{d}$ | $6.5^{def}$ | 1.0 | 0.0 | 1.0 | 0.0 |
| 80 | 89 | $0.0^{d}$ | $0.0^{f}$ | 0.0 | 0.0 | 0.0 | 0.0 |
| 90 | 96 | $0.0^{d}$ | $0.0^{f}$ | 0.0 | 0.0 | 0.0 | 0.0 |
| 100 | 95 | $0.0^{d}$ | $0.0^{f}$ | 0.0 | 0.0 | 0.0 | 0.0 |

* -45 Days after planting; **- 60 Days After Planting

UT- Untreated; Data with same letter designation are not significantly different according to Duncan's Multiple Range Test at P=0.05%

**Effect of discontinuous microwaving on bacterial wilt incidence**

| Microwave Treatment (Seconds) | Temperature (°C) | *Cumulative wilt incidence (%) |
|---|---|---|
| Unexposed | 26 | 0.0 |
| 0 | 27 | 57.0 |
| 1 X 10 S | 30 | 26.7 |
| 2 X 10 S | 35 | 13.2 |
| 3 X 10 S | 42 | 19.1 |
| 4 X 10 S | 45 | 0.0 |
| 5 X 10 S | 47 | 0.0 |

Disease noticed after 45 days

* - 120 Days After Planting

## Screening for Bacterial wilt resistance

**i. Standardization of methods for screening ginger (*Zingiber officinale* Rosc) for bacterial wilt resistance:** Currently the bacterial wilt management emphasizes on selection of disease free seed rhizomes, rhizome treatment by hot air or hot water or rhizome solarization, periodical rouging of infected plants and crop rotation with non-host plants to reduce the disease causing potential of soil. Though effective in disinfecting the ginger rhizomes from *Ralstonia solanacearum*, none of these strategies practically arrested the spread of disease in the field during peak monsoon season, which is highly congenial for horizontal disease spread across the region. Exploitation of host resistance for management of bacterial wilt can be one of the important ecofriendly disease control strategies.

To locate resistance against bacterial wilt **a reliable screening procedure becomes vital**. Three *in vivo* methods viz., pseudostem inoculation, soil inoculation, rhizome inoculation and a novel *in vitro* method i.e., direct incorporation of bacterial cells in the medium when the plantlets are 2-3 leaf stage were evaluated by using different concentration of bacterial cells for screening ginger germplasm for bacterial wilt resistance. Among the different methods evaluated, pseudostem inoculation resulted in wilting of plants in 5-7 days, followed by the soil inoculation method in 7-10 days, rhizome inoculation method in 45-60 days and the *in vitro* method in 10-14 days.

**Evaluation of different method for screening ginger for bacterial wilt resistance**

| Method | Infectivity titer of *Ralstonia solanacearum* | No of days for symptoms expression | Nature of Symptoms | Remarks |
|---|---|---|---|---|
| Stem inoculation after pinprick | 100 cells | 5-7 days | Typical wilt | Rapid assay but depend on injury |
| Soil inoculation | 106 cells per gram of soil | 7-10 days | Typical wilt | Repeated inoculation with pathogen is needed |
| Rhizome inoculation | 106 cells per ml of suspension | 40-45 days | Typical wilt | Long incubation period |
| Direct inoculation in tissue cultured medium | 100 cells/bottle of 100 ml medium with 20 plantlets | 10-14 days | Only yellowing, no wilting | Screening of large collection is possible |

## 8221 Achievements in terms of targets fixed for each activity

Diseases are one of the major constraints on production of ginger, and of these bacterial wilt or 'Mahali' or 'Ginger blast') is one of the most important. Bacterial wilt of ginger inflicts serious economic losses in many ginger-growing countries on small and marginal farmers who depend on this crop for their livelihood. Bacterial wilt of ginger is caused by a prokaryote, *Ralstonia solanacearum*, with a wide host range including both dicots and monocots. Achievements of the project are furnished below

### 1. Pathogen characterization, diversity and pathogenicity

Biovar 3 of *Ralstonia solanacearum* as causal agent of bacterial wilt of ginger was found in Kerala and Karnataka. The incidence of biovar 4 of *R. solanacearum,* which was relatively less virulent in ginger, was less frequent in Kerala. *Ralstonia solanacearum* from other hosts such as tomato, *Chromolaena*, chilli and potato were non-pathogenic on ginger. It is interesting to note that the *R. solanacearum* isolates from *Chromolaena*, a common weed of ginger fields were not pathogenic on ginger though they belonged to biovar 3. *Zingiber zurumbet,* a close relative of edible ginger is susceptible to bacterial wilt caused by ginger strains of *R. solanacearum*. The molecular analysis by rep-PCR and ITS-PCR revealed that the ginger strains isolated from different locations during different years were having 100% similarity according to Dice's coefficient. The analysis further revealed that the genetic diversity of *Ralstonia* is very low within ginger. The analysis could identify the host, biovar, pathogenic behavior and geographical origin of *Ralstonia solanacearum* strains

### 2. Pathogen detection by serological and DNA based methods

Techniques have been standardized to detect the pathogen in soil as well as in rhizomes using post enrichment ELISA. The PCR-based method for detection of the bacterium in soil has been based on universal primers specific for *R.solanacearum*. The post enrichment ELISA could detect as low as 40 cells per g of ginger tissue

### 3. Screening of ginger germplasm for bacterial wilt tolerance

*In vitro* and *in vivo* techniques are available for screening germplasm for bacterial wilt tolerance in ginger. Almost all cultivated edible ginger is susceptible to bacterial wilt. Over 600 accessions screened for bacterial wilt tolerance using soil inoculation method were found to be

susceptible to the disease. Screening *in vitro* by incorporating the toxic metabolites of *Ralstonia* in the culture medium has been used as a method of selection; however surviving plantlets were found to be susceptible to bacterial wilt in the field. An efficient *in vitro* screening technique for tolerance to bacterial wilt has been developed at the Indian Institute of Spices Research, Calicut. Live bacterial cells are added to tissue culture bottles containing ginger plantlets; this method enables screening of large numbers of plantlets in tests of two weeks duration. Susceptible plants became chlorotic.

**4. Development of disinfection technology for management of bacterial wilt of ginger.**

Thermal inactivation point for *R. solanacearum* is 46-50°C at 30 minutes of continuous exposure *in vitro*. Basic data on effect of heat on stability and viability of Ralstonia solanacearum was generated in this project, which eventually helped in securing a project from International Foundation for Science, Sweden

**8222 Questions- Answered**

- Which of the biotype is dominating in India?
- What are the morphological and growth characters of *Ralstonia solanacearum*?
- Is there any diversity?
- Is it rhizome borne?
- Is it possible to disinfect rhizomes using heat?
- What is its effect on the rhizome intactness?
- Is it possible to detect the pathogen in rhizomes and soil?
- Is it possible to unambiguously identify *Ralstonia solanacearum?*
- Is there any resistance in ginger to bacterial wilt?
- Is it possible to develop a screening method for ginger against bacterial wilt resistance?
- Is there any alternative host for ginger strain of *Ralstonia solanacearum*?

**8223 Process/ Product/ Technology/ Developed**

- Large collection of *Ralstonia solanacearum* was added to the repository
- Method for detection of the bacterium in rhizome and soil was developed
- Method for isolation of DNA from soil was developed
- Method for screening large collection of ginger against bacterial wilt was standardized
- Technology for small-scale disinfection of ginger rhizomes using microwave was standardized

**8224 Practical Utility** (not more than 150 words)

Biovar 3 of *Ralstonia solanacearum* as causal agent of bacterial wilt of ginger was found in Kerala and Karnataka. The incidence of biovar 4 of *R. solanacearum,* which was relatively less virulent in ginger, was less frequent in Kerala and other parts of India. *Ralstonia solanacearum* from other hosts such as tomato, *Chromolaena*, chilli and potato were non-pathogenic on ginger. It is interesting to note that the *R. solanacearum* isolates from *Chromolaena*, a common weed of ginger fields were not pathogenic on ginger though they belonged to biovar 3. *Zingiber zurumbet,* a close relative of edible ginger is susceptible to bacterial wilt caused by ginger strains of *R. solanacearum*. The molecular analysis by rep-PCR and ITS-PCR revealed that the ginger strains isolated from different locations during different years were having 100 % similarity. Techniques have been standardized to detect the pathogen in soil as well as in rhizomes using post enrichment ELISA. The PCR-based method for detection of the bacterium in soil has been based on universal primers specific for *R.solanacearum*. The post enrichment ELISA could detect as low as 40 cells per g of ginger tissue. *In vivo* techniques were developed for screening germplasm for bacterial wilt tolerance in ginger. Method of disinfecting ginger using rhizome heat treatment was found.

**8225 Constraints, if any**

Nil

**823 Publications and Material Development**

(One copy each to be supplied with this proforma.)

8231 Research papers

| Authors | Year | Title | Journal |
|---|---|---|---|
| 1. Kumar A. and M.Anandaraj | 2006 | Method for isolation of soil DNA and PCR based detection of ginger wilt pathogen, *Ralstonia solanacearum* | *Indian Phytopathology* 59 (2) in press |
| 2. Kumar, A. | 2006 | Methods for screening ginger (*Zingiber officinale* Rosc) for bacterial wilt resistance | *Indian Phytopathology* 59 (2) in press |
| 3. Kumar, A and Y.R. Sarma. | 2004 | Characterization of *Ralstonia solanacearum* causing bacterial wilt of ginger in India. | *Indian Phytopathology* 57: 12-17 |
| 4. Kumar, A., Sarma, Y.R., and Anandaraj, M. | 2004 | Evaluation of genetic diversity of *Ralstonia solanacearum* causing bacterial wilt of ginger using REP-PCR and PCR-PFLP. | *Current Science* 87: 1555-1561 |

| | | | |
|---|---|---|---|
| 5. Kumar, A, Y.R. Sarma and S. Priou | 2002 | Detection of *Ralstonia solanacearum* in ginger rhizomes using post-enrichment NCM-ELISA | *Journal of Spices and Aromatic Crops* 51:35-40 |
| 6. Dake. G.N and Manoj, P.S | 1995 | Bacterial wilt of *Kaempferia galanga* L caused by *Pseudomonas solanacearum* (Smith) Smith. | *J. Spices and Aromatic Plants* 4(2): 159 |
| 7. Dake, G.N | 1995 | Diseases of Ginger (*Zingiber officinale* Rosc) and their management | *J. Spices and Aromatic Plants* 4(1): 40-48 |

### 8232 Popular articles

| Name | Year | Title | Periodical |
|---|---|---|---|
| G.N.Dake | 1994 | Integrated management of bacterial wilt of ginger | *Spice India* 7(11): 14-16 |

### 8233 Reports

| Sl No | Title | Published by | Year |
|---|---|---|---|
| 1 | Kumar, A and A.C.Hayward (2005) Bacterial Diseases of Ginger and Their Control. In: Ginger: The genus Zingiber (Eds. P.N.Ravindran and K.N.Babu), CRC Press, USA, pp: 341-366 | CRC Press, USA | 2005 |
| 2 | Kumar, A. et al (2004) Evaluation of genetic diversity of rhizobacteria obtained from spice crops. In: Agricultural Bioinformatics workshop | IISR, Calicut | 2004 |
| 3 | Kumar, A. (2003) Microbial Genomics In: Training Manual on Bioinformatics and Biotechnology tools and applications (Eds. S.J.Eapen et al) Department of Biotechnology, 2-23rd Dec 2003, IISR, Calicut | Department of Biotechnology | 2003 |
| 4 | Kumar, A., Anandaraj, M. and V. A. Parthasarathy (2003) Computational tools for molecular analysis of bacterial diversity: *Ralstonia solanacearum* – a case study In: *Proceeding of Workshop on Agricultural Bioinformatics* (Eds. S.J.Eapen, K.N.Babu and A.I. Bhat) 29-30 Oct 2003, Calicut p: 56-59 | Department of Biotechnology | 2003 |
| 5 | Kumar, A. (2003) Microbial Genomics. In: *Training Manual on Bioinformatics and Biotechnology- Tools and Applications.* (Eds. S.J.Eapen) IISR, Calicut, p: 52-57 | IISR, Calicut | 2003 |
| 6 | Kumar, A. (2003) Molecular approaches for identification and detection of bacterial pathogens affecting spice crops. In: *Training Manual on Techniques in biochemistry and biotechnology* (Eds. J.K. George and A. I. Bhat) IISR, Calicut, p: 184-188 | IISR, Calicut | 2003 |

| | | | |
|---|---|---|---|
| 7 | Kumar, A and M. Anandaraj, (2005) Biocontrol, Biofertilizer production and indexing plant for viruses sponsored by SDC-IC- NGO Programme, Kerala (6-10th Feb 2005) | IISR, Calicut | 2005 |
| 8 | Sharma, Y.P., Kumar, A., Yadav, D.S., Sasikumar, B., Tripathi, A.K., Anandaraj, M., Sanwal, S.K and V.A.Parthasarathy 2006 *Production and Marketing of Ginger- Technical Bulletin No-24.* Published by ICAR-RCNEH, Barapani and IISR, Calicut | ICAR-RCNEH, Barapani | 2006 |
| 9 | Krishnamurthy. B. and A. Kumar, (2004) Summer training on Techniques in biochemistry and biotechnology for summer trainees (5th May to 4th June 2004) | IISR, Calicut | 2004 |
| 10 | Dake, G.N., Anandaraj, M., Raju, C.A & Iyer, R 1994. Effect of seed treatment and lining material on rhizome rot of ginger in storage. In: International Symposium on Plantation crops (PLACROSYM-X1) | NRCSCALICUT | Nov 30- Dec 3, 1994 |
| 11 | Dake, G.N. 1994 Diseases of ginger and their management In: national seminar on diseases of spices | NRCS, Calicut | April 7-8 1994 |

**8234 Seminars, conferences and workshops (relevant to the project) in which the scientists have participated. (List abstracts forwarded)**

| Sl no | Title | Title | Year |
|---|---|---|---|
| 1 | Methods for screening ginger for bacterial wilt resistance. IISR, Calicut | Commercialization Of Spices, Medicinal And Aromatic Crops (SYMSAC-1) | 1-2 Nov 2004, |
| 2 | Ecofriendly approaches for plant disease management | CAS in Botany, Chennai | 22-24 Jan 2001 |
| 3 | Dake, G.N., Anandaraj, M., Raju, C.A & Iyer, R 1994. Effect of seed treatment and lining material on rhizome rot of ginger in storage. In: International Symposium on Plantation crops (PLACROSYM-X1) | NRCS, CALICUT | Nov 30- Dec 3, 1994 |
| 4 | Dake, G.N. 1994 Diseases of ginger and their management In: national seminar on diseases of spices | NRCS, Calicut | April 7-8 1994 |
| 5 | Symposium on Recent developments in the diagnosis and management of Plant diseases for meeting global challenges | Indian Phytopathological Society, UAS Dharwad | 18-20th December 2003 |

**824 Infrastructural facilities developed** (Details of field, laboratory, notebooks and final material and their location)

- Three green houses were established in IISR, Chelavoor and IISR, Peruvannamuzhi for conducting ginger pot culture experiments.
- Several field trials were conducted in IISR, Peruvannamuzhi

- Three note books and final culture collections kept in the division under my custody (The cultures are kept in minus 80°C)
- Many post graduate students were trained during the project period on the basic bacteriological techniques
- Information generated from the project was exploited for obtaining fund from IFS in the form of a new project on rhizome treatment.

**825 Comments / Suggestions of Project Leader regarding possible future line of work that may be taken up arising out of this Project**

- The rhizome treatment methodology can be scaled up to accommodate large volume of rhizome material
- Resistance may be searched from other members of the Zingiberaceae members
- Collection from other parts of the country such as northeastern states of India can be compared with the collection from south India

**Part-IV : Project Expenditure**
**(Summary)**
**Year**

**830 Total Recurring Expenditure**

8301 Salaries: (Designation with pay scale)

| | | Estimated | Actual |
|---|---|---|---|
| i) | Scientific | 1200000 | 100000 |
| ii) | Technical | 400000 | 300000 |
| iii) | Supporting | 150000 | 150000 |
| iv) | Wages | - | - |
| **Sub-Total** | | 1750000 | |
| **8302 Consumables** | | | |
| i) | Chemicals | 300000 | 300000 |
| ii) | Glassware | 300000 | 300000 |
| iii) | Others | - | |
| Sub-Total | | 2,0,0000 | |

| | | |
|---|---|---|
| 8303 | Travel | |
| 8304 | Miscellaneous (Other costs) | - |
| 8305 | Sub-Total (Recurring) | 23,50,000 |
| **831** | **Total Non-Recurring** | **Nil** |
| **823** (830 and 831) | **Total** | 23,50,000 |

**Part-V: Declaration**

This is to certify that the final report of the Project has been submitted in full consultation with the Project workers as per the approved objectives and technical programme and the relevant records, note-books, materials are available for the same.

Signature of the Project Investigator: A. Kunire

Co-Investigators 1. M. Anandaraj

2. R. Suseela Bhai

Signature & Comments of the Head Of the Division/ Section: The work has been completed as per the technical programme. All the laboratory records and cultures are maintained in the Division.

Signature & Comments of the Joint Director (Research): Sreevasthayam

Signature & Comments of the Director: 26/8/06

[V. A. Parthasarathy]
Director
Indian Institute of Spices Research
Calicut - 673 012, Kerala

*Paper No. 4 (Poster)*

## EFFECT OF SEED TREATMENT AND LINING MATERIAL ON RHIZOME ROT OF GINGER IN STORAGE

G.N. DAKE, M. ANANDARAJ, C.A. RAJU[1] and ROHINI IYER[2]

*National Research Centre for Spices, Calicut 673 012, Kerala, India.*
*[1]Present address : Central Tobacco Research Institute, Rajahmundry 533 101, Andhra Pradesh, India.*
*[2]Present address : Central Plantation Crops Research Institute, Kasaragod 671 124, Kerala, India.*

Seed rhizomes of ginger (*Zingiber officinale* Rosc.) have to be stored to use it as seed material during the next season. During storage, the rhizomes get deteriorated if colonized by micro-organisms and cause heavy losses. The results of trials on storage involving seed treatment with fungicides and lining materials are presented. Two fungicides, Bavistin 50 WP (0.3%) and Dithane M-45 (0.3%) and three lining materials viz., sand, saw dust, and paddy husk were evaluated.

The highest percentage of healthy rhizomes was obtained with Dithane M-45 (0.3%) as dip treatment for 20 min, and the lowest percentage of infected rhizomes was observed in rhizomes stored in pits lined with saw dust.

*Paper No. 5 (Poster)*

## EFFECT OF SEED DRESSING FUNGICIDES AND ORGANIC AMENDMENTS ON RHIZOME ROT OF TURMERIC

T.A. SREERAMA SETTY, E. MOHAN, P.S. HERLE and N.S. PARAMESHWAR

*University of Agricultural Sciences, Regional Research Station, Brahmavar, Karnataka, India.*

Rhizome rot of turmeric (*Curcuma longa* L.) caused by *Pythium* spp. leads to considerable economic losses. To develop control measures, field trials were conducted with fungicides viz., Blitox, Emisan, Captan and different formulations of metalaxyl along with soil amendments such as press mud and neem cake. The results showed that metalaxyl formulations were superior to control in reducing disease incidence and increasing the yield. Among soil amendments, press mud gave higher yield with reduced disease incidence followed by neem cake.

# INTEGRATED MANAGEMENT OF BACTERIAL WILT OF GINGER

## INTRODUCTION

In India ginger (*Zingiber officinale* Rosc.) is infected with bacterial wilt caused by *Pseudomonas solanacearum* (E. F. Smith), which is the most serious disease causing heavy losses in yield. A severe outbreak of bacterial wilt caused by *P. Solanacearum* biotype III was noticed during 1978 at Ambalavayal in Wynad district which spread into other major ginger growing areas of Kerala.

## SYMPTOMATOLOGY

The conspicuous are flaccidity and curling of leaf margins. Yellowing starts from lower most leaf and gradually progresses to upper leaves. The pseudostem at collar region becomes water soaked and breaks away from the rhizome at ground level. In the advanced stage, plants exhibit severe yellowing and wilting symptoms. Vascular tissues of affected pseudostems show dark streaks. The affected pseudostem or the rhizome when pressed gently, exudes the milky ooze from the vascular strands, since both fungal and bacterial infection occurs simultaneously in nature. Wrong identity and thereby the wrong choice of the chemical quite often render control measures ineffective. The soft rot can be distinguished from bacterial wilt by the absence of milky bacterial ooze when the rhizome or pseudostem is cut transversely.

## CONSTRAINTS

Since the disease is both soil and seed borne, it is less amenable to control. In many ginger growing tracts especially in Wynad district of Kerala, much of the available land is already infected. This is particularly true in small holdings where land scarcity restricts crop rotations. The benefits of expensive pathogen free seed rhizomes are negated with the limited land available for growing ginger, already infested as a result of previous crops or weed hosts. Moreover, *P. solanacearum* is carried over long range vegetative propagating materials. Use of such unregulated infected ginger rhizomes as planting material has amplified and extended a severe disease problem in Kerala.

Absence of a foolproof technique to detect seed borne inoculum in apparently normal rhizomes is another handicap. Ideally, the bacterial wilt can be avoided by ensuring that the soil and seed rhizomes are free of the pathogen. At present, farmers rely mostly on presence or absence of symptoms to indicate whether soils and harvested rhizomes are pathogen free. In practice, routine techniques used to detect the bacterium in soil and plant materials have lacked the level of sensitivity required for reliable seed rhizome selection. The movement of infected seed rhizomes from infected to non-infected areas continues to abet the large scale spread of *P.solanacearum* throughout Kerala.

Ginger is propagated exculsively by vegetative means because of lack of seed set. Hence, the conventional approach in ginger breeding and selection for disease resistance are

especially difficult. Poor genetic base with little variability for disease resistance and absence of seed set are a few of the hurdles in the crop improvement programme.

Antibiotics streptomycin sulphate/streptocycline (200 ppm) are effective to inhibit the bacterial growth *in vitro* but are ineffective *in vivo* because of fairly heavy and well distributed showers during the crop growth period June-October. The bacterium *P.solanacearum* gets inhibited at a particular concentration only. Therefore, if there is heavy downpour after drenching, the applied/drenched antibiotics either get diluted or washed away, resulting in poor control. Bacteriocides so for tried as seed treatment as well as drenches were found less effective and uneconomical. Being a soil and seed borne disease, it is very difficult to eliminate bacterium through chemicals. Moreover it is not cost effective.

In Kerala, the crop is rainfed and cultivated from sea level to an altitude of 1500 m. The crop at sprouting stage is more vulnerable to infection because of its tender and succulent tissues. A warm and humid climate predisposes the plant to infection. Bacterial wilt of ginger in the field may either originate from infected seed rhizomes or soil. Once the plant is infected, it results in total loss of clumps. The spread is non random, typical of soil borne disease. The spread is through soil water and along the gradient of the field. But water splashes also aid the spread even against the gradient. It is clear that the warm humid climate and rainfall help to produce bacterial ooze more rapidly from infected pseudostems and in disease spread to the adjacent plant within the bed through film of water from clump to clump as the distance between plant to plant is short. The bacterial ooze gets mixed in film of water and gets dispersed alongwith gradient slope. The severity of infection depends on quantity of inoculum produced at the start of initial infection, favourable weather for pathogen and subsequent infection and rain splash to dispear the bacterium during this period.

## MANAGEMENT STRATEGIES

### CULTURAL METHODS

1. The disease is severe when a ginger is grown every year on the same land because of persistence of the pathogen in soil. Rotate ginger with crops which are not susceptible to bacterial wilt. More over, being an exhaustive crop, it is not desirable to grow ginger in the same site year after year.

2. Poor drainage and water stagnation predispose the crop to infection. Heavy soils with high moisture holding capacity are conductive for disease development. The disease incidence is more during June-October coinciding with south-west monsoon. Therefore, well drained seed bed sites should be selected. Provision of adequate drainage channels in the plot is a must to avoid disease incidence and spread.

3. Healthy rhizomes from disease free ginger area are to be selected as seed rhizomes. At the time of harvest, rouging of suspected infected clump is a must to prevent carry over of inoculum to subsequent ginger crop.

4. Treat the selected seed rhizomes with streptocycline (200 ppm) for 30 minutes, drain off excess solution and shade dry the rhizome.

5. The root knot nematode infestation in ginger enhances development of bacterial wilt. Care should

be taken to control nematode infestation.

6. Many weeds are getting infected with bacterium with or without showing any symptoms. Therefore, ginger field should be kept free from weed hosts to minimize infection. Mulching and earthing up are to be carried out at 40 and 90 days after planting, immediately after weeding and application of fertilizers. If sufficient care are not taken properly, the disease may spread through contaminated equipments/ implements. All tools and equipments used in the seed beds must be disinfected for effective reduction in spread of disease and minimize loss in yield, otherwise inoculum potential will reach excessive levels leading to severe crop loss.

7. Phytosanitary measures are to be takenup once the disease is noticed in the field. Several times the outbreak in poorly managed infected seed beds leads to high inoculum potentials at test sites. The diseased clumps should be removed and soil surrounding this should be drenched with suitable antibiotics as spot application to minimize further spread of the disease.

## CHEMICAL CONTROL

Studies carried out so far indicated that treatment of seed rhizomes with streptocycline (200 ppm) and subsequent drenches with 10 litres/3sq m kept the disease under check for three months, but subsequently they succumbed to disease. Soil treatment with streptocycline, bordeaux mixture (1%) or application of bleaching powder was partly effective but not cost effective.

## RESISTANCE

Disease resistance has not been reported so far in the germplasm material tested against *P. solanacearum*

Studies are in progress for evolving disease resistant types by inducing somclonal variation through callus and cell culture and also to identify resistance to *P. solanacearum* using toxins to screen the cultures and callus for resistance.

## BIOLOGICAL CONTROL

Efforts are on to identify avirulent strains of *P.solanacearum* for using in biological control of the disease. There is scope for utilizing biological control agents as complementary strategies to manage bacterial wilt caused by *P.solanacearum* (biotypes III&IV), in combination with resistant cultivars, crop rotation with non host crops, nematode and weed control, establishment of strict quarantine measures (restriction on movement of infected seed rhizomes), improved seed storage and farm management practices.

## CONCLUSION

Under the above situation, integrated management strategy incorporating one of the resistant cultivars and adopting appropriate cropping sequences and cultural practices can alter suitability of the micro-environment conducive to the pathogen and hence reduce disease incidence. Efficiency of a particular system is location specific, hence, it must be adopted to suit local climate, soil type, pathogen strain, farming system and the socio-economic situation.

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# DISEASES OF GINGER AND THEIR MANAGEMENT

G N Dake

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Ginger crop suffers from many diseases of fungal and bacterial origin. Of them, soft rot caused by Pythium aphanidermatum and bacterial wilt by Pseudomonas solanacearum are of economic importance because of their potential to cause great losses to ginger production.

The constraints in disease management and the strategies that have been developed to control the diseases involving cultural, chemical and biological methods combined with soil solarization are discussed. Future options for the management of ginger diseases by integration of conventional strategies with modern biotechnological means for effective control measures are also discussed.

# Bacterial wilt of *Kaempferia galanga* L. caused by *Pseudomonas solanacearum* (Smith) Smith[1]

G N DAKE & P S MANOJ [2]

*Indian Institute of Spices Research*
*Calicut - 673 012, Kerala, India.*

ABSTRACT

*Pseudomonas solanacearum* is reported for the first time causing bacterial wilt of Kaempferia (*Kaempferia galanga*) from Kerala, India.

Key words : bacterial wilt, *Kaempferia galanga*, *Pseudomonas solanacearum*.

Kaempferia ('Kacholam') (*Kaempferia galanga* L.) is cultivated throughout the plains of India for its aromatic rhizomes. During September 1994, a few plants of *K. galanga* were found showing wilt symptoms at Athiyodi in Kozhikode District of Kerala State, India. The initial symptoms appeared as water soaked lesions on petioles and spread to all leaves resulting in death of the plant. The rhizomes when cut open transversely exuded ooze profusely. The rhizomes and roots of affected plants exhibited varying degrees of rotting.

The pathogen was isolated on nutrient agar. The isolates were further restreaked on triphenyl - tetrazolium chloride medium and light pink centered colonies were maintained on Yeast Glucose Carbonate Agar in sterile distilled water at 4°C in refrigerator. Koch's postulates were proved on healthy plants with a bacterial suspension of $10^8$ cfu/ml. Based on morphological, cultural and physiological characters (Sands 1990) the pathogen was identified as *Pseudomonas solanacearum* (Smith) Smith. Further, the strain was characterized as *P. solanacearum* biovar 3 on the basis of differences in biochemical reactions (Hayward 1964). Even though the pathogen has been reported on a number of Zingiberaceous hosts, this is the first report of the pathogen on this plant.

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# Diseases of ginger (*Zingiber officinale* Rosc.) and their management

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ABSTRACT

Ginger (*Zingiber officinale* Rosc.) suffers from 24 diseases of fungal, bacterial, viral and mycoplasmal origin. Of them, soft rot (*Pythium aphanidermatum*), yellows (*Fusarium oxysporum* f. sp. *zingiberi*), bacterial wilt (*Pseudomonas solanacearum*), *Phyllosticta* leaf spot (*Phyllosticta zingiberi*) and storage rot by many pathogenic, saprophytic fungi and bacteria are of economic importance because of their potential to cause great losses to ginger production. The constraints in disease management and the strategies that have been developed to manage the diseases involving cultural, chemical and biological methods combined with soil solarization are discussed. Future options for the management of ginger diseases by integration of conventional strategies with modern biotechnological means for effective control measures are also discussed.

Key words: diseases, ginger, management, *Zingiber officinale*.

## Introduction

Ginger (*Zingiber officinale* Rosc.) is an important commercial crop grown in Kerala, Karnataka, Tamil Nadu, West Bengal, Bihar, Uttar Pradesh, Himachal Pradesh, Madhya Pradesh, Meghalaya, Sikkim and Orissa for its aromatic rhizomes which are used both as spice and medicine.

Ginger is affected by many diseases (Iyer 1988). Of them, rhizome rot, bacterial wilt, yellows, *Phyllosticta* leaf spot and storage rot are major diseases which cause economic loss and are dealt in this paper.

## Distribution and crop losses

Rhizome rot caused by *Pythium* spp., yellows by *Fusarium* spp. and bacterial wilt by *Pseudomonas solanacearum* are serious diseases in most of the ginger growing areas and cause great losses to ginger, because once the plants are infected, they result in total loss of clumps. In Kerala, bacterial wilt and rhizome rot are prevalent in major ginger areas (Dake & Edison 1988).

## Epidemiology

The pathogens responsible for soft rot,

bacterial wilt and yellows are soil inhabitants invaders/ dwellers having high degree of competitive saprophytic ability. Being seed and soil borne in nature, the infection of these diseases in the field may either originate from infected seed rhizomes or soil. The spread of the disease is through soil, rain, irrigation water or rain splash to the adjacent plant within a bed as well as along the gradient of the field.

## Effect of climatic factors

The ginger crop is rainfed as well as irrigated. A warm and humid climate predisposes the plant to infection at sprouting stage, because of its tender and succulent tissues. The spread is typical of soil borne diseases because of fairly heavy and well distributed showers during the crop growth period June to October

### Diagnosis

The fungal (*Pythium* spp., *Fusarium* spp.) and bacterial (*P. solanacearum*) infections occur simultaneously in field (Dake & Edison 1989). Proper diagnosis of diseases is essential for their management and prevention (Dake, Ramachandran & Sarma 1988). The first conspicuous symptom of bacterial wilt (to differentiate it from fungal infection) is flaccidity and curling of leaf margins downward. The colour of leaves remain dark green in bacterial wilt whereas in case of fungal infection the infected plants turn to pale yellow. The fungal infection can also be distinguished from bacterial wilt by the absence of milky bacterial ooze when the rhizomes or pseudostems are cut transversely.

### *Phyllosticta* leaf spot

Leaf spot of ginger caused by *Phyllosticta zingiberi* Ramkr. is observed on leaves especially when the crop is grown under exposed conditions. The disease starts as water soaked, oval to elongated spots and later turn as whitish spots surrounded by dark brown margin with an yellowish halo. The pycnidia appear on the mature lesions and remain viable for about 14 months in leaf debris. The spores ooze out into water drops on the leaves and get dispersed through rain splashes (Brahma & Nambiar 1982 & 1984)

Dohroo *et al.* (1986) reported that none of the ginger types screened was found resistant to *P.zingiberi*. However, Premanathan, Peethambaran & Abi Cheeran (1982) found that the cultivars Maran and Karakkal are comparatively resistant to *Phyllosticta* leaf spot. The disease can be managed by one or two sprays of Bordeaux mixture (1%) (Ramakrishnan 1942; Sohi, Sharma & Varma 1973).

### Storage rot

Seed rhizomes of ginger have to be stored for about 5 months from harvest in December to planting in April - May. During storage, the rhizomes are subjected to moisture loss and also deterioration if colonised by microorganisms (Haware & Joshi 1974; Sarma & Nambiar 1974). To check such deterioration and moisture loss during storage, several practices have been recommended (Joshi & Sharma 1982; Dake *et al.* 1989).

### Disease management

An inegrated disease management programme involving cultural, chemical and biological methods combined with disease resistance is called for, to minimise crop losses and thus increasing the yield of ginger.

*Cultural*

Selection of seed material

These diseases apparetly perpetuate through infected rhizomes and this serves as primary source of inoculum in the newly cultivated field of ginger. The use of rhizomes from disease free areas to prevent carry over of inoculum to subsequent ginger crops has been recommended to control soft rot caused by *Pythium* spp. (Park 1941), yellows by *Fusarium oxysporum* f. sp. *zingiberi* (Rana 1991) and bacterial wilt by *P. solanacearum* (Pordesimo & Raymundo 1963).

Crop rotation

Soil borne diseases are severe when ginger is grown every year on the same land because of the persistence of the pathogen in soil. Pordesimo & Raymundo (1963) suggested crop rotation to control bacterial wilt of ginger. Quimio and Chan (1979) found that rice and corn are reliable rotation crops with susceptible host species to minimize the incidence of bacterial wilt.

Organic amendments

The incorporation of various organic amendments was found effective in reducing the incidence of soft rot caused by *P. aphanidermatum* and increase in yield (Balagopal *et al.* 1974; Ghorpade & Ajiri, 1982). Ghorpade & Ajiri (1982) and Thakore *et al* . (1987) reported that amendments of oil cakes made from *Azadirachta indica, Calophyllum inophyllum, Pongamia glabra, Hibiscus subdariffa* and *Brassica campestris* were effective in reducing the incidence of rhizome rot caused by *F. solani,* and increasing yield of ginger crop.

Suppressive soils

Lee, Cheong & So (1990) reported that higher clay content and lower pH in soil from Eunhari is suppressive to *P. zingiberum* and *F. oxysporum* f. sp. *zingiberi* than the conducive soils in Korea. Power (1983) reported that bacterial wilt never occurs on the sea - shell ridges of the coastal plain of Surinam.

Elimination of weed hosts

Many weed hosts of *P. solanaccarum* are symptomless carriers, wherein bacteria survive in the rhizospheres of these weed hosts (Quinon, Aragaki & Ishii 1964; Ishii & Aragaki 1963; Zehr 1969; Moffett & Hayward 1980). Pegg & Moffett (1971) and Indrasenan *et al.* (1981) suggested removal of weed hosts of *P. solanaccarum* to check the disease spread.

Soil solarization

Soil solarization has been successfully utilized using solar heating by polythene mulching for 40 days in April - May. It was found that disease incidence was reduced and germination percentage and yield of ginger were increased in solarized plots compared to non- solarized plots (NRCS 1993).

Planting in raised beds

Poor drainage and water stagnation predispose the crop to infection. Well drained raised beds and provision of adequate drainage channels in the fields are recommended.

Phytosanitation

Phytosanitary measures are to be taken once the diseases are noticed in the field. Roguing diseased plants and destroying them will help in reducing the disease. All the tools used for earthing up of infected beds are to be disinfected to check the spread of inoculum to healthy beds.

*Chemical*

Soft rot

Treating seed rhizomes with Dithane M - 45 (0.3%) for 30 min and soil drenching with the same fungicide at same concentration have been recommended for the control of soft rot (NRCS 1986). In pot culture experiment, application of metalaxyl formulations, namely, Ridomil 5 G (soil application) and Apron 35 WS (Seed treatment) gave best control of rhizome rot in *Pythium* infected soil (Ramachandran, Dake & Sarma 1989).

Yellows

Haware & Joshi (1974) recommended dipping seed rhizomes in fungicidal suspension of Dithane M - 45 (0.3%) or Benelate for the control of rhizome rot caused by *F. oxysporum* f. sp. *zingiberi*. Rajkumar & Pandey (1989) found best control of rhizome rot caused by *F. oxysporum* when seed rhizomes were treated with Topsin M-70 (1%) combined with soil drench with formaldehyde (4%).

Bacterial wilt

Dake, Ramachandran & Sarma (1988) reported that treatment of seed rhizomes with streptocycline 200 ppm and soil drenching with streptocycline or application of bleaching powder was partially effective to keep the disease under check for 3 months. Ishii & Aragaki (1963) observed that soil fumigation with methyl bromide at 1.362 kg/ 1.21 sq m checked the disease.

*Biological*

Soft rot

*In vitro* antagonistic effect of *Trichoderma* spp. against *Pythium* spp. was reported by Thomas (1939). The disease incidence of *Pythium* rot was less and yields were higher in beds treated with *Trichoderma* spp. and *Gliocladium virens* compared to beds that received Dithane M - 45 and untreated control in soil solarized plots (NRCS 1993).

Yellows

The use of some strains of fluorescent Pseudomonads against *Pythium* spp. and *Fusarium* spp. is well documented (Hagedora, Gould & Bardinelli 1989; Kaiser, Hannan & Weller 1989; Howell & Stipanovic 1980). However further studies are required to test their efficacy in suppressing diseases in ginger.

Bacterial wilt

Sekhawat *et al.* (1992) showed the possibilities of biological managment of potato bacterial wilt using strains of *Bacillus* spp., *B. subtilis*, *Pseudomonas fluorescence* and actinomycetes. Kempe & Sequeira (1983) and McLanghlin & Sequeira (1988) used the antagonisitic avirulent mutants of *P. solanaccarum* to induce resistance against *P. solanaccarum* causing bacterial wilt in potato.

Storage

Seed treatment with *Trichoderma* spp. was effective in controlling rhizome rot of ginger in storage (Bhardwaj *et al.* 1988).

## Resistance

None of the varieties screened against *Pythium* spp. (Nybe & Nair 1979; Sarma, Nambiar & Brahma 1980), *Fusarium* spp. (Rana & Arya 1991) and *P. solanaccarum* (Indrasenan *et al.* 1982) was resistant. However the cultivars Maran, Nadiya and Narasapattom were found resistant to moderately resistant to *P.*

*aphanidermatum* (Indrasenan & Paily 1973; Balagopal *et al.* 1974). In artificially inoculated field conditions, China, Rio-de-Janeiro, Jorhat, Thingpui, Maran, Tura and Amadi were reported resistant to *P. solanacearum* (Sinha *et al.* 1990). Attempts are being made to select toxin resistant cells by culturing ginger cells in the presence of toxic compounds isolated from *P. solanacearum* and *P. aphanidermatum* (NRCS 1993).

## Constraints

*Production of disease free seed rhizomes*

Non-availability of disease free planting material is a major constraint in the cultivation of ginger and there are at present no agencies involved for producing and distribution of quality seed rhizome material.

*Lack of disease detection technique*

At present no technique is available to detect seed borne pathogens. Farmers very often find it difficult to raise disease free planting material and rely mostly on presence or absence of symptoms to indicate whether the harvested rhizomes are pathogen free or healthy.

*Resistant varieties*

None of the varieties available for cultivation are resistant to soft rot, yellows and bacterial wilt. Moreover ginger is propagated exclusively by vegetative means, because of lack of seed set, and the conventional approach for breeding and selection for disease resistance is also a stumbling block in the development of varieties resistant to these diseases.

*Lack of control measures*

The etiology of these soil borne diseases is well understood but there is no effective control measure to save the crop in the field. Once the plants/clumps get infected it results in complete rotting. The technology in respect of management of these soil borne diseases has to be improved to increase productivity of ginger.

## Future strategies

1. Production and distribution of disease free seed material is one of the best methods to promote production. Certified seed plots for this purpose have to be established in disease free locations.

2. Development of sensitive techniques such as DNA probes for the detection and differentiation of pathogens involved in rhizome rot complex.

3. Breeding for disease resistant is difficult through conventional breeding methods due to absence of seed set. Exploitation of somaclonal variation for *in vitro* selection for disease resistance has to be done to incorporate resistance in integrated disease management programmes.

4. Soil solarization, which is a new approach for disinfestation of soil has to be incorporated in disease management programmes involving resistance, biological, chemical and cultural methods of management of diseases.

5. The integration of conventional strategies with modern biotechnological means for effective management of disease is essential. The potential of *Gliocladium*, *Trichoderma*, *Bacillus* and fluorescent *Pseudomonas* spp. in combating these soil borne diseases of

ginger are well documented. Manipulation of these strains for greater effectivity and field stability has to be exploited.

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# Detection of *Ralstonia solanacearum* in ginger rhizomes using post-enrichment NCM-ELISA

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**Abstract**

Bacterial wilt, caused by *Ralstonia solanacearum*, is an important production constraint in ginger. The pathogen is seed rhizome borne and the low level of inoculum is very difficult to be detected and monitored by conventional methods. Pathogen detection and selection of healthy planting material is an important prerequisite for the production of a healthy crop of ginger in the field. In the present work we have evaluated the suitability of NCM-ELISA kit, developed at International Potato Center (CIP), Lima, Peru, for detecting bacterial wilt pathogen in ginger. The result indicated that the antibodies developed at CIP for potato strain of *R. solanacearum* was sensitive enough to detect *R. solanacearum* from ginger, chilli, *Chromolaena* and tomato. The sensitivity of the kit was determined to be 42 cells per ml of ginger extract when ELISA was performed after enrichment in selective medium. We have also tested the specificity of antibodies and found that the antibodies were specific for *R. solanacearum*. The extraction protocol (citrate buffer at pH 5.6) developed for potato was found to be suitable for ginger also.

**Key words:** bacterial wilt, ginger, NCM-ELISA, *Ralstonia solanacearum*, rhizome borne disease.

## Introduction

Bacterial wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* 1996 is one of the important diseases that limits the production of ginger in India and many other tropical countries. Bacterial wilt is particularly severe in countries like India, Indonesia, Malaysia and China because of unregulated movement of latently infected seed rhizomes which is mainly responsible for the spread of the disease (Hayward 1991). Ginger is cultivated in virgin soil or in soil after a reasonable period of fallow. Incidence of wilt noticed in such fields, clearly reveals the rhizome-borne nature of *R. solanacearum* in ginger. Since there are no chemicals or biocontrol agents presently available for the management of bacterial wilt, the main practical approach to ensure a healthy ginger crop is through the planting of bacterial wilt - free seed rhizomes.

Many sensitive detection techniques have been developed for monitoring pathogens in seed tubers of potato (Seal & Elphinstone 1994; Priou *et al.* 1999b). However, no such technique is available for testing ginger seed rhizomes for the presence of *R. solanacearum* in India. The technique should be sensitive enough to detect a very low level of inoculum in the seed rhizome and also the technique should be easy to be performed even in poorly equipped

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laboratories. In the present work we have tested the suitability of the post-enrichment NCM-ELISA kit developed at the International Potato Centre (CIP), Lima, Peru for the detection of *R. solanacearum* in latently infected potato tubers, to detect the pathogen in ginger seed rhizome. The objectives of the work were to test (i) the ability of antibodies produced at CIP against potato isolates of *R. solanacearum* to detect isolates of *R. solanacearum* from ginger and other hosts and (ii) the suitability of the pathogen extraction protocol developed for potato tubers to ginger rhizome.

## Materials and methods

### R. solanacearum *strains*

Twenty strains of *Ralstonia solanacearum* were isolated from ginger, tomato and *Chromolaena* (Table 1). These isolates belonged to biovar III with the exception of some isolates obtained from Sikkim that were aberrant biovar III because they did not utilize dulcitol as a carbon source. The ginger strains were isolated from the major ginger growing locations in Kerala, Assam, Sikkim, West Bengal and Andhra Pradesh. Typical colonies of *R. solanacearum* (white fluidal colony with pink centre) from pure cultures on Kelman's medium (Kelman 1954) were maintained at 4° C in sterile distilled water and also in 20% glycerol at - 80° C.

### *NCM-ELISA*

In order to know the specificity of the antibodies produced at CIP to recognize ginger isolates, pure cultures of 20 ginger isolates of *R. solanacearum* (Table 1) were tested using NCM-ELISA described by Priou & Gutarra (1998).

An 8 x 12 cm of 0.45 mm pore size nitro cellulose membrabe (NCM) (Biorad) was immersed for 5 minutes in 30 ml Tris Buffered Saline buffer (TBS) (0.02 mol $l^{-1}$ Tris-HCl [pH 7.5], 0.05 mol $l^{-1}$ NaCl, 0.01% $NaN_3$) for NCM-ELISA. Twenty µl of the samples were put on the membrane manually, transferred to a dry filter paper and air-dried for at least 60 minutes. The NCM dotted with the samples was incubated for 1 h in 30 ml of the blocking solution (2% non-fat powdered milk in TBS buffer) in a petridish (15 cm dia.), with gentle rotary agitation (50 rpm) and the membrane was incubated for 2 h with gentle agitation in 30 ml of the antibody solution (100 ml of *R. solanacearum*-specific antiserum diluted 1 : 1000 was added to another 30 ml of the same blocking solution). The membrane was washed to remove the unbound *R. solanacearum* antibodies with 30 ml T-TBS (TBS with 0.05% Tween-20) three times for 3 minutes each with constant agitation at 100 rpm. Then, the membrane was incubated for 1 h with gentle agitation with 30 ml of the conjugated solution, containing goat-antirabbit antibodies conjugated to alkaline phosphatase (Biorad), diluted 1 : 4000 in 30 ml of the blocking solution. The membrane was rinsed three times for 3 minutes each with T-TBS, with constant agitation (100 rpm) to remove the unbound conjugate. During the last washing, the colour development (substrate) solution was prepared by adding drop by drop while agitating, first 100 ml of NBT (p-nitro blue tetrazolium) solution and then 100 ml of BCIP (p-toluidine salt of 5-bromo, 4-chloro, 3-indolyl phosphate) solution in a dark flask containing 25 ml of substrate buffer (0.1 mol $l^{-1}$ Tris base, 0.1 mol $l^{-1}$ NaCl, 0.005 mol $l^{-1}$ $MgCl_2$, $6H_2O$, pH 9.6). The membrane was then incubated with 25 ml of the substrate solution with gentle agitation for 5 to 30 minutes. The reaction was stopped by discarding the substrate solution and by rinsing the membrane thoroughly with tap water. The membrane was then placed on filter paper sheets to dry.

### *NCM-ELISA of ooze from infected plants*

Twenty ml of bacterial oozes obtained from different infected hosts were dot-blotted on nitrocellulose membrane and the ELISA was performed as described above. The test was also performed with extracts from healthy ginger samples from different localities as well as from healthy plants adjacent to severely wilted plants.

### *Sensitivity of the test*

In order to test the presence of low numbers of the bacterium, enrichment was performed by

**Table 1.** Isolates of *Ralstonia solanacearum* tested using CIP NCM-ELISA kit

| Isolate | Crop | Location | Biovar | Serological Reaction |
|---|---|---|---|---|
| GRS Tms | Ginger | Thamarassery, Kerala | III | + |
| GRS Ktm1 | Ginger | Kothamangalam, Kerala | III | + |
| GRS Pul | Ginger | Pulpally, Kerala | III | + |
| GRS Vyt | Ginger | Vythiri, Kerala | III | + |
| GRS Ktm2 | Ginger | Kothamangalam, Kerala | III | + |
| GRS EW | Ginger | Sikkim | III dul | + |
| GRS SW 18 | Ginger | Sikkim | III dul | + |
| GRS NWR | Ginger | Sikkim | III dul | + |
| GRS ISPS | Ginger | Sikkim | III dul | + |
| GRS White | Ginger | Sikkim | III dul | + |
| GRS Zah | Ginger | Zahirabad, Andhra Pradesh | III | - |
| GRS Mdm | Ginger | Madakimalai, Kerala | III | - |
| GRS Jor | Ginger | Jorhat, Assam | NT | - |
| GRS Kki | Ginger | Kahikuchi, Assam | III | + |
| GRS Soma | Ginger | Vythiri, Kerala | III | + |
| GRS Tms M1 | Ginger, Spontaneous EPS⁻ mutant | Thamarassery, Kerala | III | + |
| GRS Vyt Rif$^R$ | Ginger, Spontaneous rifamycin resistant mutant | Vythiri, Kerala | III | + |
| GRS Tms M2 | Ginger, Spontaneous EPS⁻ mutant | Thamarassery, Kerala | III | + |
| TRS Cal | Tomato | Peruvannamuzhi, Kerala | III | + |
| ERS Cal | *Chromolaena* | Peruvannamuzhi, Kerala | III | + |

+ Purple colour comparable to that of positive control (loaded with 20 ml of a water suspension of *R. solanacearum* potato strain CIP204, biovar 2A, at $10^8$ cells $ml^{-1}$), - No colour development, NT: Not tested

incubating the extract in selective medium (Elphinstone *et al.* 1996) at 30° C with constant agitation (Priou *et al.* 1999a & b). A water suspension of *R. solanacearum* strain GRS Km1 (Biovar III) at 4.15 x $10^8$ cells per ml was serially diluted until a final concentration of 0.0415 cells per ml of ginger extract was obtained. Five hundred ml of diluted *R. solanacearum* suspension was added to 500 ml of SMSA. The broth was incubated at 30° C with constant agitation for three days. One set of tubes was frozen away immediately after addition of bacterial cells and also after every 24 h upto 72 h. Twenty ml of the culture was dot-blotted on to NCM and the assay was performed as described earlier. The extract of ginger rhizome in citrate buffer without any enrichment was included as a check.

*Specificity of NCM-ELISA*

Specificity of Rs. antibodies raised against potato strain of *R. solanacearum* was tested using 121 bacterial isolates obtained from Bacterial Repository, Indian Institute of Spices Research, Calicut. These isolates were isolated from rhizosphere soils or infected plants of ginger, black pepper and cardamom from states of Kerala, Karnataka, Tamil Nadu, Sikkim, West Bengal, Himachal Pradesh and Assam. A loopful of bacterium was inoculated in 5 ml of nutrient broth for 12-15 h at 28° C with constant agitation and incubated. Well grown culture (20 ml) was dot blotted on to nitrocellulose membrane and ELISA was performed as mentioned above.

## Results and discussion

The results obtained clearly indicated the ability of the antibodies included in the kit to detect

**Table 2.** Specificity of NCM-ELISA kit developed for detection of *Ralstonia solanacearum*

| Source | No. of isolates tested | No. of typical *R. solanacearum* | No. of isolates closely related to *R. solanacearum** | No. of isolates showed positive reaction |
|---|---|---|---|---|
| Ginger (*Zingiber officinale*) | 60 | 20 | 18 | 38 |
| Black pepper (*Piper nigrum*) | 51 | 0 | 0 | 9 |
| Cardamom (*Elettaria cardamomum*) | 1 | 0 | 0 | 0 |
| Ageratum (*Ageratum conyzoides*) | 2 | 0 | 2 | 0 |
| Tomato (*Lycopersicon esculautum*) | 1 | 1 | 0 | 1 |
| Potato (*Solanum tuberosum*) | 1 | 1 | 0 | 1 |
| *Chromolaena* (*Chromolaena odorata*) | 1 | 1 | 0 | 1 |
| Capsicum (*Capsicum annuum*) | 1 | 1 | 0 | 1 |
| Teak (*Tectona grandis*) | 1 | 0 | 1 | 1 |
| Neem compost | 2 | 0 | 0 | 0 |
| Total | 121 | 24 | 21 | 52 |

* Based on colonies developed on Kelman's medium

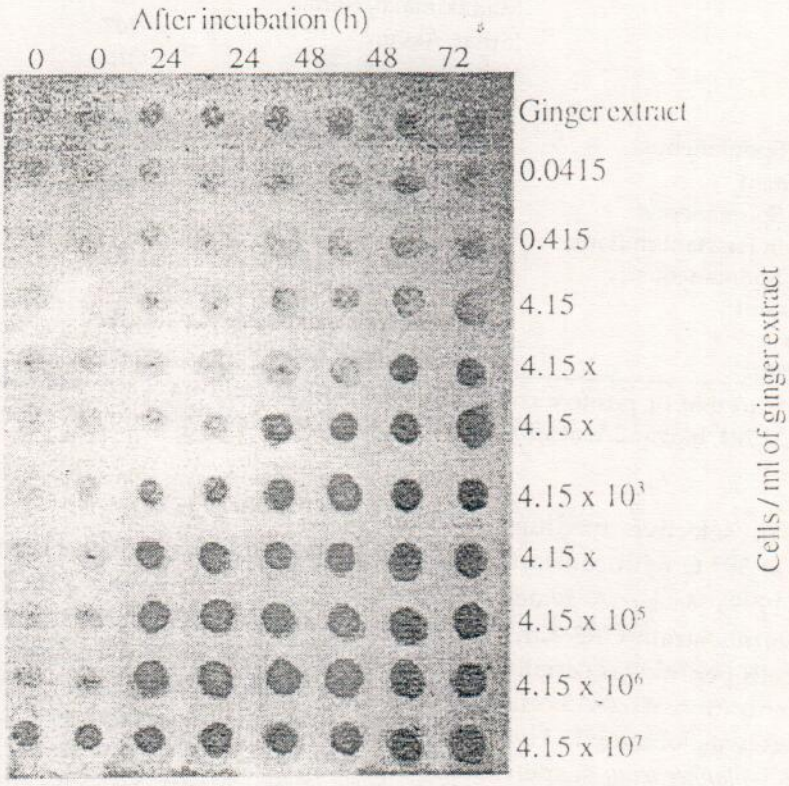


Fig. 1. NCM-ELISA after enrichment in selective medium

strains of *R. solanacearum* isolated from ginger, tomato and *Chromolaena* (Table 1). The colour development was comparable to that of positive control strips provided with the kit, loaded with water suspensions of *R. solanacearum* at $10^8$ to $10^5$ cells $ml^{-1}$. It is interesting to note that the antibodies could also detect aberrant isolates with dulcitol negative phenotype. Bacterial ooze from all the seven locations had a positive reaction, which testify for the suitability of the antibodies to detect *R. solanacearum* in plant material. The kit could detect *R. solanacearum* not only from ginger but also from tomato, *Chromolaena* and *Ageratum*.

The results of the specificity of NCM-ELISA kit are presented in Table 2. It is clear from the data that the ginger strains tested positively with the antibodies. Of the 50 ginger isolates assayed, 38 of them gave positive reaction. Among the 38 isolates 20 are typical *R. solanacearum* as identified on Kelman's medium, the remaining 18 isolates were very closely related to *R. solanacearum* in colony characters. Nine of the black pepper isolates had given positive reaction with *R. solanacearum* specific antibodies. These bacteria could be closely related to *R. solanacearum*. Other isolates, which gave positive reaction, were obtained from bacterial wilt affected teak, tomato, *Capsicum*, *Chromolaena*, potato and *Ageratum*. Except the isolates obtained from *Ageratum*, all others tested positive with NCM-ELISA.

The sensitivity of the assay is presented in Fig 1. The non-enriched extract when dot-blotted, could detect the bacteria at a concentration of $7.7 \times 10^5$ cells per ml of extract. However, the kit could detect as low as 41.5 cells of bacteria in the ginger extract when the extract was incubated in selective medium for 72 h at 30° C, while for potato extracts a sensitivity of 2-10 cells $ml^{-1}$ was obtained after only 48 h of enrichment (Priou *et al.* 1999a & b). When the kit was evaluated using field samples, positive result was noticed with typically wilted samples and also with extracts dot blotted from healthy rhizomes collected from plants adjacent to the wilted plants. Several detection techniques have been suggested for detection of *R. solanacearum* from plant materials. Some of the techniques do not require isolation, culturing and purification of bacterium (Black & Elphinstone 1997). Such methods are advantageous as very often it is difficult to isolate or purify *R. solanacearum* from ginger, soil and other agricultural samples. Enzyme linked immunosorbent assay (ELISA) is one of the direct detection methods that can be performed in resource poor laboratories with limited facilities (Seal 1997). In conclusion, our results demonstrate that the kit developed at CIP for potato tubers could be used for monitoring *R. solanacearum* in latently infected seed rhizomes of ginger with a fair degree of accuracy.

## Acknowledgements

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# Characterization of *Ralstonia solanacearum* causing bacterial wilt in ginger

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**ABSTRACT: Bacterial wilt pathogen *Ralstonia solanacearum* isolated from ginger, tomato, *Chromolaena*, chilli and potato, characterized for biovar, pathogenicity, infectivity titer, and intrinsic antibiotic resistance. The isolates were also characterized on the basis of their membrane protein pattern and amenability of the isolates for serological detection (NCM-ELISA) using *R. solanacearum* specific antibodies. All the ginger isolates were highly fluidal with characteristic spiral pink center on 2,3,5-triphenyl tetrazolium chloride-amended medium. Among the isolates characterized nine belongs to biovar 3 while one from potato to biovar 2. Among the various isolates tested, only ginger ones except one from Assam induced cent percent wilting in ginger cv. *Himachal* within a week. Plants were wilted even at a concentration of 3.2 X $10^2$ cfu $ml^{-1}$ in stem inoculation while in soil inoculation it was $10^5$ cfu $ml^{-1}$. All the isolates were resistant to antibiotics tetracycline, polymyxin B sulphate and chloramphenicol and isolates GRS Tms and its spontaneous mutant were resistant to Rifamycin. Isolates were detected with NCM-ELISA and biovars on the basis of membrane protein pattern on SDS-PAGE and biovar specific protein from *R. solanacearum* could be isolated.**

**Key words:** *Ralstonia*, ginger, NCM-ELISA, membrane protein

Ginger (*Zingiber officinale* Rosc.) is an important spice crop that supports the livelihood of many farmers in Kerala, Karnataka, Himachal Pradesh, Meghalaya, Sikkim, West Bengal and other North Eastern states of India. Apart from being used as vegetable, ginger is grown for several value added product. Among the major production constraints bacterial wilt or '*Mahali*' or blast of ginger caused by *Ralstonia solanacearum* Yabuuchi (Smith) is very serious. The disease is endemic in majority of the ginger growing areas viz., Kerala, Sikkim and many other northeastern regions of the country causes yield loss up to 100 per cent under conducive conditions. (Dohroo 1991; Mathew *et al.,* 1979; Sarma *et al.,* 1978). The disease has also been reported from other parts of world viz., Queensland (Hayward *et al.,* 1967), Hawaii (Ishii and Aragaki, 1963), Mauritius and Malaya (Orian, 1953), Korea Republic (Choi and Han, 1990), China (Zheng -Xian Ming *et al.,* 1995) and Indonesia (Mulya *et al.,* 1990).

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Though the causal organism has been identified as *Ralstonia solanacearum* the characteristic features of *R. solanacearum* causing bacterial wilt in ginger are lacking in India. The present article deals with the pathogenic behavior, intrinsic antibiotic resistance, amenability of the isolates for serological detection and the strain differentiation by membrane protein pattern of *R. solanacearum* collected from different ginger growing areas of India and its relationship with strains of *R. solanacearum* isolated from tomato (*Lycopersicon esculantum),* chilli (*Capsicum annuum)*, potato (*Solanum tuberosum*) and eupatorium *(Chromolaena odorata*).

## MATERIALS AND METHODS

### Collection and phenotypic characterization of *Ralstonia solanacearum*

The pathogen *Ralstonia solanacearum* was isolated from wilted ginger plants collected from different locations of Kerala, Assam and West Bengal following standard procedure (Kelman, 1954; Mehan, 1995). *R. solanacearum* colonies appeared

after 36 hours of incubation at 28 °C as typical white fluidal with spiral pink center were purified. A loopful of bacterial growth was suspended in sterile distilled water and kept at 4 °C for short-term storage, while at –80 °C in 20 per cent glycerol for long-term storage.

For routine work media used was sucrose peptone agar (Mehan, 1995), CPG medium (Kelman, 1954), King's B medium (King *et al*., 1954) and semi selective medium (Hara and Ono, 1983). Catalase and salt tolerance tests were performed in nutrient broth. Biovar characterization of *R. solanacearum* was performed following the methods of Hayward (1960) with slight modification, i.e. use of microfuge tubes (1.0 ml) and microtiter plates (200 µl) along with conventional test tubes (3 ml) with bromothymol blue as pH indicator.

### Variation in virulence of *R. solanacearum* and infectivity titer

Pathogenicity of *R. solanacearum* was tested on 45 days old ginger plants by stem inoculation method. One hundred microlitre of inoculum ($10^8$ cfu $ml^{-1}$) multiplied on CPG medium was placed on pinprick portion in between bottom leaf sheath and pseudostem near collar region and ten such inoculated plants were kept at 30°C. For infectivity titer assay, cells of *R. solanacearum* multiplied in sucrose peptone broth were washed free of medium (5000 rpm/20min), resuspended in sterile distilled water and 10-fold dilutions of cell suspension were made up to $10^{-9}$. One hundred microlitres of diluted cell suspension from $10^{-8}$, $10^{-6}$ and $10^{-4}$ was spread plated on sucrose peptone agar for estimation of colony forming units of *R. solanacearum* and another 100 µl was inoculated at base between last leaf sheath and pseudostem of 45 days old ginger plants. In the next experiment 1ml of bacterial suspension from different dilutions were poured around the base of the plant. The data on wilt incidence was recorded until the disease symptom appeared in all the inoculated plants.

### Intrinsic antibiotic resistance of *Ralstonia solanacearum*

For antibiotic resistance Kanamycin (50 µg $ml^{-1}$), Nalidixic acid (40µg$ml^{-1}$), Chloramphenicol (20 µg $ml^{-1}$), Streptomycin (100 µg $ml^{-1}$), Ampicillin (100 µg$ml^{-1}$), Gentamycin (10µg$ml^{-1}$), Tetracycline (15 µg $ml^{-1}$), Rifamycin (50 µg $ml^{-1}$) and Polymyxin B sulphate (10 µg $ml^{-1}$) were incorporated in sucrose peptone agar. A loopful of bacterial suspension was streaked on such amended medium, the plates were incubated at 30°C and growth was recorded after 48 h and compared with growth on unamended media

### Serological diagnosis of *R. solanacearum*

Serological diagnostic kit (CIP–NCM-ELISA) obtained from International Potato Center, Lima, Peru was used to know the amenability of these isolates for serological detection. Using antibodies rose against potato isolates of *R. solanacearum*, ELISA was performed on nitrocellulose membrane as per the protocol of Priou *et al,* (1999).

### Characterization of *R. solanacearum* based on membrane protein pattern

Isolation of membrane protein from ten isolates of *R. solanacearum* was carried out following the protocols suggested by Dianese and Dristig (1994). Isolates of *R. solanacearum* were grown overnight in medium 523 of Kado and Heskette (1970) at 30°C on a rotary shaker at 150 rpm. Late logarithmic phase cells were harvested by centrifugation at 13200g for 20min at 4°C and then washed twice in cold buffer (3.3mM Tris-Cl, pH 7.4). The cells were subjected to lysis in lysozyme (100 µg $ml^{-1}$ of 10mM Tris-Cl + 0.75M sucrose, pH 7.4). After incubation for 10min in ice the products were homogenized in polytron homogenizer (Kinematica). The fraction containing total cell membrane was centrifuged at 36900g for 60 min to obtain membrane protein. The isolated protein was harvested in 50 µl buffer (3.3mM Tris-Cl, pH-7.4 + 0.25mM sucrose) and quantified (Bradford 1976). The isolated protein was separated in discontinuous PAGE system with a stacking gel containing acrylamide (5%) on top of a acrylamide analytical gel (12%) both containing SDS (0.1%).

## RESULTS AND DISCUSSION

Fluidal colonies with characteristic pink centre appeared after 48 h on CPG medium. The colony morphology and key biochemical characters i.e. absence of growth on NaCl amended medium, catalase positive reaction, growth at 37°C, absence of fluorescence on Kings medium B and orange

fluorescence on Nile blue amended medium confirmed the identity of bacterium as *Ralstonia*. Further characterization revealed that isolate from ginger, *Chromolaena*, chilli and tomato belongs to *R. solanacearum* biovar 3, while potato to biovar 2 (Table 1). Earlier Hayward *et al,* (1967) identified biovar 3 and biovar 4 as wilt causing bacteria from ginger in Queensland.

Observations on colony morphology on different media revealed that the colonies were irregular, white and fluidal with incubation period of 48-72 hours on CPG and SMSA. Strains from ginger and other hosts could be differentiated on CPG medium on basis of colony fluidity. Colonies of ginger strains were highly fluidal with characteristic spiral pink centre whereas in the case of other strains fluidity and pink centre was less conspicuous. The colonies were irregular, highly fluidal, white and without pigmentation on King's B while on sucrose peptone agar colonies these were round to irregular,

**Table 1.** Details of *Ralstonia solanacearum* isolates obtained from ginger and other hosts

| Isolate* | Crops & Location | Colony character on CPG medium | Pathogenicity to ginger | Biovar** |
|---|---|---|---|---|
| GRS Tms | Ginger, Thamarassery, Kerala | Incubation time: 36-72 h White, Fluidal, spiral pink centre, irregularity shaped. | Highly pathogenic | 3 |
| GRS Tms $Rif^R$ | Spontaneous rifamycin resistant mutant of GRS Tms, Thamarassery, Kerala | Incubation time: 48-72 h White, Fluidal, spiral pink centre, irregularly shaped. | Highly pathogenic | 3 |
| GRS Pul | Ginger, Pulpally, Kerala | Incubation time: 48-72 h White, Fluidal, spiral pink centre, irregularly shaped. | Highly pathogenic | 3 |
| GRS Kki | Ginger, Kahikuchi, Assam | Incubation time: 48-72 h White, less fluidal, spiral pink centre, irregularly shaped | Non pathogenic | 3 |
| GRS Vyt | Ginger, Vythiri, Kerala | Incubation time: 48-72 h White, highly fluidal, spiral pink centre, irregularly shaped | Highly pathogenic | 3 |
| GRS Ktm | Ginger, Kothamangalam, Kerala | Incubation time: 48-72 h White, Fluidal, spiral pink centre, irregularly shaped | Weakly pathogenic | 3 |
| TRS Cal | Tomato, Calicut, Kerala | Incubation time: 48-72 h White, Highly fluidal, dark pink centre, irregularly shaped, rarely round. | Non pathogenic | 3 |
| ERS Cal | *Chromolaena*, Calicut, Kerala | Incubation time: 48-72 h White, Highly fluidal, dark pink centre, irregularly shaped, elongated colonies, rarely round. | Non pathogenic | 3 |
| PRS Pun | Potato, Pundibari, West Bengal | Incubation time: 48-72 h White, Highly fluidal, spiral pink centre, irregularly shaped, rarely round | Non pathogenic | 2 |
| CRS Avl | Chilli, Ambalavayal, Kerala | Incubation time: 48-72 h White, Highly fluidal, dark pink centre, irregularly shaped, rarely round. | Non pathogenic | 3 |

*All isolates are resistant to Chloramphenicol (20 $\mu$g $ml^{-1}$), Tetracycline (15$\mu$g $ml^{-1}$) and Polymyxin (10$\mu$g $ml^{-1}$)
** All isolates reacted strongly with Rs specific antibodies as observed in NCM-ELISA

**Table 2.** Infectivity titer of *Ralstonia solanacearum* in ginger

| Inoculum concentration (cfu/ml) | Pseudostem inoculation | | Soil inoculation | |
|---|---|---|---|---|
| | Initiation of disease (Days) | wilt (%) | Initiation of disease (Days) | wilt (%) |
| 3.2 x $10^8$ | 6 | 100(8) | 12 | 100(19) |
| 3.2 X $10^7$ | 6 | 100(9) | 12 | 30 |
| 3.2 X $10^6$ | 7 | 100(20) | 13 | 30 |
| 3.2 X $10^5$ | 7 | 100(15) | 14 | 10 |
| 3.2 X $10^4$ | 8 | 80 | * | * |
| 3.2 X $10^3$ | 8 | 80 | * | * |
| 3.2 X $10^2$ | 9 | 80 | * | * |

Figures in parenthesis indicate number of days to wilt all the inoculated ginger plants.
*- No wilt incidence

creamy white and fluidal. All the pathogen strains were resistant to chloramphenicol (20 $\mu$g $ml^{-1}$), tetracycline (15 $\mu$g $ml^{-1}$) and polymyxin (10 $\mu$g $ml^{-1}$). Among the strains, GRS Tms and its spontaneous mutant could resist antibiotic rifamycin at 50 $\mu$g $ml^{-1}$ (Table 1).

### Pathogenicity of isolates

Among the different *Ralstonia solanacearum* strains tested, all the ginger ones except one from Assam induced wilt symptoms in ginger (Table 1). *Ralstonia solanacearum* from tomato, *Chromolaena*, chilli and potato were found non-pathogenic to ginger. It is interesting to note that the *R. solanacearum* from *Chromolaena*, a common weed of ginger fields was not pathogenic on ginger though it belonged to biovar 3. Wilting of plants started 6-7 days after inoculation and all the inoculated plants wilted with in 8-20 days (Table 2). Zehr (1970) has also found the wilting of ginger plants with in 10 days of inoculation with virulent isolates of *R. solanacearum*. However, Hayward *et al,* (1967) grouped the ginger isolates in to two groups i.e., the group 1, which is biovar 4, induced wilt in 14 and 21 days of stem and root inoculation while in group 2 (belong to biovar 3) plants wilted over a period of 6 weeks. But in the present investigation in India, biovar 3 was found quick wilting pathogen as it took only 6 days to initiate wilting.

### Infectivity titer of *Ralstonia solanacearum* in ginger

Wilting started after 6 days of inoculation at the concentrations of 3.2 x $10^8$ and 3.2 x $10^7$ cfu $ml^{-1}$ when the inoculum was placed in between bottom leaf sheath and pseudostem. At concentrations of 3 x $10^8$ cfu $ml^{-1}$ the wilting started after 6 days and complete wilting was observed on 8$^{th}$ day (Table 2). However, in the case of inoculum poured around the base of the plant after pinprick, wilting was noticed after 12$^{th}$ day and there was no wilting below the concentration of 3.2 x $10^5$ cfu $ml^{-1}$.

### Reactions of *R. solanacearum* with antibodies

NCM-ELISA originally developed for detection of *R. solanacearum* in potato seed tubers (Priou *et al.,* 1999) was adopted and compared with positive control strips provided in the kit (Fig. 1). All the

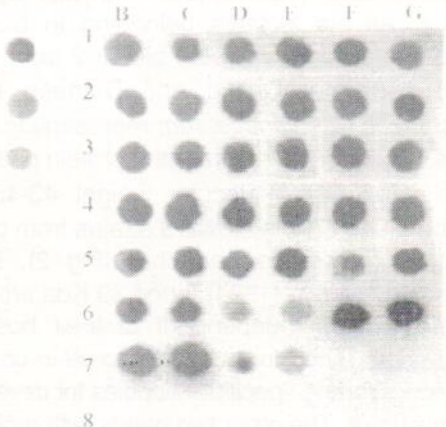

**Fig. 1.** NCM-ELISA of different isolates of *R. solanacearum*. Dark spot indicates positive serological reaction with Rs specific antibodies

**Table 3.** SDS-PAGE analysis of membrane protein of *R. solanacearum*

| Isolate | Yield µg/ml | Rf value* | Molecular Weight (Kd) |
|---|---|---|---|
| Gin Tms/GRS Tms | 27.45 | 0.54, 0.57, 0.73 | 42.7, 40.5, 26.8 |
| GRS Tms Rif$^R$ | 27.30 | 0.54, 0.57, 0.73 | 42.7, 40.5, 26.8 |
| GRS Pul | 23.9 | 0.54, 0.56, 0.73 | 42.7, 41.2, 26.8 |
| GRS Kki | 35.4 | 0.52, 0.56, 0.73 | 45.4, 41.2, 27.4 |
| GRS Vyt | 32.2 | 0.54, 0.56, 0.72 | 43.5, 41.6, 27.3 |
| GRS Ktm | 32.1 | 0.54, 0.57, 0.72 | 42.7, 39.8, 26.3 |
| TRS Cal | 31.9 | 0.54, 0.57, 0.73 | 42.7, 39.8, 26.3 |
| ERS Cal | 27.4 | 0.53, 0.56, 0.72 | 44.9, 40.7, 26.5 |
| PRS Pun | 34.2 | 0.47, 0.56, 0.72 | 52.1, 41.5, 26.5 |
| CRS Avl | 23.9 | 0.54, 0.56, 0.72 | 43.6, 41.5, 26.5 |

ginger, tomato and *Chromolaena* strains gave positive colour reaction at a concentration of $10^8$ cfu/ml, which broaden the scope of the kit for strains isolated from other hosts.

**Membrane protein pattern of the pathogen**

Ten isolates representing biovar 3 and biovar 2 were characterized using membrane protein pattern. (Table 3). The isolated membrane protein was separated in discontinuous PAGE system and the banding pattern was analyzed using Alpha imager gel documentation system. All the isolates were resolved in to two groups. Similarity index using unbiased Pair Group Method with Arithmetic Averages (UPGMA) was calculated and cluster analysis was performed using NTSyspc (Numerical Taxonomy and Multivariate Analysis System) software. All the isolates belonging to biovar 3 formed one cluster, while the biovar 2 from potato formed separate. Dristig and Dianese (1990) characterized *R. solanacearum* representing all the biovars based on their membrane protein pattern. A protein band with molecular weight 43-45 Kda could be found only in biovar 3 strains from ginger, tomato, *Chromolaena* and chilli (Fig 2). Earlier Dianese and Dristig (1994) found 39 Kda protein in all three biovars independent of their host and locality origin. The biovar specific protein could be exploited for raising specific antibodies for developing a diagnostic kit. The other two bands with molecular weights, 41 Kda and 27 Kda, could be species-specific proteins. A band with molecular weight of 51 Kda found only in biovar 2 which could be specific for this biovar.

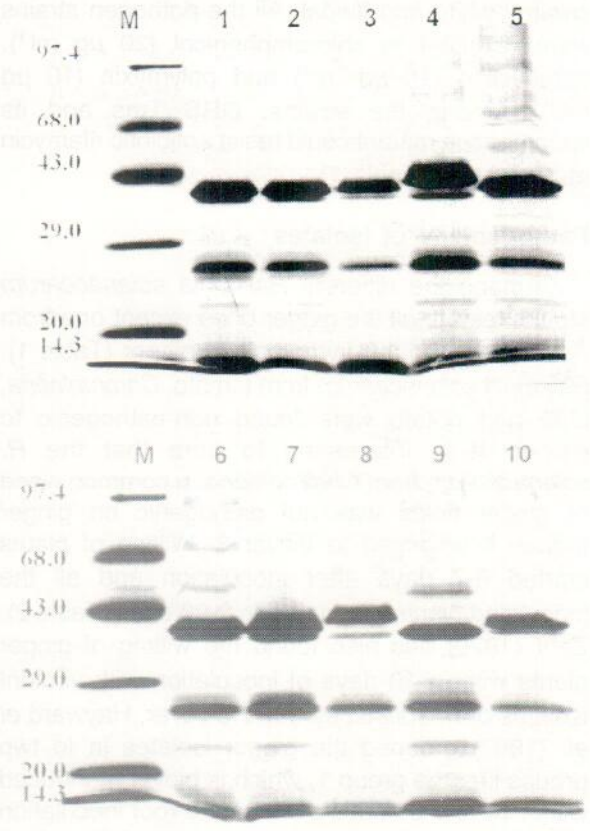


**Fig. 2.** Membrane protein pattern of *R. solanacearum* isolates. M- Protein marker (Kda), 1. GRS-Tms, 2. GRS Tms Rif$^R$ 3. GRS-Pul, 4. GRS-Kki, 5. GRS-Vyt, 6. GRS-Ktm 7. TRS-Cal, 8. ERS-Cal 9. PRS Pun 10. CRS Avl
1-5: Biovar 3 from ginger, 6: Biovar 3 (ginger), 7: Biovar 3 (tomato), 8: Biovar 3 (Chromolaena), 9: Biovar 2 (potato), 10: Biovar 3 (capsicum)

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# Evaluation of genetic diversity of *Ralstonia solanacearum* causing bacterial wilt of ginger using REP–PCR and PCR–RFLP

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**Thirty-three strains of *Ralstonia solanacearum* Yabuuchi (Smith) isolated from ginger, paprika, chilli, tomato, *Chromolaena* and potato from Kerala, Karnataka, West Bengal and Assam in India, were phenotypically and genotypically characterized. Phenotypic characterization for biovar revealed the predominance of biovar 3 in India. Molecular analysis by REP–PCR, ITS–PCR and RFLP–PCR classified the strains into three clusters at 70% similarity, where ginger strains are grouped in Clusters I and II. Strains from potato (biovar 2) clustered in the III cluster. Molecular analysis also revealed that ginger strains isolated from different locations during different years had 100% similarity according to Dice's coefficient. The analysis further revealed that the genetic diversity of *Ralstonia* is very low within ginger, confirming that the pathogen population is of clonal lineage and is distributed through 'rhizome transmission' of the inoculum between locations and also between seasons within the locality.**

BACTERIAL wilt caused by *Ralstonia solanacearum* Yabuuchi (Smith) is a disease widely distributed in tropical, sub-tropical and temperate regions worldwide. The host range of the pathogen is very wide and ginger is one of the important hosts of the pathogen. Geographical distribution of the pathogen is expanding in the recent years. Bacterial wilt of ginger is reported from India, China, Japan, Indonesia, the Philippines, Hawaii and many other ginger-growing countries. In India the disease is found in Kerala, Karnataka, Himachal Pradesh, Sikkim, West Bengal, Assam and other North Eastern States. In contrast to the report from Queensland, the strains causing bacterial wilt of ginger in India belong to biovar 3 that causes wilt in 5–7 days in 45-day-old ginger plants[1]. The pathogen is primarily rhizome-borne and it is believed to be transmitted to many ginger-growing areas through latently infected rhizomes and secondary spread within the field and neighbouring localities is through rain splashes and run-off water in the field.

*R. solanacearum* belongs to the rRNA homology group II pseudomonads based on rRNA : DNA homology[2] and to the $\beta$-sub class of Proteobacteria. *R. solanacearum* exhibits both phenotypic and genotypic diversity. The species is divided into five races and 5 biovars based on its host range and also on difference in the oxidation/utilization of certain carbon sources respectively[3]. A number of different phenotypic and genotypic methods are presently being employed for the identification and classification of bacteria, including plant pathogenic bacteria like *Ralstonia*. Each of these methods permits a certain level of phylogenetic classification from the genus, species, subspecies, biovar to the strain level. Moreover, each method has its advantages and disadvantages with regard to ease of application, reproducibility, requirement for equipment and level of resolution[4]. Modern phylogenetic classification is based on 16S rRNA sequence analysis[5,6]. There are also highly discriminatory PCR fingerprinting methods for *R. solanacearum*, ranging from amplification with 8–10 base pair primer (RAPD) to using longer primers that target repeated sequences such as tRNA gene consensus primer[7] or bacterial repetitive elements[8]. Cook *et al.*[9] have assessed the diversity of the pathogen according to RFLP using *h*ypersensitive *r*esponse and *p*athogenicity (*hrp*) genes as probes. The RFLP technique revealed the presence of two major geographical origins of the strains, viz., American origin consisting of biovars 1 and 2, and Asian origin consisting of biovars 3, 4 and 5. Recently, a study by Poussier *et al.*[10] using PCR–RFLP of the *Hrp* gene region, AFLP and 16s rRNA sequence analysis allowed identification of the African subdivision.

REP–PCR fingerprinting makes use of DNA primers complementary to the naturally occurring, highly conserved repetitive DNA sequence present in multiple copies in the genome of most Gram-negative and Gram-positive bacteria[11,12]. Though the bacterial wilt pathogen of ginger has been isolated and identified unambiguously, the genetic diversity of the pathogen in India is not known. With the background information, an attempt was made to analyse the genetic and phenotypic diversity of *R. solanacearum* isolated from different ginger-growing states as well as isolates collected during different years (1998–2002) from a bacterial wilt endemic location in India. Few strains isolated from other crops, viz. paprika, chilli, tomato, *Chromolaena* and potato found on ginger fields or nearby fields have also been included in the analysis.

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## Materials and methods

### *Strains used and biovar characterization*

All the strains used in the present study were isolated on CPG medium[13] (g $l^{-1}$ cassamino acid, 1; peptone, 10; glucose, 5; pH 7.2) from typical bacterial-wilt affected plants. The list of strains is furnished in Table 1. For determination of biovar, the method described by Hayward[3] was adopted.

### *Determination of pathogenicity and biovar*

For pathogenicity assay virulent colonies of *R. solanacearum*, as identified on CPG medium, were multiplied in sucrose peptone broth (g $l^{-1}$ sucrose, 20; peptone, 10; $K_2HPO_4$, 0.5; $MgSO_4$, 0.25; pH 7.2) for two days. The cells were pelleted at 10000 *g* for 20 min at 4°C, resuspended in water and poured around the base of the 45-day-old ginger cultivar *Himachal*, as water suspension at a concentration of $10^9$ cells per ml of water. The inoculated plants were grown at 28 ± 2°C and were closely monitored for wilt disease.

### *DNA isolation*

Genomic DNA from *R. solanacearum* was isolated and used as template in the REP–PCR fingerprinting. Briefly, bacteria cells multiplied in sucrose peptone broth were pelleted and washed three times in sterile solution of 10 mM $MgSO_4$ to remove traces of the medium. The bacterial cells were then lysed in *N*-cetyl-*N*,*N*,*N*-trimethyl-ammonium bromide (CTAB) buffer (Tris-Cl, 100 mM; EDTA, 100 mM; $Na_2HPO_4$, 100 mM; NaCl, 1.5 M; CTAB, 1%; proteinase K, 20 µg; lysozyme, 100 µg) at 37°C for 30 min before further lysis at 65°C for about 2 h in the presence of sodium dodecyl sulphate (15 mg per tube). Further, the lysate was clarified and DNA precipitated by adopting standard DNA isolation protocol. The isolated DNA was dissolved in TE buffer (Tris-Cl, 10 mM; EDTA, 0.1 mM; pH 8.0), its purity checked, quantified and concentration was adjusted to get 100 ng of DNA per µl of sterile distilled water

### *PCR amplification*

The protocol developed by Louws *et al.*[8] was followed for amplification of genomic DNA of *R. solanacearum*. Am-

**Table 1.** Strains used in the study

| Strain | Host | Location | Year | Biovar | Reaction to ginger |
|---|---|---|---|---|---|
| GRS PTD | Ginger | Poothady, Wyanad, Kerala | 2000 | 3 | Pathogenic |
| GRS GH | Ginger | Vythiri, Wyanad, Kerala | 1999 | 3 | Pathogenic |
| GRS GH1 | Ginger | Vythiri, Wyanad, Kerala | 1999 | 3 | Pathogenic |
| GRS MK | Ginger | Mullamkolli, Wyanad, Kerala | 2000 | 3 | Pathogenic |
| GRS TNY | Ginger | Thaniyood, Wyanad, Kerala | 2001 | 3 | Pathogenic |
| GRS Vy | Ginger | Vythiri, Wyanad, Kerala | 1999 | 3 | Pathogenic |
| GRS PUL | Ginger | Pulpally, Wyanad, Kerala | 1998 | 3 | Pathogenic |
| GRS MEEN1 | Ginger | Meenangadi, Wyanad, Kerala | 2002 | 3 | Pathogenic |
| GRS PUL02 | Ginger | Pulpally, Wyanad, Kerala | 2002 | 3 | Pathogenic |
| GRS MEEN2 | Ginger | Meenangadi, Wyanad, Kerala | 2002 | 3 | Pathogenic |
| GRS PER | Ginger | Peruvannamuzhi, Kerala | 2000 | 4 | Weakly pathogenic |
| GRS CHE | Ginger | Chemanoda, Kerala | 2000 | 3 | Pathogenic |
| GRS PER1 | Ginger | Peruvannamuzhi, Kerala | 2000 | 4 | Weakly pathogenic |
| GRS PER2 | Ginger | Peruvannamuzhi, Kerala | 2001 | 4 | Weakly pathogenic |
| GRS PER2A | Ginger | Peruvannamuzhi, Kerala | 2001 | 4 | Weakly pathogenic |
| GRS PER02 | Ginger | Peruvannamuzhi, Kerala | 2002 | 3 | Pathogenic |
| GRS 117 | Ginger | Kothamangalam, Kerala | 2001 | 3 | Pathogenic |
| GRS HIM | Ginger | Kothamangalam, Kerala | 2001 | 3 | Pathogenic |
| GRS KM | Ginger | Kothamangalam, Kerala | 1999 | 3 | Pathogenic |
| GRS KMR | Ginger | Kothamangalam, Kerala | 2001 | 3 | Pathogenic |
| GRS KMP | Ginger | Kothamangalam, Kerala | 2000 | 3 | Non-pathogenic |
| GRS TMS | Ginger | Thamarasery, Calicut, Kerala | 1998 | 3 | Pathogenic |
| GRS TMS RIF | Ginger | Mutant of GRS TMS | 1998 | 3 | Pathogenic |
| GRS KAR | Ginger | Hattur, Coorg, Karnataka | 2000 | 3 | Pathogenic |
| GRS KK | Ginger | Kakikuchi, Assam | 2000 | 3 | Non-pathogenic |
| GRS KK1 | Ginger | Kakikuchi, Assam | 2000 | 3 | Non-pathogenic |
| CRS AVL | Chilli | Ambalavayal, Wyanad, Kerala | 1999 | 3 | Non-pathogenic |
| CRS PER | Paprika | Peruvannamuzhi, Kerala | 1999 | 3 | Non-pathogenic |
| CRS PER1 | Paprika | Peruvannamuzhi, Kerala | 2000 | 3 | Non-pathogenic |
| CRS PER2 | Paprika | Peruvannamuzhi, Kerala | 2000 | 3 | Non-pathogenic |
| ERS CAL | *Chromolaena* | Peruvannamuzhi, Kerala | 1999 | 3 | Non-pathogenic |
| TRS CAL | Tomato | Peruvannamuzhi, Kerala | 1999 | 3 | Non-pathogenic |
| PRS PUN | Potato | Pundibari, West Bengal | 1999 | 2 | Non-pathogenic |

**Table 2.** Sequence of primers used in the study and temperature conditions for REP and ITS–PCR

| BOX: 5′-CTACgAAggCgACgCTgACg-3′ | ERIC I R 5′-ATgTAAgCTCCTggggATTCAC-3′<br>EICR 2: 5′-AAgTAAgTgACTggggTgAgCg-3′ | REP IR: 5′-IIIIICgICgICATCIggC-3′<br>REP 2I: 5′-ICgICTTATCIggCCTAC-3′ | ITS all F: 5′-TAgGCgTCCACACTTATCggT-3′ |
|---|---|---|---|
| | Initial denaturation 95°C for 7 min | | Initial denaturation 96°C for 5 min |
| 94°C for 1 min | 94°C for 1 min | 94°C for 1 min | 94°C for 30 s |
| 53°C for 1 min | 52°C for 1 min | 40°C for 1 min | 59°C for 30 s |
| 65°C for 8 min | 65°C for 8 min | 65°C for 8 min | 72°C for 30 s |
| | Final extension 65°C for 16 min | | Final extension 72°C for 10 min |
| Versalovic *et al.*[11] | Versalovic *et al.*[16] | Versalovic *et al.*[16] | Fegan *et al.*[25] |

plification was performed in 25 µl of reaction mixture containing Gitschier buffer[14] [83 mM $(NH_4)_2SO_4$; 335 mM Tris-HCl, pH 8.8; 33.5 mM $MgCl_2$; 33.5 µM EDTA; 150 mM β-mercapto-ethanol; DMSO, 10%; BSA, 10 µg; dNTP mix, 25 mM each; primers (Table 2), REP1R and REP2I, ERIC1R and ERIC2, BOXAIR, 10 pmol (Genei, Bangalore); DNA polymerase, 2U and DNA 100 ng. PCR amplification for 16S–23S rRNA gene intergenic spacer regions was done in 20 µl of reaction mixture containing PCR buffer, 1× (Genei, Bangalore); $MgCl_2$, 3 mM; dNTP mix, 0.2 mM; Taq DNA polymerase, 0.5 U; primer, 10 pmol and template DNA, 100 ng.

REP–PCR was performed in thermal cycler (Eppendorf, Germany) using PCR conditions furnished in Table 2 and the final PCR products were resolved in 2.0% agarose in 1× Tris acetate EDTA buffer at 4°C for 16 h at 4 V/cm. The gel was stained with ethidium bromide, photographed on UV transilluminator and the results documented in Alpha imager 2002 for analysis.

### *Restriction analysis of ITS–PCR products*

ITS–PCR products were restricted with *Msp*I by adopting the manufacturer's instruction (Promega Corporation, USA). Briefly, 20 µl of PCR product was restricted for 6 h. The digested and undigested amplified fragments were resolved in 2.5 and 1.4% agarose gel respectively, in 1× Tris acetate EDTA buffer at 4°C for 16 h at 4 V/cm. The gel was stained with ethidium bromide, photographed on UV transilluminator (Alpha Innotech, USA) and the results were documented in Alpha imager 2002 for analysis.

### *Data analysis and interpretation of results*

Comparison of banding pattern generated from each strain allowed identification of strains which were considered as haplotypes. REP- and ITS-PCR fingerprints were scored manually as 1 for the presence of a band and 0 for absence of a band, assuming that bands with molecular size in different individuals were homologous. The final binary data were analysed using NTSys software and similarity coefficient was calculated. The tree plot was constructed by unweighted pair group arithmetic average.

## Results

PCR primers targetted to conserved repetitive sequences were used to investigate the genetic relationship among strains of *R. solanacearum* that cause vascular wilt diseases of ginger. Isolation on CPG medium amended with 2,3,5-tetrazolium-chloride allows identifying colonies of *R. solanacearum* among other bacteria by their fluidal, smooth, white appearance with red central whirling pattern[13]. Interestingly, the colony of *R. solanacearum*, irrespective of its host and geographical origin, looks strikingly similar on this medium. Though a useful phenotypic character, this conventional bacterial identification is not useful to know the diversity of the pathogen vis-à-vis the infraspecific subgroup affecting the hosts within or between the species.

Among the isolates characterized only ginger strains were found to be pathogenic on ginger, with few exceptions (Table 1). None of the isolates obtained from other hosts could wilt ginger plants. The phenotypic characterization

**Table 3.** Diversity of *Ralstonia solanacearum*

| Biovar | No. of strains | Host plant | Location | Pathogenicity |
|---|---|---|---|---|
| 3 | 28 | Ginger, tomato, *Chromolaena*, chilli, paprika | Kerala, Karnataka, Assam | Only ginger strains are pathogenic |
| 2 | 1 | Potato | West Bengal | Non-pathogenic on ginger |
| 4 | 4 | Ginger | Kerala | Weakly pathogenic on ginger |

**Table 4.** Number of polymorphic bands obtained using different primers

| Primer | Number of amplified products |
|---|---|
| REP | 18 |
| ERIC | 19 |
| BOX | 13 |
| ITS ALL F | 2 |
| ITS ALL F+ *Msp*I restriction | 5 |

for biovar revealed the predominance of biovar 3 over biovar 4 in Kerala, India (Table 3). Differential disease reactions could be observed in ginger when two biovars (biovar 3 and biovar 4) were inoculated, where biovar 3 was found to be more virulent than biovar 4. Strains used in the present investigation were collected mainly from ginger (26) followed by paprika (3) tomato (1), chilli (1), *Chromolaena* (1) and potato (1). The strains represent majority of the ginger-growing area, viz. Kerala (29), Karnataka (1), West Bengal (1) and Assam (2) in India.

The number of amplified products of genomic DNA is furnished in Table 4. The REP primer generated more PCR-amplified fragments than the other two primers (Figures 1 and 2). Composite dendrogram constructed from the cluster analysis of REP, ERIC, BOX and ITS all F primer PCR pattern showed that the ginger strains and other strains could be divided into three clusters at similarity coefficient of 70%. Cluster I consists of strains of biovars 3 and 4 from ginger and other hosts. Cluster II includes biovar 3 from Assam isolated from ginger and biovar 3 from Wyanad isolated from chilli. The third cluster consists of a strain from potato that belongs to biovar 2. Ginger strains isolated from Wyanad District, Kerala, the endemic area for bacterial wilt, over the last five years, have 100% similarity to each other. Interestingly, an isolate collected from Karnataka is clustered along Kerala strains with 100% similarity. Within cluster 1 there are sub-clusters in conformity with their host origin, location and pathogenic behaviour. Isolates obtained from Peruvannamuzhi, Kerala are clustered together, which tested positive for biovar 4 and are weakly pathogenic on ginger. Similarly, isolates from wilted paprika from Peruvannamuzhi are clustered together with 100% similarity to each other, whereas a strain isolated from bacterial-wilt-affected local chilli from Wyanad is clustered separately. Pooled analysis of data generated from REP–PCR and PCR–RFLP analysis could cluster isolates in conformity with their biovars, pathogenic behaviour and geographical origins as indicated by the clustering pattern of the strains in the pooled dendrogram (Figure 3).

## Discussion

Genetic diversity of phytopathogenic bacteria has been studied by PCR-based approaches to generate evidence of genome plasticity, ecological distribution, dispersal and evolution. Knowledge on the existence of variability in the pathogen population is important for plant breeding and the consequent crop improvement programme. Ginger is one of the few monocots affected by wilt caused by *R. solanacearum* in several tropical countries. In India, biovar 3 is predominant over biovar 4 and it induced wilt in ginger within 5–7 days in stem inoculation of pathogen and in 7–10 days under soil inoculation[1]. The incidence of biovar 4 is less frequent in India, which further confirms that biovar 3 is more versatile in its adaptation to varying environmental conditions and is less influenced by the vagaries of soil edaphic factors. We also found that biovar 3 of *R. solanacearum* is more aggressive than biovar 4 on ginger as evident from its quick induction of wilt in ginger. The ginger isolates of *R. solanacearum* are grouped into two types based on their incubation period[15]. Group I is mostly biovar 4, where ginger plants wilted in 14 and 21 days of stem and root inoculation respectively, while in the group II, mostly biovar 3, ginger plants wilted over a period of 6 weeks. In contrast to the report from Queensland[15], we report here *R. solanacearum* biovar 3 as most aggressive pathogen in ginger.

REP–PCR analysis using primer sets (REP, ERIC and BOX) of highly conserved repetitive sequences[16] as well as RFLP–PCR using ITS primer and restriction using *Msp*I showed differential banding patterns among *R. solanacearum* strains of different biovars and hosts. The analysis clearly reveals the potential of the REP–PCR to cluster the strains of *R. solanacearum* to their pathogenic behaviour as indicated in the dendrogram, where highly pathogenic isolates are clustered together with 100% similarity coefficient (Figure 3). However, in a similar study Jaunet and Wang[17] could not correlate biovar or geographic origin of tomato strains of *Ralstonia* to the UPGMA clusters derived from RAPD, REP–PCR, or composite data. Using REP–PCR, strains of *Xanthomonas* are differentiated and the potential of REP-PCR in discriminating the strains is proved in several publications[18]. Low genetic variability or diversity among

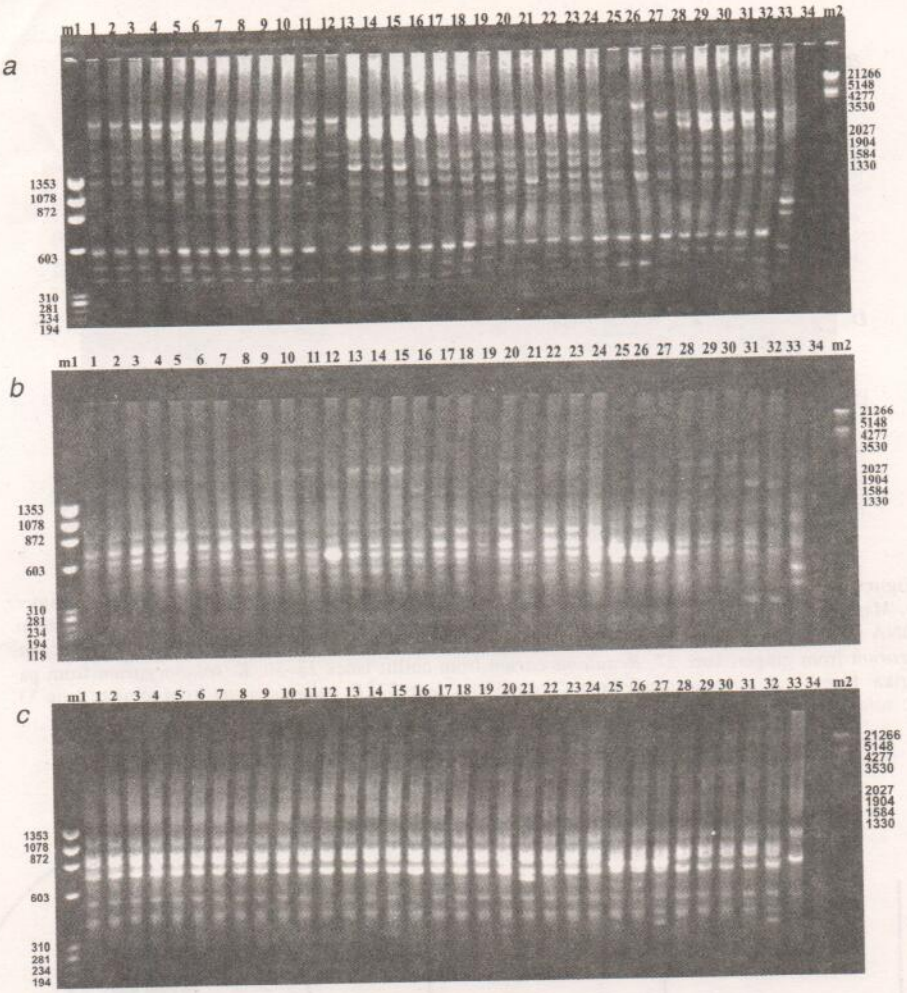


**Figure 1.** REP–PCR analysis (*a*) ERIC–PCR analysis (*b*) and BOX–PCR (*c*) analysis of ginger strains of *R. solanacearum*. m1, DNA size marker-$\phi$X174 *Hae*III digest (Bangalore Genei, India); lanes 1–26, Isolates of *R. solanacearum* from ginger; lane 27, *R. solanacearum* from chilli; lanes 28–30: *R. solanacearum* from paprika; lane 31, *R. solanacearum* from Chromolaena; lane 32, *R. solanacearum* from tomato; lane 33, *R. solanacearum* from potato; lane 34, Negative control, m2, DNA size marker-$\lambda$ DNA *Eco*RI and *Hin*dIII digest (Bangalore Genei, India).

ginger strains of *Ralstonia* could be due to lack of selection pressure from the ginger genotypes to evolve new strains/biotypes/races in *Ralstonia*, which is evident from the absence of resistance in ginger germplasm to bacterial wilt disease, as none of the 600 accessions of ginger genotypes screened for disease resistance showed any degree of resistance to bacterial wilt[19]. Absence of host resistance could be attributed to the lack of variability in ginger strains of *R. solanacearum*. This has been earlier proved using molecular tools in two pathosystems[20,21]. High diversity in population of fungal pathogen *Cephalosporium maydis* is attributed to the widespread cultivation of resistant cultivars of maize[20]. Similar observation was made in the population of another fungal foliar pathogen, *Colletotrichum lindemuthianum* in a legume crop *Phaseolous vulgaris*[21]. Probably the narrow genetic base of ginger in India is one of the reasons for prevalence of single virulent lineage of *R. solanacearum*.

Besides, *R. solanacearum* could not be detected in soil or ginger periderm collected from bacterial-wilt-affected area (data not shown) using Rs-specific DAS-ELISA[22]. This along with the data generated from the present diversity analysis indicates that the pathogen is not under evolutionary pressure from the soil environment to evolve into new biotypes or races. However, *Ralstonia* is reported to survive in the rhizosphere of many weed hosts in Queensland[23,24].

High similarity among the biovar 3 strains could be due to the prevalence of rhizome transmission of the pathogen from one location to another, which indicates the population is clonally propagated and transmitted. Large-scale cultivation of ginger in Indian states is mainly through the rhizome material brought from the predominant ginger-growing locations, particularly Kerala and northeastern States, which are incidentally endemic areas for bacterial wilt of ginger. It is likely that the pathogen is transmitted

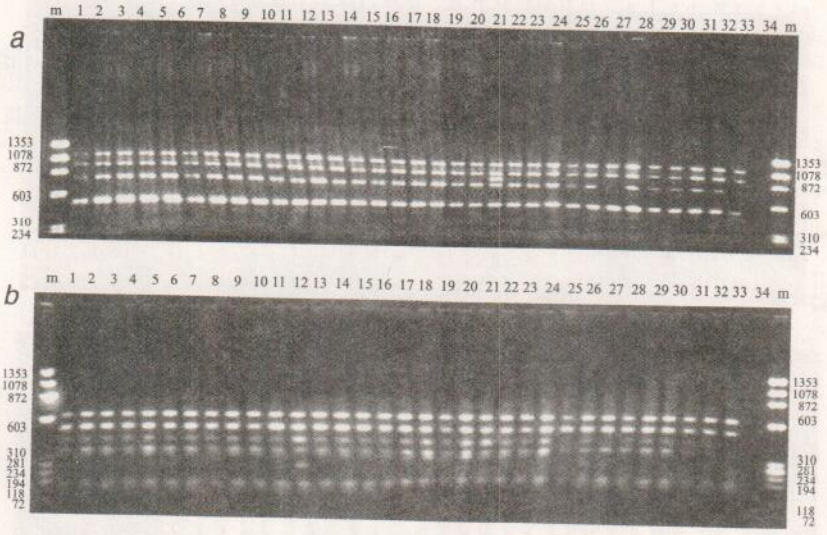


**Figure 2.** *a*, Amplification of genomic DNA of isolates of *R. solanacearum* using ITS all F primer. *b*, *Msp*I restriction profile from ITS amplified fragments of genomic DNA of *R. solanacearum*. m, DNA size marker-$\phi$ X174 *Hae*III digest (Bangalore Genei, India); lanes 1–26, Isolates of *R. solanacearum* from ginger; lane 27, *R. solanacearum* from chilli; lanes 28–30, *R. solanacearum* from paprika; lane 31, *R. solanacearum* from Chromalaena; lane 32, *R. solanacearum* from tomato; lane 33, *R. solanacearum* from potato; lane 34, Negative control.

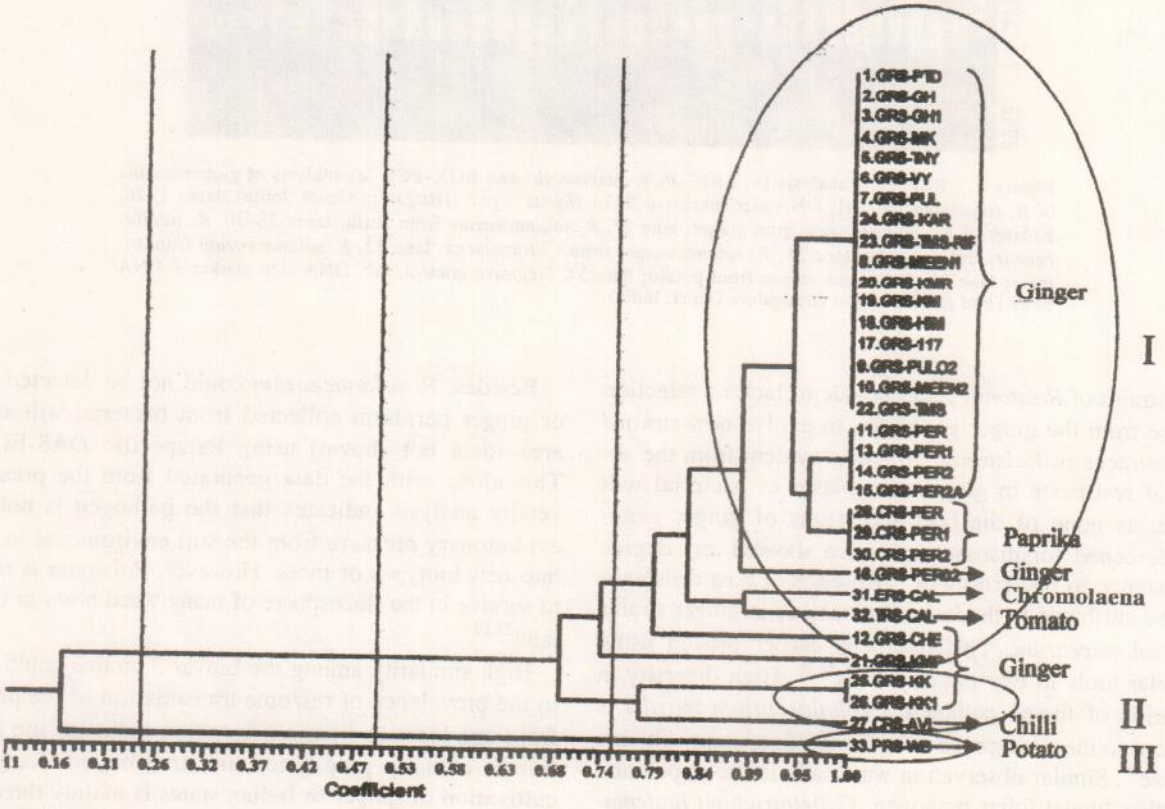


**Figure 3.** Dendrogram derived by unweighted pair group method with arithmetic mean showing similarity among isolates of *R. solanacearum* using REP–PCR, ITS–PCR and *Msp*I restriction of ITS amplified products of genomic DNA. Clustering at 70% similarity coefficient. Cluster I, All ginger strains + other hosts; Cluster II, Ginger strains from Assam + chilli from Kerala; Cluster III, Potato strain.

along with the rhizome material used for planting in these localities as evident from the prevalence of identical haplotypes of *R. solanacearum* in geographically and chronologically separated isolations (Figure 3). Serological evidence for rhizome transmission of *R. solanacearum* is reported in ginger, where the apparently healthy rhizomes collected from bacterial-wilt-affected field tested positive for *R. solanacearum* using Rs-specific antibodies in DAS–ELISA[22,24].

The findings of this study along with other serological evidence clearly indicate that the ginger strains of *Ralstonia* analysed are genotypically identical and probably transmitted from one location to another through a protected carrier, most likely the rhizome.

## Conclusion

Diversity of *R. solanacearum* causing bacterial wilt of ginger and other hosts in India was analysed using REP–PCR and RFLP–PCR. The molecular tools could cluster the highly pathogenic isolates in a cluster at 100% similarity coefficient in conformity with their host origin and biovar. High level of similarity (100%) among the ginger strains from geographically and chronologically separated isolations indicated that the isolates of biovar 3 of *R. solanacearum* were lineages of single virulent strain and inter-state rhizome transmission could be one of the possible means of pathogen spread across the States.


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# 9 Bacterial Diseases of Ginger and Their Control

*A. Kumar and A.C. Hayward*

Ginger (*Zingiber officinale* Rosc.) is an important source of spice and essential oil, and both products are obtained from the underground stem or rhizome, which also serves as planting material. Apart from India, ginger is also grown in China, Hawaii (USA), Indonesia, Jamaica, Japan, Malaysia, Nigeria, Queensland (Australia), Sierra Leone, and the Philippines. In India, Kerala, Karnataka, Himachal Pradesh, Sikkim, Meghalaya, Assam, and other northeastern states cultivate this crop very extensively. India contributes up to 45 percent of total global production of ginger (Peter, 1997). Here ginger is cultivated in an area of about 58.1 thousand hectares with a total production of 889.4 thousand tons. Diseases are one of the major constraints of production of ginger, and of these bacterial wilt (Figure 9.1) (also called "Mahali" or "Ginger blast") is one of the most serious. Apart from wilt, rotting of bacterial origin has been recorded very infrequently (Choi and Han, 1990; Nnodu and Emehute, 1988; Sarmiento, 1959). The rot diseases are classified as bacterial soft rot and bacterial rhizome rot depending on the causative organism (Choi and Han, 1990).

## Crop Loss and Distribution

Bacterial wilt of ginger inflicts serious economic losses in many ginger-growing countries on small and marginal farmers who depend on this crop for their livelihood. Although it is difficult to estimate the economic losses that can be attributed directly or indirectly to bacterial wilt, it ranks as one of the most serious and damaging diseases of bacterial origin in the world in terms of the actual number of plants killed each year in major crops such as banana, ginger, groundnut, potato, tobacco, and tomato (Sequeira and Kelman, 1976). The disease is endemic on other host plants in most of the ginger-growing regions in the world. Under conducive conditions, it causes loss in yield up to 100 percent in many ginger-growing states in India (Thomas, 1941; Sarma et al. 1978; Mathew et al. 1979; Dohroo 1991; Dake, 1995). According to an Indonesian report, bacterial wilt of ginger is estimated to cause annual losses up to 75 billion rupiah (Supriadi, 2000). Bacterial wilt of ginger is widespread and exceedingly destructive in several countries, a situation made worse by the easiness with which the pathogen is carried within the planting material.

Ever since the first report by Thomas of bacterial wilt of ginger from the Malabar region in the Madras presidency in 1941, voluminous information about the disease has accumulated, which is an indirect reflection of the economic importance of the disease. Since then the disease has been reported in Australia (Hayward et al., 1967; Pegg and Moffett, 1971), China (Li et al., 1994), Hawaii (Rosenberg, 1962; Quinon et al., 1964), Indonesia (Sitepu et al., 1977, Mulya et al., 1990), South Korea (Choi and Han, 1990), Malaysia

*Figure 9.1* Bacterial wilt of ginger.

(Lum, 1973), Mauritius (Orian, 1953), Nigeria (Nnodu and Emehute, 1988), and the Philippines (Zehr, 1969, 1970). The disease spreads rapidly when conditions of high temperature and rainfall are favorable for the disease development. The disease devastated the ginger crop in an area of 5 ha at the Horticultural Research Station, Ambalavayal, India, in 1978 (Mathew et al., 1979).

## Symptoms of the Disease

A wilting and yellowing of the lower leaves, which extends upward until all the leaves appear golden yellow in appearance is the first recognizable symptom of bacterial wilt in ginger. As the disease progresses, the pseudostem becomes water soaked and readily breaks away from the underground rhizome. The vascular tissue of the stem darkens to a black color and symptoms progress very rapidly until the ginger plant collapses (Pegg et al., 1974). Diseased rhizomes are usually darker than healthy ones and have water-soaked areas with pockets of milky exudates. When diseased rhizomes or pseudostems are cut, white milky exudates flows freely from the cut surface (Figure 9.2). High concentrations of bacteria in the vascular tissue deprive the plant of water and nutrients from the soil, which adversely affects plant development and ultimately results in death (Buddenhagen and Kelman, 1964). Infected ginger plants become stunted and chlorotic and the lower leaves dry out gradually before the plant is finally killed. The inner core of the rhizome, including the vascular tissue, is rotten leaving the outer epidermis intact. However, it is not clear whether the rotting is due to the primary pathogen or caused by secondary saprophytic microflora or microfauna.

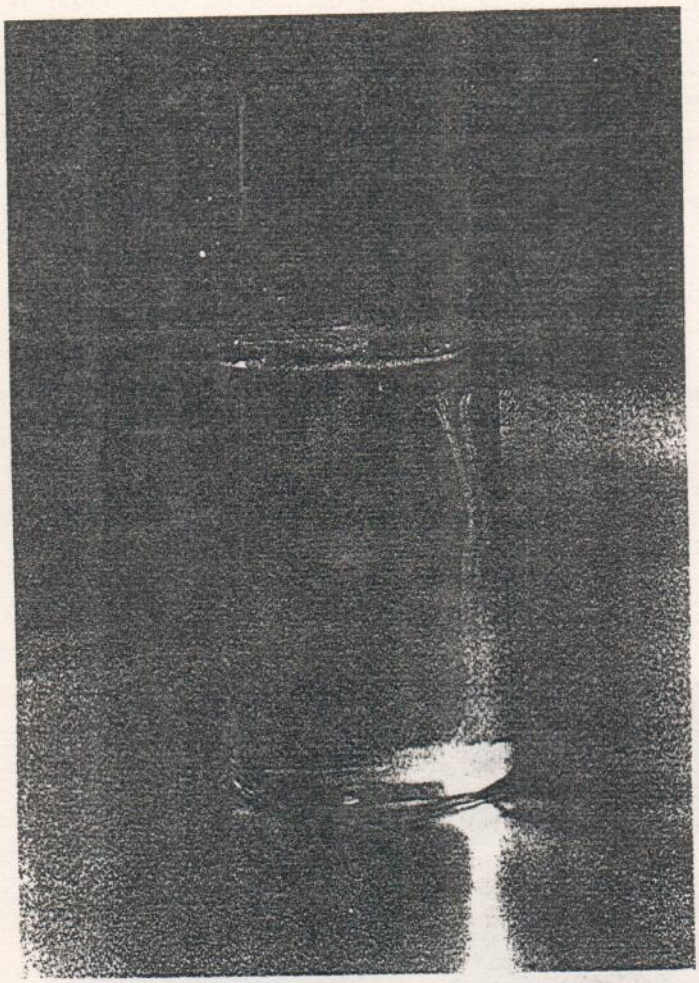


*Figure 9.2* Bacterial ooze from the cut end of the pseudostem of ginger.

In susceptible host plants this pathogen disrupts water transport, alters physiology, and induces a severe, usually total, bacterial wilt (Hayward, 1991). Complete wilt commonly occurs 3 weeks after inoculation. The most notable symptom of the disease is rapid wilting of foliage primarily due to vascular dysfunction (Denny et al., 1990). Intercellular spaces of the root cortex and vascular parenchyma are subsequently colonized and cell walls are disrupted, facilitating spread through the vascular system (Vasse et al., 1995). In xylem vessels, bacterial populations reach very high levels ($>10^{10}$ cells/cm of stem in tomato), concomitant with wilting and plant death. The bacterium then returns to the soil, living as a saprophytic organism until it infects a new host plant. Studies using artificial inoculation methods and avirulent mutants suggest that the production of copious amounts of extracellular polysaccharide is the key factor in virulence and the major requirement for infection via roots as well as wilting and killing of the host plant.

## The Pathogen

Bacterial wilt of ginger is caused by a prokaryote, *Ralstonia solanacearum* Yabuuchi (Smith), (synonym *Pseudomonas solanacearum* E.F. Smith), that has a wide host range including both dicots and monocots. *R. solanacearum* (Yabuuchi et al., 1992, 1995) is gram negative, rod shaped, and motile with one or more polar flagella or nonmotile without flagella. Erwin Frink Smith originally described the pathogen as *Bacillus solanacearum* in 1896. *R. solanacearum* belongs to the rRNA homology group II pseudomonads based on rRNA:DNA homology (Palleroni et al., 1973) and to the beta subclass of Proteobacteria. The bacterium is endemic in most subtropical and tropical regions of the world. Colonies of *R. solanacearum* on tetrazolium medium are distinguished from other bacterial colonies by their fluid smooth white appearance with red central whirling pattern (Figure 9.3) (Kelman, 1954). Conventionally, strains of *R. solanacearum* are grouped into races based on host range and biovars based on the ability to oxidise or utilize selected sugars (Buddenhagen et al., 1962; Hayward, 1960, 1964). The two groupings have been used in epidemiological studies, although they are imperfectly correlated, except that biovar 2 is equivalent to race 3. Almost all isolates from naturally infected ginger have proven to be either biovar 3 or biovar 4; they show variable degrees of pathogenicity to ginger. Isolates from ginger in Queensland showed a differential disease reaction correlated with biovar (Hayward et al., 1967). Biovar 4 produced a wilt on ginger in 14 or 21 days after stem and root inoculation, respectively, whereas biovar 3 isolates produced a wilt 6 weeks postinoculation. Biovar 4 was accordingly described as producing a rapid wilt and biovar 3 a slow wilt. The relationship between virulence on ginger and biovar is not consistent. In India, for example, where biovar 3 has been

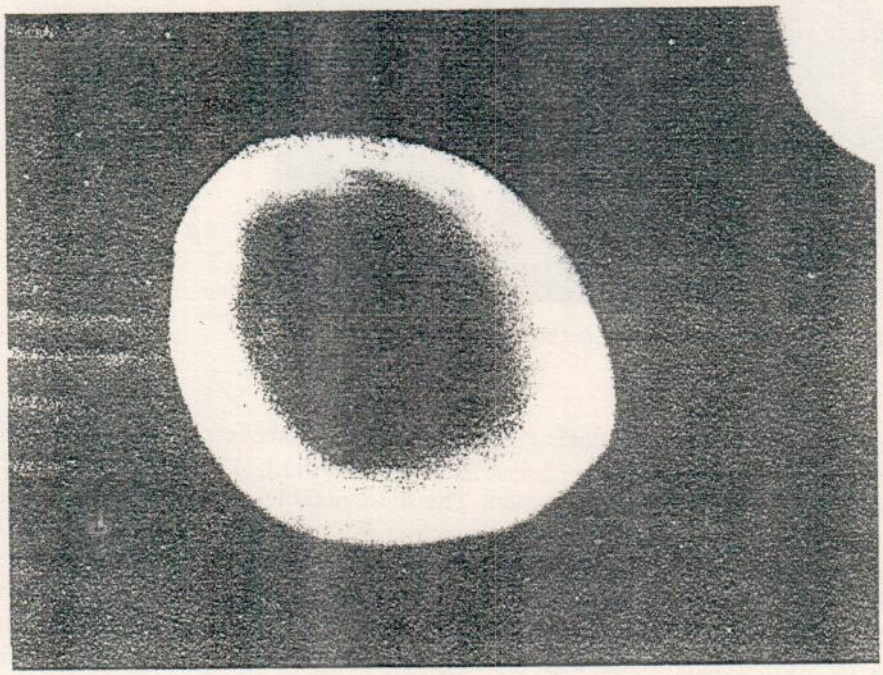


*Figure 9.3* Typical colony of *R. solanacearum* causing bacterial wilt of ginger.

identified on ginger (Sarma et al., 1978), a rapid wilt of ginger was produced on inoculation.

Studies have shown that biovar 3 isolates from ginger cause slow browning of the leaves over a period of 6 weeks, whereas biotype 4 isolates from ginger caused typical wilting in *Lycopersicon esculentum* L., *Solanum tuberosum* L., *Zinnia elegans* Jacq, *Capsicum frutescens* L., *Physalis peruviana* L., and *Solanum melonga* L. in 7 to 14 days. *R. solanacearum* from other hosts such as tomato, *Chromolaena*, chili, and potato was nonpathogenic on ginger. It is interesting to note that the *R. solanacearum* isolates from *Chromolaena*, a common weed of ginger fields, were not pathogenic on ginger even though they belong to biovar 3. Similarly, isolates from potato, tomato, and capsicum were not pathogenic to ginger (Kumar and Sarma, 2004). However, in India biovar 3 causes wilt in ginger in 5 to 7 days after artificial stem inoculation and in 7 to 10 days following soil inoculation of the pathogen (Kumar and Sarma, 2004). Biovar 4 is encountered infrequently in India. Zheng and Dong (1995) reported the predominance of biovar 3 in China among 129 isolates of *R. solanacearum* causing bacterial wilt of plants including ginger.

*R. solanacearum* is a heterogeneous species showing significant genotypic and phenotypic diversity (Cook et al., 1989; Hayward, 1991, 1994). In Hawaii, strains affecting *Strelitzia* (bird of paradise) and ginger, both relatives of banana, were affected by two different strains, neither of which could wilt triploid banana (Quinon et al., 1964). Two clusters within strains of *R. solanacearum* have been reported based on restriction fragment length polymorphism (RFLP) (Cook et al., 1989); Division 1 includes biovars 3, 4, and 5 and Division 2 biovars 1, 2, and N2. Division 1 was referred to as an Asian subdivision and Division 2 as an American subdivision because of the geographic origin of the isolates represented. A subcluster of *R. solanacearum* isolates belonging to Division 2 (Cook et al., 1991) has recently been recognized by Taghavi et al. (1996) based on 16S rRNA gene sequence information; this subcluster contains isolates from Indonesia and also *Pseudomonas syzygii* and the blood disease bacterium. This close relationship of *R. solanacearum*, the blood disease bacterium, and *P. syzygii* led Taghavi et al. (1996) to coin the term "*R. solanacearum* species complex" to describe the complexity of the species as revealed by RFLP analysis and sequencing of conserved genes. The use of 16S rRNA gene sequences for the classification and identification of *R. solanacearum* has shown up to 99 percent similarity between isolates. *R. solanacearum* isolates from ginger similarly showed a very high degree of homology (Kumar et al., 2004). Other measures of genetic diversity are needed before it can be concluded that there is limited genetic diversity among ginger isolates. Wilt of the ornamental gingers—*Hedychium flavum*, *H. coronarium*, and *H. gardenarianum*—is caused by *R. solanacearum* and the strains causing wilt are similar (Aragaki and Quinon, 1965). *Zingiber zurumbet*, a close relative of edible ginger, is susceptible to bacterial wilt caused by ginger strains of *R. solanacearum* (Figure 9.4). Isolates of *R. solanacearum* from ginger on tetrazolium medium are similar in appearance. This and other evidence suggests that strains of *R. solanacearum* from ginger may have evolved in a particular location and then spread to other parts of the world through planting material. All of the ginger strains belong to the Asian division of Cook et al. (1989).

Bacterial soft rot and rhizome decay is caused by either *Pectobacterium (Erwinia) carotovorum*-subsp. *carotovorum* or *Pectobacterium (Erwinia) chrysanthemi* (M. Stirling, personal

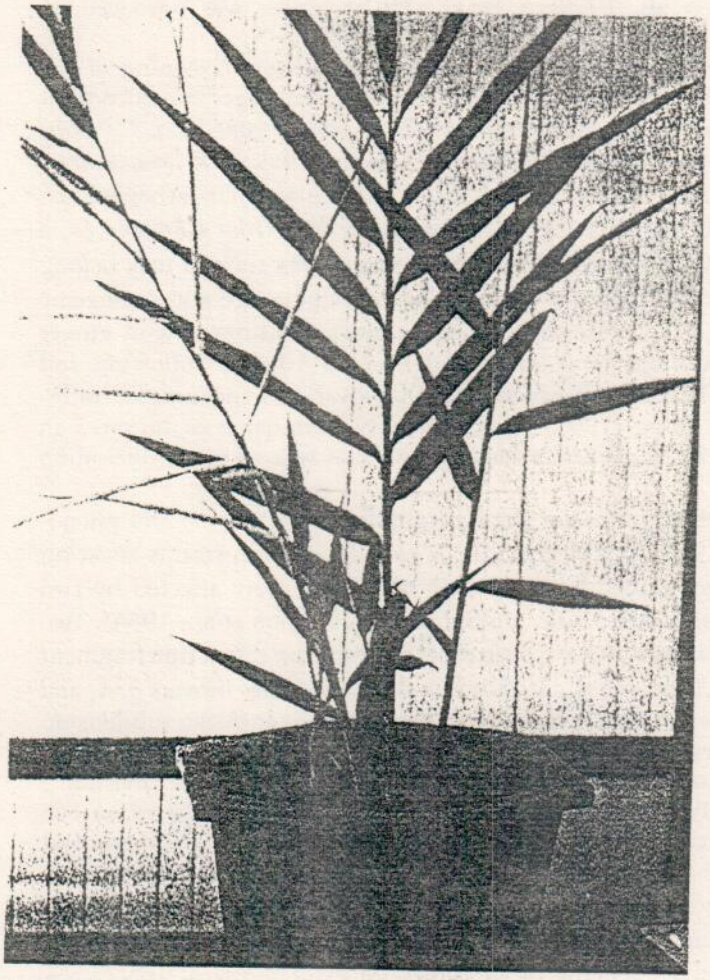


*Figure 9.4* Susceptibility of *Z. zurumbet* to *R. solanacearum*.

communication) and bacterial rhizome rot is caused by *Pseudomonas marginalis* in Korea (Choi and Han, 1990) and Nigeria (Nnodu and Emehute, 1988). However, their importance under field conditions is not known. Bacterial rot caused by *Pseudomonas zingiberi* has been reported from China (Li et al. 1994).

*R. solanacearum* is considered to be one of the most important plant pathogenic bacteria as it causes great economic losses worldwide (Hayward, 1991). The bacterium has an unusually wide host range; plant species susceptible to the pathogen have been observed to occur in over 50 plant families (Hayward, 2000). The host range includes solanaceous plants (tomato, potato, tobacco, eggplant), leguminous plants (such as groundnut, French bean), monocotyledons (mainly banana, ginger), and several tree and shrub hosts (such as mulberry, olive, cassava, eucalyptus). Recently it was shown that certain ecotypes of the model plant *Arabidopsis thaliana* are also susceptible to the pathogen (Deslandes et al., 1998).

## Epidemiology and Modes of Infection and Transmission of Bacterial Wilt on Ginger and Other Hosts

*R. solanacearum* can survive in the soil for long periods in the absence of host plants. There are conflicting reports on the longevity of *R. solanacearum* strains in soil, especially in the absence of protected sites (Graham and Lloyd, 1979) and on its resistance to desiccation. Soil types have been differentiated as being either conductive or suppressive to bacterial wilt (Hayward, 1991); their indirect influence on soil moisture determines the population size of antagonistic microorganisms, which affect, in turn, the persistence of *R. solanacearum.* Many workers have stressed the complexity of the epidemiology of bacterial wilt and involvement of many interacting factors (Kelman, 1953; Buddenhagen and Kelman, 1964; Hayward, 1991). Dissemination on infected vegetative planting material is of major importance in the case of banana, ginger, and potato and the potential for spread may be local or international. Some early evidence indicated that true seed might serve as a means of dispersal of the pathogen. In the case of groundnut, the pathogen is potentially seedborne, but early attempts to demonstrate seed transmission proved negative (He, 1990). It now appears that the pathogen does not survive in the dry seed normally used for planting. However, at high seed moisture content as in succulent plants like ginger rhizomes, survival and transmission are possible. When moist seeds from wilted groundnut plants are harvested and planted immediately in sterile soil, wilting of seedlings occurs. If the moisture content of harvested seed is above 10 percent, then survival is possible in storage; below this level there is no prolonged survival (Zhang et al., 1993a). Irrigation and the "wetting and drying" cycles that occur in the soil may tend to move cells up and down the soil profile and at times concentrate them in the rhizosphere. Also, the application of fertilizers during the growing season may influence root growth and the rhizosphere dynamics of the bacterium. Brown rot of potato caused by *R. solanacearum* race 3 (biovar 2) occurs in some cool temperate environments in which latent infections commonly occur in progeny tubers (Ciampi and Sequeira, 1980; Hayward, 1991). There is no comparable evidence of latent infection of either banana or ginger being affected by bacterial wilt under relatively cool conditions. Rhizomes used as planting material may show no obvious symptoms and serve as a means of dissemination of the disease.

Vaughan (1944) proved that apparently healthy tomato seedlings taken from infected seedbeds were responsible for dispersal of the pathogen when replanted in areas hundreds of miles away. Insect dissemination of *R. solanacearum* to banana (Moko disease) is uniquely important in that disease. In banana insects carry bacteria mechanically from the ooze issuing from diseased banana inflorescences to healthy inflorescences (Buddenhagen and Elsasser, 1962). There is little evidence of insects being of the same importance in dissemination of *R. solanacearum* on other hosts. However, it should be noted that a related pathogen, *P. syzygii*, the cause of Sumatra disease of cloves in Java and Sumatra, is transmitted by tube-dwelling cercopids of the genus *Hindola* (Homoptera: Cercopoidea: Machaerotidae). Few insect pests affect ginger, but their role in disease spread in the field cannot be ruled out. Leaf infection has been reported in a few instances in nature and can occur by inoculation under conditions of high humidity and temperature (Hayward and Moffett, 1978; Moffett et al., 1981). Aerial transmission through rain splash dispersal of epiphytic populations on tobacco leaves has been described in Japan (Hara and Ono, 1985). The worldwide distribution and damaging nature of bacterial

wilt on many crops suggests that *R. solanacearum* is an ecologically competent pathogen able to survive in the absence of its host, and in some circumstances overwinter in temperate zones and survive the dry season in tropical areas (Persley, 1986).

The modes of invasion of *R. solanacearum* differ from those of most other bacterial plant pathogens. Since ginger is planted as broken seed pieces termed "seed rhizomes," the soilborne bacterial inoculum has ample opportunity to invade the cut ends of the rhizomes during plant emergence. Infection occurs through wounds in roots or rhizomes or at sites of secondary root emergence. The bacterium colonizes the intercellular spaces of the root cortex and vascular parenchyma and produces extracellular enzymes that break down the pectic compound in the host plant cell wall and middle lamella facilitating spread through the vascular system (Vasse et al., 1995). In xylem vessels bacterial populations rapidly reach a very high level (Figure 9.5) ($10^{10}$ cells/cm of stem in tomato) concomitant with wilting and plant death. The bacterium is then released to the soil, living as a saprophyte until able to infect new host plants. Infection from rhizomeborne inoculum is assumed as a result of simultaneous multiplication of bacterium and plant cells and eventual blockage of the vascular elements by the bacterial cells.

Latently infected planting material is the major means of dispersal of *R. solanacearum* between locations, states, countries, and continents (Hayward, 1991). Traditionally, ginger is cultivated in previously fallowed soil or virgin soil. The occurrence of bacterial wilt in such fields is indirect evidence of the rhizomeborne nature of *R. solanacearum* in ginger (Pegg et al., 1974; Indrasenan et al., 1981; Kumar et al., 2002). Bacterial wilt of banana has long been known to be passively disseminated, primarily on seed pieces, by root wounding during transplanting or by tools and by root-to-root spread (Rorer, 1911; Sequeira, 1958). Serological evidence for the rhizomeborne nature of *R. solanacearum* in ginger has been reported (Prasheena 2003, Supriadi et al., 1995). In vegetatively propa-

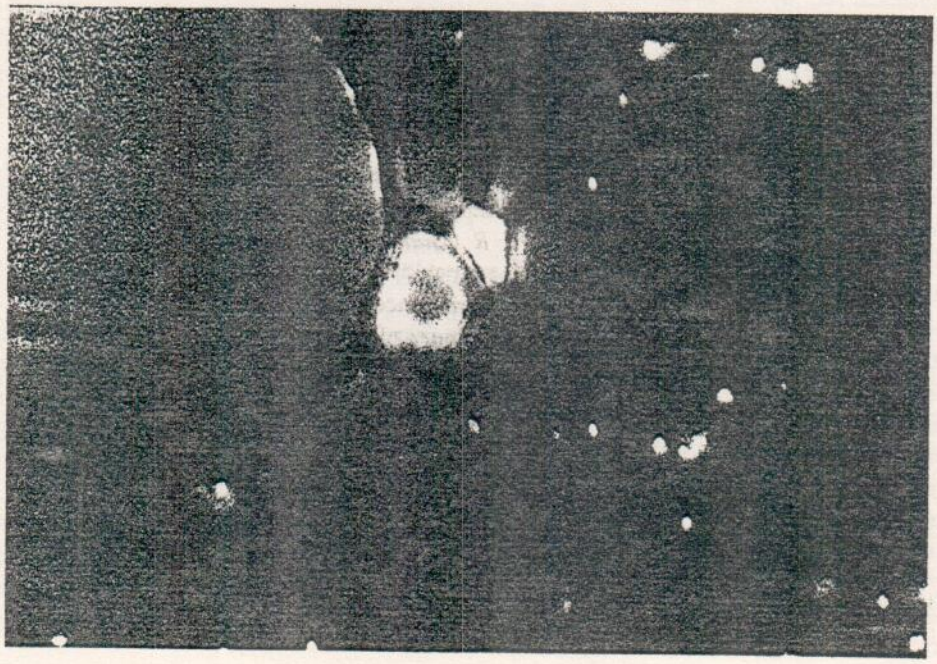


*Figure 9.5* Colonization of xylem element in ginger by *R. solanacearum*.

gated crops such as potato, banana, and ginger, infected planting material is the major means of dispersal of *R. solanacearum* from place to place and also from season to season (Buddenhagen, 1961). Spread of the potato race in tubers is important in parts of Africa, Australia, and South America, and this race has been introduced in England and Sweden in tubers from Egypt and other Mediterranean countries (Anonymous, 1960). Since the pathogen is mainly transmitted through tuber seed, the use of healthy planting material is the most effective means to control the disease (Hayward, 1991).

Root-to-root spread of the bacterium has been recorded (Kelman and Sequeira, 1965) but there is little evidence of long-distance spread from field-to-field except in circumstances where floodwater is responsible for the movement of infested soil and infected plant debris (Kelman, 1953). A synergistic interaction between the root knot nematode, *Meloidogyne incognita,* and bacterial wilt has been reported. It has been shown that the intensity of bacterial wilt on tomato increased with an increased number of nematodes in the field (Hutagalung and Widjaya, 1976). However, nematicide application did not result in bacterial wilt control.

## Survival of *R. solanacearum* in Soil, Planting Material, and Weeds

In Mauritius bacterial wilt is known to be endemic all over the island, including forest soils and sugarcane fields (Ricaud and Felix, 1971). Soil contaminated with bacterial wilt even to a high level may not give a high wilt incidence when susceptible plants are grown without adequate moisture, which confirms the role of high soil moisture in bacterial wilt incidence (Felix and Ricaud, 1978). Long-term survival of *R. solanacearum* in soil has long been attributed to the weed population in the field prior to cultivation. There is evidence of the saprophytic and parasitic survival of the bacterium in the rhizosphere of certain weeds in Queensland (Pegg and Moffett, 1971; Moffett and Hayward, 1980). However, there are few reports on the role of weedborne populations of the bacterium in actual bacterial wilt epidemics.

Being a vascular pathogen, it is presumed that *R. solanacearum* survives in ginger rhizomes at a very low inoculum level without affecting the normal state of the ginger. In general, the bacterial wilt pathogen will not multiply in intact rhizomes during storage unless the dormancy is broken and sprouting initiated. *R. solanacearum* can survive better under conditions of high soil moisture than in desert areas even under irrigation (Buddenhagen and Kelman, 1964). *R. solanacearum* survives in soil as well as the seed piece, and this forms a potential source of primary inoculum for the ensuing crop (Indrasenan et al., 1981). The potato race (race 3) is a low-temperature–adapted pathovar and it survives at cool temperatures in plant debris and latently infects potato tubers. *R. solanacearum* race 1, also a pathogen of potato, differs from race 3 in geographical distribution and the ability to survive under different environmental conditions (Graham et al., 1979). Alternative weed hosts and nonhost plants play an important role in the survival of *R. solanacearum* in the absence of susceptible crops (Granada and Sequeira, 1983). *R. solanacearum* has the ability to invade the roots of resistant cultivars of host species, such as tomato and persimmon, and nonhosts, such as bean and corn, without any symptoms. Disease control is made difficult by the ability of the pathogen to survive in the absence of a susceptible host (Granada and Sequeira, 1981). Lum (1973) found that both biovars 3 and 4 survived in soil for 20 months during a severe drought in Malaysia.

## Control of Bacterial Wilt of Ginger

Various control measures has been tried to combat the disease with limited success. Bacterial wilt is a major problem and one of the constraints in the production of ginger and other vegetable crops because of its wide host range, the genetic variability it exhibits, and the complexity of its epidemiology and modes of transmission. The general strategies for management of bacterial wilt are: selection of healthy rhizome material from a disease-free area; selection of field with no previous history of bacterial wilt; preplant treatment of rhizomes by application of heat or chemicals; strict phytosanitation in the field, including restrictions on movement of farm workers and irrigation water across the field; clean cultivation and minimum tillage; crop rotation with nonhost plants such as paddy and maize; insect pest and nematode control in the field; and soil amendments, including biological control agents. Some of these control methods are considered in greater detail in the following.

### *Selection of Healthy Rhizome Material from Disease-Free Area*

The use of rhizomes collected from previously disease-affected areas as planting material invariably results in severe disease when such material is planted in virgin soil or fallowed soil or even soil that has been rotated with nonhost crops. This experience emphasizes the need for pathogen-free seed in order to prevent disease outbreaks. In the absence of effective chemical and biological control methods, the best possible approach would be planting of pathogen-free rhizomes in pathogen-free soil in order to avoid or prevent the occurrence of bacterial wilt epidemics (Pordesimo and Raymundo, 1963; Supriadi, 2000). Techniques have been standardized to detect the pathogen in rhizome using nitrocellulose membrane-enzyme linked immunosorbent assay (NCM-ELISA) (Kumar et al., 2002) (Figure 9.6). In spite of the availability of excellent pathogen-detection technologies to detect the pathogen in rhizomes, soil, and irrigation water, their use in the indexing of planting material is negligible among ginger farmers. Although very sensitive and selective, these techniques for detection of *R. solanacearum* in ginger rhizomes are not readily adapted to the processing of large volumes of planting material; they are almost impractical under the farming conditions of developing nations in Asia and, therefore, they have not been adopted. The only method that has been used is selection of seed rhizomes from disease-free fields is visual inspection. This unscientific method of planting material selection often results in severe epidemics of bacterial wilt disease in India and other southeast Asian nations. Moreover, pathogen-free rhizomes are not readily available to all farmers owing to scarcity of seed material during peak seasons of planting, especially in crops like ginger, which require 1 ton of seed rhizome per acre of land.

### *Selection of Field with No Previous History of Bacterial Wilt*

Site selection is one of the most important factors that contribute to the successful control of bacterial wilt of ginger. It has been observed that a soil with no history of bacterial wilt often results in healthy crops of ginger if the rhizomes are free from the pathogen. Traditionally, ginger is cultivated in a previously fallowed soil, virgin forest soil, or rubber plantations after 20 to 25 years of rubber crop in the Kerala state of India. This long crop rotation often results in a healthy crop of ginger. Another alternative is to plant underneath perennial trees or in social amenity forests with

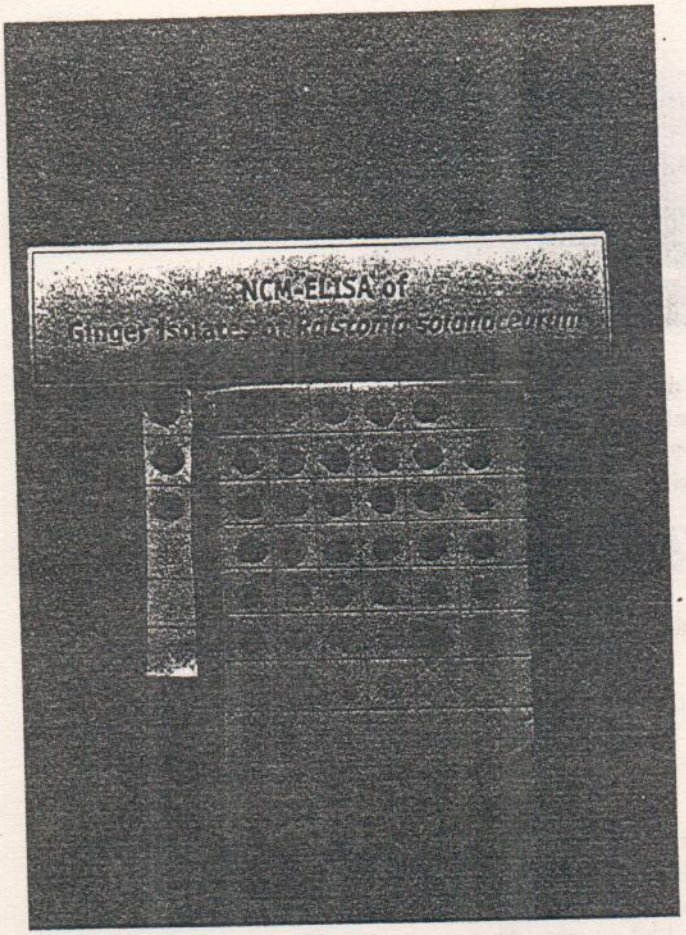
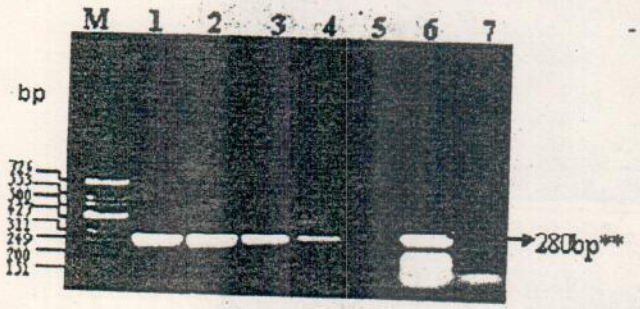



*Figure* 9.6 NCM-ELISA of ginger isolates of *R. solanacearum*.

regulated shade (Supriadi, 2000). French (1994a,b) has pointed out that pathogen-free soil and the use of certified seed tubers contribute most to the avoidance of brown rot of potato, and it is likely that the same is true for the avoidance of bacterial wilt of ginger. Soil can also be indexed for the presence of the pathogen by sensitive methods like the polymerase chain reaction (PCR). Techniques have been standardized to detect the pathogen in soil using double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and PCR (Kumar et al., 2002; Priou, 2001; Priou et al., 1999, 2002). The PCR-based method for detection of the bacterium in soil has been based on universal primers specific for *R. solanacearum* (Opina et al., 1997, Kumar, unpublished data) (Figure 9.7).

### *Heat Treatment of Soil by Solarization*

Solarization of soil prior to planting has been widely used to control soilborne pathogens and pests in various crops including potato, ginger, onion, carrot, and peanut, without consequential damage to the environment as occurs when methyl bromide is used for




M: DNA size marker (bp) Phi X 174 Hinf digest,
Lane 1-4: DNA from soil contaminated with *R. solanacearum* Biovar III,
Lane 6: Positive control (pure DNA of *R. solanacearum* Biovar III),
Lane 7: Negative control (DNA of Soil free from *R. solanacearum*).

Rs Forward Primer1: 5'-gTC gCC gTC AAC TCA CTT TCC-3';
Rs Reverse Primer 2: 5'-gTC gCC gTC AgC AAT gCg gAA TCg-3'
(Opina *et al* 1997)

** Amplification of 280bp DNA sequence indicates presence of *R. solanacearum* in soil

*Figure 9.7* PCR-based assay for the detection of *R. solanacearum* (Rs) in soil using RS specific primers.

shallow-rooted, short-season crops (Katan and DeVay, 1991; Stapleton, 1994). Solarization does not leave any toxic residue. It is a hydrothermal process dependent for success on moisture of the sample for maximum heat transfer. In more temperate regions soil is covered with clear plastic in order to trap solar radiation and raise the temperature sufficiently to suppress or eliminate soilborne pathogens and pests (Katan 1981; Katan and DeVay, 1991; Kumar et al., 2003). Polyethylene is a suitable cover because it transmits the germicidal component of sunlight. Solarization can be effective against a broad spectrum of soilborne diseases caused by pests such as fungi, nematodes, and bacteria. But the effectiveness of this method is directly linked to climate (Katan and DeVay, 1991). Solarization causes complex biological, physical, and chemical changes that improve plant growth, quality, and yield for up to several years (Stapleton, 1994). Solarization has already proven to be an effective pest control tool for tomato, pepper, and eggplant production in northern parts of Florida and California (Gamliel and Stapleton, 1993). The success of soil solarization is based on the fact that most plant pathogens and pests are mesophiles, which do not produce heat-resistant spores, and they are unable to survive for long periods at high temperature. Death of the organisms at high temperature involves inactivation of enzyme systems, especially respiratory enzymes (DeVay et al., 1990). The greater the temperature, the less time needed to attain a lethal effect. Solarization studies have shown that: solarization reduces or eliminates pathogens and pests prior to planting, crop yields can be significantly increased, and the beneficial effect of solarization can extend through several growing seasons (Afek et al., 1991).

### *Chemical Treatment of Soil and Rhizomes for Bacterial Wilt Control*

Hartati and Supriadi (1994) provide evidence of the activity of streptomycin and oxytetracycline both on the surface and inside tissue of ginger rhizomes soaked in solutions of antibiotics. Similar observations were made by Mulya et al. (1986) for effective bacterial wilt management in ginger. Disease control using commercial chemicals such as antibiotics, fertilizers, and fungicides has not been very successful. Fumigants such as chloropicrin have been used with some success for the control of bacterial wilt of tomato (Enfinger et al., 1979), but in general fumigation is not economically feasible over large areas. Ishii and Aragaki (1963) suggested soil fumigation with methyl bromide at 3 lb/100 $ft^2$ to get good control of bacterial wilt of ginger. The bactericide Kekuling, applied to the rhizosphere of ginger five times during growth of the crop as a wettable powder formulation at dilutions of 1:500 to 1:1200, has been reported to provide good control of bacterial wilt of ginger under field conditions in China (Zhang et al., 1993).

### *Preplant Rhizome Treatment by Heat*

Disinfection of seed pieces prior to planting is an important approach to the control of bacterial wilt of ginger; as noted earlier, contaminated planting material is one of the primary inoculum sources for field infection. Since no chemical and biological control approaches are available for control, a possible alternative is heat treatment to inactivate or kill bacteria, fungi, and nematodes (Janse and Wenneker, 2002). Pathogens are killed either directly by heat or weakened by sublethal heat to the extent that they are unable to damage the crop. Heat can be induced in rhizomes by hot water (Tsang and Shintaku, 1998), hot air, water vapor, solar energy, and microwaves (Kumar et al., 2003). Of these, hot air, hot water, and water vapor have been used for many years as effective treatments for planting material to rid them of various pathogenic microorganisms including nematodes (Colbran and Davis, 1969). Each of the approaches are discussed below.

#### *Heat Induction by Hot Air*

Heat inactivation using hot air has had wide application in the control of postharvest disease and insect pests in fruits and vegetables (Couey, 1989). The use of nonsaturated heated air is a potential treatment for disinfection of ginger seed pieces. Exposure of ginger seed pieces to hot air at 75 percent relative humidity (RH) until their center temperature reaches 49°C(112°F) for 30 minutes or 50°C (122°F) for 30 minutes results in minimal injury to the host without an adverse effect either on germination or subsequent growth. Rhizomes harvested from plants grown from seed pieces inoculated with *R. solanacearum* and subsequently heat treated with hot air at 75 percent RH until their central temperature reached 49°C for 30 minutes were free of the bacterial wilt pathogen. The bacterial wilt pathogen was destroyed in ginger seed pieces treated with hot air at 75 percent RH, and this method is recommended for disinfection (Tsang and Shintaku, 1998). Heat treatment also serves to release the seed piece from dormancy. To be effective, heat treatment must be long enough to penetrate the seed piece to its full depth, but not so prolonged as to be injurious to the host. Selection of similar sized seed pieces is an aid in maintaining uniform thermal gradients in a batch. There are many examples of the use of heat to kill pathogens without affecting the viability of the planting units (Waterworth and Kahn, 1978; Kuniyasu, 1983; Shiomi, 1992; van der Hulst and de Munk, 1992; Dhanvantari and Brown, 1993; Tsang and Shintaku,

1998). In ginger planting of seed pieces after exposure to 50 °C for 30 minutes often resulted in healthy plantlets in Hawaii (Tsang and Shintaku, 1998).

*Heat Induction by Hot Water*

Soaking of ginger seed in hot water at 50°C for 10 minutes (Nishina et al., 1992; Trujillo, 1963) is the usual preplant preparation in Hawaii. Shorter exposure times give insufficient heat penetration,, and longer soaking periods result in heat injury to the seed piece and growth of stunted crops (Nishina et al. 1992).

*Heat Induction by Rhizome Solarization*

Disinfection of rhizomes with solar radiation, a method called rhizome solarization, has been developed for bacterial wilt management (Figure 9.8) (Kumar et al., 2003). This is one of the most ecofriendly and energy-efficient methods available for rhizome treatment. Rhizome temperatures of 40 and 50°C were recorded after 1 and 2 hours of solarization from 9:00 A.M. to 11:00 A.M. on a bright sunny day (January to May in India) (Prasheena, 2003). Plants emerging from solarized rhizomes often escape the disease due to in situ killing of the pathogen in the seed rhizome or in the vascular tissue itself (Kumar et al., 2003). Serological evidence for elimination of *R. solanacearum* from ginger rhizomes has been reported (Prasheena, 2003). When rhizomes are exposed to solar radiation, the rhizome temperature rises especially in the vascular region. Incidentally, the thermal inactivation point for *R. solanacearum* is 46 to 50°C at 30 minutes of continuous exposure in vitro (Kumar et al., 2003). However, data obtained

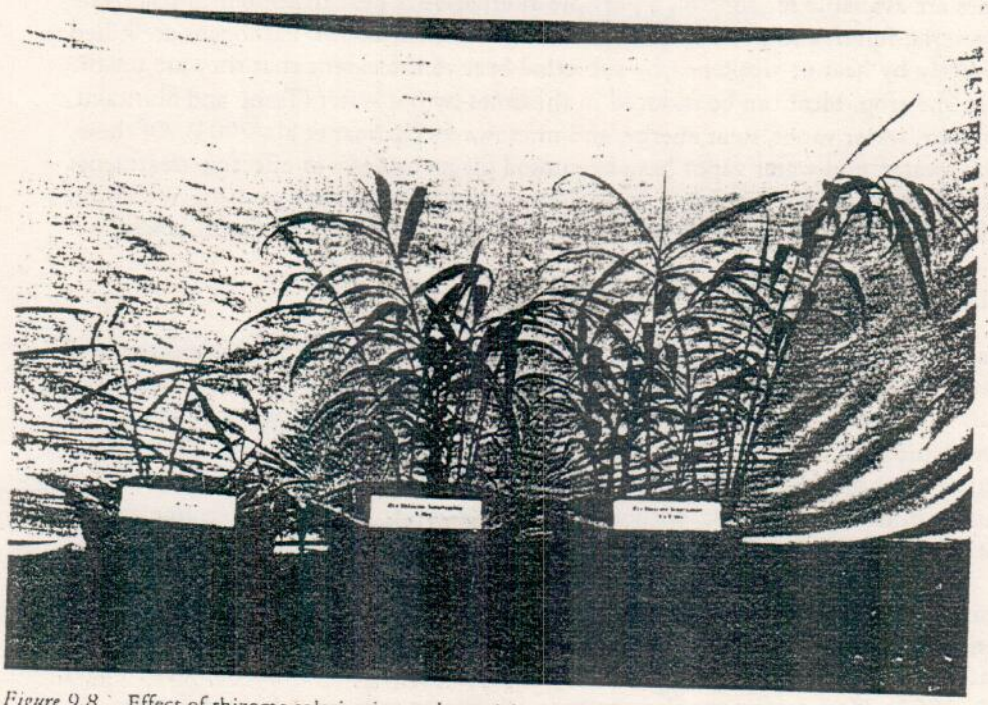


*Figure 9.8* Effect of rhizome solarization on bacterial wilt incidence in ginger 1. Untreated rhizomes. 2 & 3-Solarized rhizomes.

from studies in vitro with bacterial suspensions are not comparable to the situation where the bacterium is well protected in the vascular tissues of ginger rhizomes. To achieve the requisite temperature inside the vascular tissue or in a site where the pathogen is located, 2 hours of rhizome solarization are sufficient. A temperature of over 49°C was recorded in almost all the locations. The consistency with which the rhizome temperature increases in the rhizome once again confirms the effectiveness of rhizome solarization for heat induction in rhizomes (Prasheena 2003). However, one of the major sources of variability in heat build up vis-à-vis the fate of pathogen, *R. solanacearum,* is variation in the size and shape of the rhizome. As the size increased the heat build-up was also increased. Larger rhizomes recorded 1 to 3°C higher temperatures than the smaller rhizomes. The variation in the heat build up in the rhizome could be due to the fact that the larger seed rhizome has a larger surface area to trap the sunlight that, in turn, results in a higher temperature in the rhizome (Prasheena, 2003). The relationship between rhizome size and heat build-up has been recorded (Tsang and Shintaku, 1998).

Rhizomes collected from ginger plants emerged from solarized infected rhizomes tested negative for *R. solanacearum* in NCM-ELISA (Kumar et al., 2003). This result corroborates the finding of Tsang and Shintaku (1998) that the bacterial wilt pathogen was killed due to heat exposure as assayed by PCR using primers specific for *R. solanacearum*. This could be due to heat killing of the microbial cells on ginger rhizomes including *R. solanacearum* as the rhizome temperature recorded after 2 hours of rhizome solarization was 50°C. The effect of rhizome solarization on microbial populations has been reported (Prasheena 2003). Tsang and Shintaku (1998) reported that *R. solanacearum* was eliminated from ginger rhizomes when the rhizome was exposed to heat for 30 minutes at 50°C. Negative results obtained in postenrichment *double antibody sandwich* (DAS)-ELISA for *R. solanacearum* in solarized rhizomes confirms that the bacterium does not survive in solarized rhizomes (Table 9.1) (Anila, 2003; Prasheena, 2003). The assay clearly indicates that rhizome solarization is capable of disinfecting the rhizomes infected by *R. solanacearum* either artificially or naturally. The temperature generated inside the rhizome may have decreased the numbers of viable bacteria in the rhizome. As surface

*Table 9.1* Fate of *R. solanacearum* in solarized ginger rhizomes as detected by DAS-ELISA

| | | A405 value[a] | | | |
|---|---|---|---|---|---|
| | | *RS inoculated* | | *Uninoculated* | |
| *Treatment* | *Rhizome temperature (°C)* | *Outer surface* | *Vascular tissue* | *Outer surface* | *Vascular tissue* |
| Unexposed | 31.4 | 0.273 (0.103) | 1.450 (0.413) | 0.309 (0.196) | 0.428 (0.033) |
| 1 h solarized (10:30—11:30 am) | 50.1 | 0.280 (0.004) | 0.512 (0.457) | 0.219 (0.130) | 0.669 (0.771) |
| 2 h solarized (10:30 am–12:30 pm) | 56.2 | 0.296 (0.109) | 0.80 (0.264) | 0.214 (0.098) | 0.356 (0237) |
| Negative control value: 0.333 | | | | | |

[a]Mean of two readings. Data in parentheses are standard deviation values.
A405 values greater then three times that of negative control are positive samples.
Absorbance was read at 405 nm 1 hr after adding the substrate solution.

washings of the unexposed rhizomes show a negative result for the presence of *R. solanacearum*, it has been concluded that *R. solanacearum* survives in the vascular tissues of inoculated or infected rhizomes.

*Heat Induction by Microwaves*

The effect of microwave treatment on microorganisms appears to be related to heat induction (Vela and Wu, 1979). However, some metabolic effect not related to heating may occur (Barker and Fuller, 1969). The microwave oven has been used as a research tool in several different investigations (Diprose et al., 1978). Susceptibility of microorganisms on seed to microwave heating is determined by altering the power level, amount of sample, and water content, as well as exposure time (Thomas et al., 1979; Puri and Barraclough, 1993). If the test sample (rhizome or seed) is homogeneous, the entire microwave energy penetrates all parts simultaneously and heat is generated evenly throughout the material; since the surface of ginger can lose energy by convection, conduction, or radiation, dielectric heating can result in the interior of the sample becoming hotter than the outside. Treatment of 1 kg soil for 150 seconds is sufficient to eliminate populations of *Pythium, Fusarium*, and most nematodes in soil. *Fusarium* species tolerated high-aerated steam temperatures than *Rhizoctonia* species, but *Fusarium* was less tolerant to microwave treatment (Bollen, 1969). The effect of 2,450 MHz heating on plant pathogens and soil microorganisms has been reported (Ferris, 1984). When infected rhizomes were subjected to microwaves for 30 seconds, the resulting plantlets were free from bacterial wilt under greenhouse conditions (Figure 9.9) (Kumar


*Figure 9.9* Effect of rhizome microwaving on bacterial wilt incidence in ginger. 1. Healthy control. 2. Untreated rhizomes. 3. Microwaved rhizomes.

et al., 2003). However, at longer exposure, germination of the rhizomes was adversely affected (Kumar et al., 2003).

### *Cultural Methods*

An effective method to reduce the occurrence of bacterial wilt of potato in infested potato fields in Peru is crop rotation with maize (Elphinstone and Aley, 1993). In China 3 years of rice cultivation reduced the bacterial wilt incidence of groundnut from 8.3 percent to 1.5 percent (Wang et al., 1983). In India rotation with finger millet or maize reduces wilt of eggplants and tomato (Sohi et al., 1981). In pot culture experiments soil amendments were effective in controlling bacterial wilt (Chang and Hsu, 1988), and similar results were obtained in field trials (Hartman and Yang, 1990; Elphinstone and Aley, 1993). Pegg and Moffett (1971) suggested that the grower should attempt to eradicate weeds known to harbor biovar 3, which has a wide host range including ginger. Experience in India has shown that crop rotation with nonhosts such as cereals and millets results in a reduction in the wilt incidence in the ensuing crop of ginger, but more work is required on cultural methods of disease management comparable to that done with other hosts of *R. solanacearum.*

## *Identification of Resistance Sources for Control of Bacterial Wilt of Ginger*

In vitro and in vivo techniques are available for screening the germplasm for bacterial wilt tolerance in ginger. Almost all cultivated edible ginger is susceptible to bacterial wilt. Over 600 accessions screened for bacterial wilt tolerance using a soil inoculation method were found to be susceptible to the disease. Incorporating the toxic metabolites of *Ralstonia* in the culture medium was used for in vitro selection; however, surviving plantlets were found to be susceptible to bacterial wilt in the field (A. Kumar, unpublished data). An efficient in vitro screening technique for tolerance to bacterial wilt has been developed at the Indian Institute of Spices Research, Calicut. Live bacterial cells are added to tissue culture bottles containing ginger plantlets; this method enables screening of large numbers of plantlets in tests of 2 weeks' duration. Susceptible plants became chlorotic (Figure 9.10). A differential reaction of ginger accessions to bacterial wilt was reported by Indrasenan et al. (1982).

### *Biological Control*

Successful biological control agents have the ability to compete with other members of the soil microflora and also to produce antibiotics or induce a response in the host that favors growth of the biological control agent while inhibiting the growth of *R. solanacearum*. Bacterial antagonists and avirulent strains of *R. solanacearum* are effective in the control of wilt in groundnut (He, 1990). The bacterial antagonists include *Pseudomonas fluorescens* (Kempe and Sequeira, 1983; Ciampi-Panno et al., 1989; Gallardo et al., 1989), *Pseudomonas glumae* (Wakimoto, 1987; Furaya et al., 1991), *Pseudomonas cepacia* (Aoki et al., 1991), *Bacillus* species (Fucikovsky et al., 1989), and *Erwinia* species (Fucikovsky et al., 1989). Avirulent mutants of *R. solanacearum* (Chen and Echandi, 1984; Kempe and Sequeira, 1983) show promise for bacterial wilt control (Trigalet and Trigalet-Demery, 1990). Other biological agents have not been very effective in natural environments due to poor colonization and because the level of protection is not sufficient for commercial use (Chen and Echandi, 1984). Endophytic antagonists derived from wild-type strains

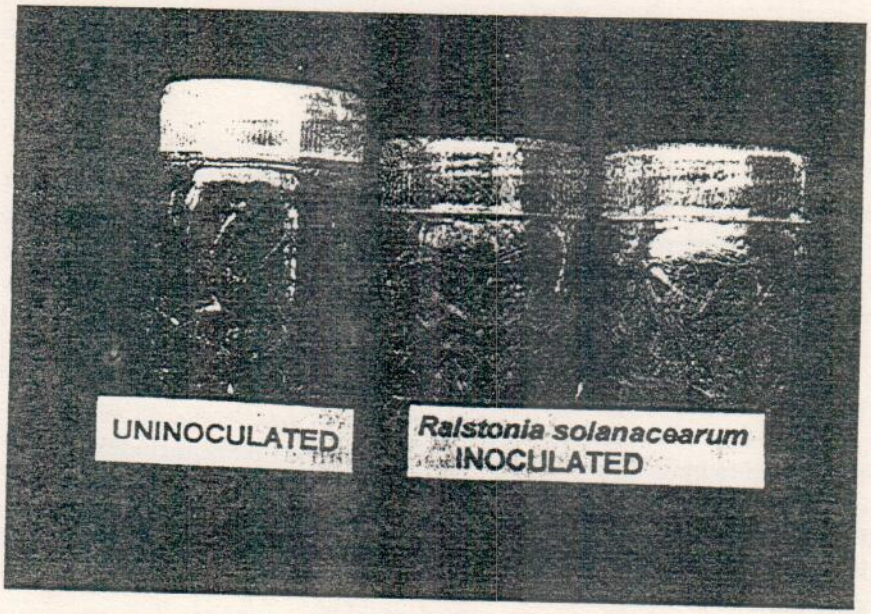



*Figure 9.10* In vitro screening technique for bacterial wilt tolerance.

are potential control agents (Frey et al., 1993). Some genetically engineered avirulent mutants of *R.solanacearum* with lesions in the *hrp* gene cluster have the ability to colonize the host plant multiplying in the rhizosphere and rhizoplane and inside the collar and lower part of the stem. These mutants induce a host defense response, and also have the ability to produce bacteriocin with a wide spectrum of activity, which makes them promising agents for the biological control of bacterial wilt under field conditions.

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