PhytofuRa
AN ICAR NETWORK PROJECT ON
Phytophthora, Fusarium & Ralstonia

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ICAR

Outreach Project on *Phytophthora, Fusarium* and *Ralstonia* Diseases of Horticultural and Field Crops

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भारतीय मसाला फसल अनुसंधान संस्थान
Indian Institute of Spices Research

OUTREACH PROJECT ON *PHYTOPHTHORA, FUSARIUM* AND *RALSTONIA* DISEASES OF HORTICULTURAL AND FIELD CROPS

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PREFACE

The Outreach Project on *Phytophthora, Fusarium* and *Ralstonia* Diseases of Horticultural and Field Crops (PhytoFuRa), a major research initiative of Indian Council of Agricultural Research during the XI Plan period, has successfully completed one more year and has been approved in the XII Plan also. This shows the good progress made under the project during the last Plan period and I congratulate and complement all my fellow investigators for their dedication and commitment.

The year 2013-14 was equally productive in many respects. A total of 304 new *Phytophthora* isolates and 154 *Ralstonia* isolates from different crops across the countrywere collected and added to the respective repositories. Studies on morphology and pathogenicity of the collected isolates were intensified. Genetic diversity in these isolates was studied using various tools such as MLST, phylotyping etc. Two more *Ralstonia* genomes were fully sequenced. With increased availability of molecular information several new diagnostic tools have been developed. Notable among these are the duplex PCR protocols for detection of *P. infestans - Alternaria solani* as well as *P. infestans – Fusarium* spp. developed at CPRI, Shimla and the multiplex PCR for simultaneous detection of *P. nicotianae* and *P. palmivora* developed at NRC for Citrus, Nagpur.

Equipped with the latest facilities, the investigators are now able to make in-depth analysis of the host –pathogen interaction and host plant resistance. Tools like qPCR, proteomics and transcriptomics are employed in *Solanum – Phytophthora, Piper - Phytophthora* and *Zingiber-Ralstonia* interactions. Potato, pigeon pea and chickpea genotypes possessing multiple disease resistance genes were identified through conventional and marker assisted selection (MAS). A SCAR marker, specific to *Phytophthora* resistance, was developed for MAS in citrus rootstock breeding programme.

Several biocontrol agents, botanicals and other non-chemical methods for managing these serious pathogens were identified and are being tested under field conditions. The project is also instrumental in developing new techniques which are destined to have a high impact in the research and development of some of the crops. A regeneration protocol for direct shoot formation in *P. colubrinum*, a rapid method for extraction of total proteins in black pepper, the single bud sprout technique in ginger and grafting of susceptible brinjal lines on resistant wild types are a few novel technologies emanated from the project during the preceding year.

The PhytoFuRa project promotes inter-institutional collaboration in an exemplary manner and facilitates close monitoring of the research progress through its dedicated portal http://www.phytofura.net.in. During the reporting period, a Sequence Repository has been launched for archiving of sequence data generated under this project.

Twenty eight high quality research articles have emanated from this project during the period and it is heartening to note that 12 of them are having a NAAS rating of >7. Since ICAR has adopted an open access policy, full texts of all the research publications are made available in a digital repository.

I humbly place this report for the scrutiny and appreciation of all our peers and colleagues. It is my privilege to profusely thank Dr. S. Ayyappan, Secretary, DARE and Director General, ICAR for his keen interest and constant encouragement. The support and guidance received from Dr. N.K. Krishna Kumar, Deputy Director General (Horticultural Sciences) and Dr. S.K. Malhotra, ADG (Hort. II) inspired and motivated us to achieve the goals. The financial support from ICAR, New Delhi is gratefully acknowledged. The pains taken by the editors in compiling and bringing out this publication deserve appreciation.

M. Anandaraj
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Director, IISR, Kozhikode

IISR, Kozhikode
20 July 2014

EXECUTIVE SUMMARY

Phytophthora

A. Biodiversity

Surveys were conducted in various districts of Kerala, Karnataka, Maharashtra, Goa, Himachal Pradesh, Uttarakhand, Uttar Pradesh and North Eastern states for the incidence of *Phytophthora* diseases and a total of 304 new *Phytophthora* isolates were collected from different crops. *Phytophthora lacustris* (formerly *P.* taxon *salixsoil*) was isolated from rhizosphere soil of a citrus orchard in Sriganganagar, Rajasthan. A cryopreservation method was standardized at IISR, Kozhikode for preserving *Phytophthora* cultures.

Studies on morphology and pathogenicity of *Phytophthora* isolates collected from diverse crops and geographical regions were continued. Among the *P. colocasiae* isolates studied, 52% were classified as sensitive, 38% were moderately resistant and 10% were resistant to metalaxyl.

The phylogenies of *Phytophthora* isolates of black pepper and *P. nicotianae* isolates were studied using a multi-gene approach using nuclear and mitochondrial genes. Five SSR markers for differentiating the Indian *P. infestans* isolates, belonging to 1a mitochondrial haplotype, were identified. Genetic diversity in *P. colocasiae* isolates was studied using ITS, start codon targeted polymorphism (ScoT) and random amplified microsatellite (RAMS) markers.

B. Diagnostics

Real-time quantitative PCR protocols were standardized for detection and quantification of *P. capsici, P. colocasiae* and *P. nicotianae*. Duplex PCR protocols for detection of *P. infestans - Alternaria solani* as well as *P. infestans – Fusarium* spp. were developed at CPRI, Shimla. ITS-RFLP analysis clearly differentiated isolates belonging to *P. palmivora, P. citrophthora* and *P. nicotianae*. A multiplex PCR for simultaneous detection of *P. nicotianae* and *P. palmivora* was developed at NRC for Citrus, Nagpur.

C. Host plant resistance

Agar bit inoculation method was found most effective for screening of castor against *P. nicotianae*. Castor and safflower cultivars resistant to *P. nicotianae* were identified.

Resistance gene candidates (RGAs) were isolated and sequenced from *Piper* spp. and wild *Solanum* spp. Defence related genes like osmotin, β - 1, 3 glucanase, defensins, PGI and PAL were studied using qPCR. Expression profiling of three putative R genes in *Piper* spp. was carried out using real time PCR. Several proteins with various biological significance were identified in the resistant black pepper variety, IISR Shakti, using 2-D proteomics coupled with mass spectrometry. A new gene construct was prepared by ligation of the restricted QTL gene sequenced from the mapping population (SC-34) and the vector for transformation of commercial potato cultivars. Potato genotypes possessing multiple disease resistance genes with late blight (R1, R2 and R3), PVY resistance (Ryadg gene) and PCN resistance (HC, H1) were identified through marker assisted selection (MAS). A SCAR marker, specific to *Phytophthora* resistance, was developed for MAS in citrus rootstock breeding programme.

D. Epidemiology

Epidemiological studies were continued in cocoa at two locations. A computer based decision support system has been developed for management of late blight disease in potato for western UP. Studies in apple nurseries and orchards showed that the collar rot disease incidence is positively correlated with rainfall, soil moisture and minimum soil temperature and was negatively correlated with soil temperature at 5 cm soil depth.

E. Disease management

Among the promising *Trichoderma* isolates, PhytoFuRa 10 followed by PhytoFuRa 8 and 15 continued to perform better across centers and crops. However, PhytoFuRa-13 was found to be the most effective against leaf blight of castor. Actinomycete, *Daldinia eschscholzii*, caused 60% mortality to *Radopholus similis* under *in vitro* conditions. Rhamnolipid based formulation at a concentration of 0.2% significantly reduced late blight lesions in detached potato leaves and disease incidence under field conditions. In apple, maximum control (>97%) of collar rot disease was recorded with four consecutive applications of biocontrol agents like *T. harzianum, T. viride* etc.

Captan 0.2% followed by Metalaxyl+ mancozeb 0.2% significantly reduced *Phytophthora* damping off and seedling blight of castor while in safflower, captan (0.2%) and dimethomorph (0.1%) were more effective. In apple, soil drenching with Curzate (0.3%) was highly effective both under nursery and orchard conditions for controlling collar rot. Combined application of *T. harzianum* or *Bacillus* with Curzate (0.3%) at pre-planting stage provided almost complete control. Talc-based formulations of two promising strains of *T. harzianum* were developed at NRC for Citrus, Nagpur.

F. Genomics and Bioinformatics

A sequence repository was designed and developed to store the sequences generated under the project. Genome assembly and annotation of *Phytophthora* isolates infecting black pepper was further refined with new *in silico* tools. Around 1440 unique domain super families and 52 effector domains related to pathogenesis present in *P. capsici* (05-06) genome were identified through comparative genomics.

G. New techniques

A regeneration protocol for direct shoot formation was standardized for *P. colubrinum*. For extraction of total proteins of black pepper, a rapid method was developed.

Fusarium

A. Biodiversity

The usefulness of finger printing by RAPD, REP, BOX and ERIC -PCRs was proved in the case of *F. oxysporum* f. sp. *psidii, F. udum* and *Fusarium oxysporum* f. sp. *ciceri* isolates. *F. oxysporum* f. sp. *ciceri* isolates (22 Nos.) were grouped into five groups based on restriction patterns of the IGS regions. Studies using host differentials showed that majority of the *F. oxysporum* f. sp. *ciceri* isolates resembled race 2. Thirty three *F. udum* isolates of pigeon pea were categorised in to seven variants using host differentials.

B. Diagnosis

An IGS region based specific marker was developed to detect *F. oxysporum* f. sp. *ciceri* using conventional and real-time PCR. Vegetative compatibility group (VCG) analysis indicated that the *F. oxysporum* f. sp. *cubensis* infecting Cavendish banana in Theni District of Tamil Nadu belongs to VCG 0124 and 0125 of race 1.

C. Host-pathogen interaction

The expression of defence relates genes like protein kinase, glucanase etc. was higher in resistant chickpea variety compared to susceptible line while MAP kinase gene was up-regulated only in the susceptible variety.

D. Host resistance

Pigeon pea lines resistant to *F. udum* and chickpea lines having multiple resistance to different races of *F. oxysporum* f. sp. *ciceri* were identified.

E. Disease management

Treatments with Captan (0.2%) and *T. harzianum* (Th4d SC) @ 2 ml/kg significantly reduced the incidence of *Fusarium* wilt disease in safflower. While in chickpea, a combination of Pusa 5SD (*T. harzianum*) + carboxin+ Pf 80 provided significantly higher seed germination and grain yield while reducing the wilt incidence due to *F. oxysporum* f. sp. *ciceri*. Several biocontrol agents and botanicals for the management of *Fusarium* wilt disease of banana were identified.

Ralstonia

A. Biodiversity

Around 154 new isolates of *R. solanacearum* were collected from Kerala, Karnataka, Meghalaya, Uttarakhand and West Bengal. MLST studies using five chromosomal housekeeping genes (*ppsA, gyrB, adk gdhA* and *gapA*) and three megaplasmid virulence-related genes (*hrpB, fliC* and *egl*) showed the close similarity of *R. solanacearum* isolates from North-East India with previously reported isolates from different parts of India. Phylotyping using different marker genes (*egl, pga* and *hrpB* genes) showed that the isolates belonging to phylotype I clustered into distinct clades.

B. Diagnostics

R. solanacearum could be detected in soil as well as in plants by DAC-ELISA using polyclonal antiserum and PCR amplification (Umaim).

C. Host-plant resistance

A total of 1201 differentially expressed genes have been identified in transcriptomes of *C. amada* and *Z. officinale* consequent to *R. solanacearum* infection, out of which 587 genes are upregulated and 613 genes are down regulated. Fifty-four differentially expressed transcription factors were also located. Chemical inducers *viz.*, ascorbic acid, isonicotinic acid, salicylic acid and β-butyric acid inhibited growth of *R. solanacearum*.

D. Disease management

Six apoplastic bacteria suppressing *R. solanacearum* infecting ginger were short-listed. Tomato plants (Arka Abha and Pusa Ruby), treated with *B. amyloliquefaciens* after 45 days of inoculation of *R. solanacearum* reduced the wilt incidence. *P. fluorescens*, alone or in combination with *T. harzianum*, reduced wilt disease in tomato. *P. fluorescens* (IHRPf24) and *Bacillus subtilis* (IHRBs39) when combined with farm yard manure and green manure gave excellent control of bacterial wilt of tomato. Essential oils of *Allium sativum, Lawsonia inermis, Piper betle* and *Syzygium aromaticum* exhibited antibacterial activity against *R. solanacearum*.

Grafts of susceptible cultivated type (Agassaim) brinjal plants on seedlings of the wild types (resistant) were resistant to wilting and endophytic colonization of *R. solanacearum*. Application of lime at 10 and 20 t/ha reduced bacterial wilt incidence in brinjal.

E. Genomics and Bioinformatics

Genomes of two isolates of *R. solanacearum* (Rs-09-161 and Rs-10-244) were Illumina sequenced and compared with the published *R. solanacearum* genomes. A total of 71 and 76 effector molecules are identified in these strains. Another highly virulent brinjal isolate was sequenced using Ion Torrent technology and annotated.

F. New techniques

A new method of transplanting technique, single bud sprout technique, has been standardized in ginger. Yield in both single sprout transplanted and direct planting methods was on par.

PhytofuRa

Phytophthora

INDIAN INSTITUTE OF SPICES RESEARCH, KOZHIKODE

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1. BIODIVERSITY

a. Collection and maintenance of *Phytophthora* isolates

Surveys were conducted in Idukki, Kasaragod and Wayanad districts of Kerala for the incidence of *Phytophthora* diseases. The survey revealed high incidence of the disease as compared to last year. Infected samples were collected and *Phytophthora* was isolated. During this period about 55 new *Phytophthora* isolates from different hosts were added to the National Repository of *Phytophthora* (Fig. 1).

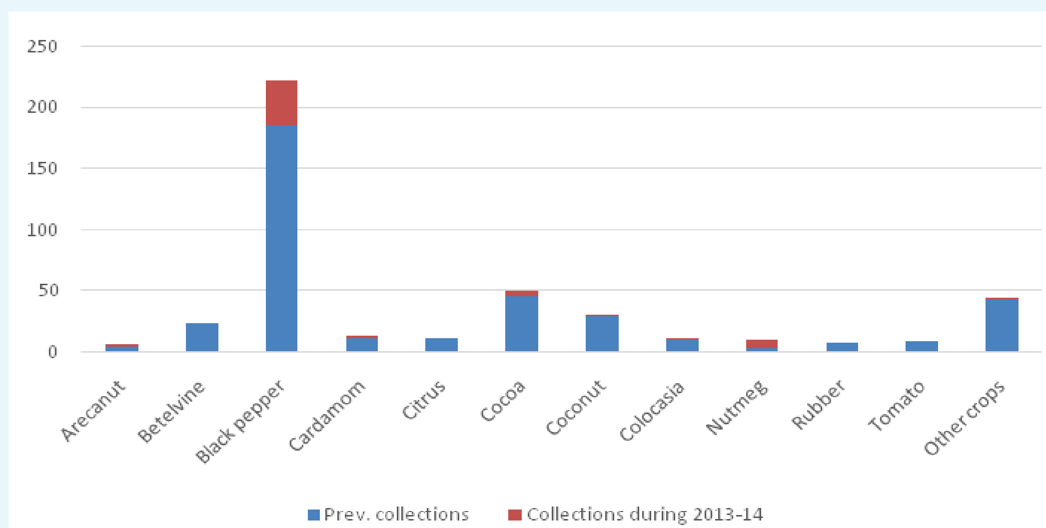


Fig. 1: Present status of *Phytophthora* collections in the repository at IISR, Kozhikode

b. Cryopreservation of *Phytophthora*

A cryopreservation protocol was standardized for long term preservation of *Phytophthora* cultures using 10% glycerol and 10% DMSO as cryoprotectants.

c. Molecular diversity

Studies on the pathogenicity and ITS sequence data have shown the existence of two groups in *P. capsici*. Dependence on the sequence information of a single gene is not always sufficient to discern the identity of an organism and also to trace its phylogeny. In this context, a multi-locus phylogenetic study using nine genes *viz*. 28S ribosomal DNA, 60S ribosomal protein L10, beta-tubulin, elongation factor 1 α, enolase, heat shock protein 90, tigA gene fusion protein, mitochondrial genome region between gene cox2 and gene *cox1* and ras-related protein (*Ypt1*) gene of 12 *Phytophthora* isolates (six isolates from each group) were amplified using PCR. The PCR products were then directly sent for sequencing except in the case of heat shock protein 90 and TigA gene fusion protein genes where direct sequencing was a failure. In the case of these two genes the purified PCR products were cloned to pTZ57R/T vector and transformed into competent *E. coli* strain DH5α using InsTAclone PCR cloning kit (Thermoscientific, USA). The isolated plasmid DNA was then sent for sequencing.

The sequence data was assembled using DNA baser V3.5.4. Similarity searches were done using BLASTn programme of NCBI. Sequences showing maximum hits were downloaded from NCBI and phylogenetic trees were constructed for each marker using neighbour-joining method with a Kimura two parameter nucleotide substitution model as implemented in MEGA version 5.1 (Fig. 2). A preliminary analysis showed wide variation among the members of the two groups. Comparative phylogenetic analysis between the sequence data of all these nine genes by *in silico* analysis is in progress.

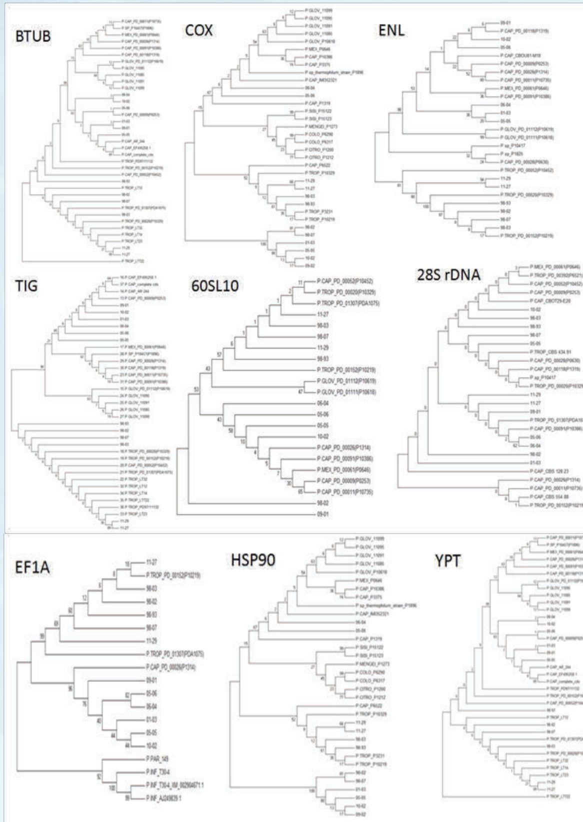


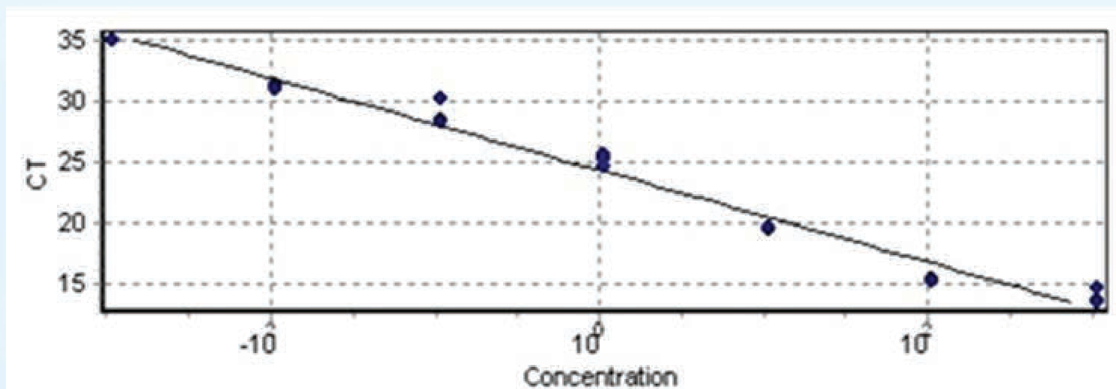
Fig. 2. Phylogenetic tree for nine individual genes of *Phytophthora capsici*

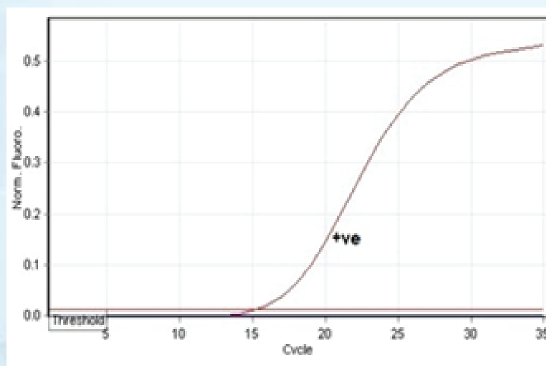
2. DIAGNOSTICS

a. Detection and quantification of *P. capsici* in soil using real time PCR

A real time PCR (qPCR) based method was developed for the detection and quantification of *P. capsici* in soil. Primers for the assay were designed from RAPD-SCAR region (Acc. FN298514.1) of *P. capsici*. The assay was standardized using genomic DNA isolated from *P. capsici* isolate 05-06 (positive) and genomic DNA from *P. nicotianae* isolate 02-21 (negative) and water control. The assay could detect *P. capsici* in positive sample without showing any amplification in negative sample and water control, thereby proving the usefulness of the assay in detection of *P. capsici*. The assay was further used for detecting the presence of *P. capsici* in soil by using soil DNA isolated from *P. capsici* inoculated (positive) and sterile soil samples (negative). Only the positive sample showed amplification.

In order to quantify *P. capsici* present in soil, a standard curve was constructed using 10 fold dilutions of genomic DNA (isolated from *P. capsici* isolate 05-06) from 1 to 10^{-7} in triplicates (Fig. 3). Using this standard curve, *P. capsici* was quantified in each sample tested.





No.	Name	Ct	Calc Conc (ng/µl)
1	Sample1	15.48	224.91
2	Sample2	-	-
3	wc	-	-

Fig. 3: Detection and quantification of *Phytophthora capsici* in soil using real time PCR. (*Top*) Standard curve, (*bottom*) Quantification in *P. capsici* inoculated soil.

3. HOST – PATHOGEN INTERACTION

a. Quantitative RT-PCR studies of transcription factors and defense genes in *Piper colubrinum*

The induction of defense related and other genes *viz.*, osmotin, β-1,3-glucanase, defensins, polygalacturonase inhibitor protein, phenylalanine ammonia lyase, were studied using real time PCR in *P. colubrinum* challenge inoculated with two strains (05-06 & 98-93) of *Phytophthora*. Plants inoculated with 05-06 strain showed high level of expression for the genes and the plants inoculated with the other strain 98-93 showed subdued expression.

b. Expression profiling of R-genes in *P. colubrinum* using quantitative RT-PCR

The expression level (fold change) of the three putative R genes; LR 2277, LR 1990 and PCR 07 was investigated at different hours post inoculation with two *P. capsici* strains *viz.*, 05-06 and 98-93. Highest level of expression was noticed in case of LR 1990 when challenged with the *Phytophthora* isolate 05-06 while LR 2277 gene expressed maximum when challenged with the isolate 98-93. The putative R gene, LR 2277 was expressed maximum in the initial period of pathogen interaction and there was a decrease in expression with time whereas, the expression of the other two genes LR 1990 and PCR 07 expression was maximum at 16 hpi (Fig. 4).

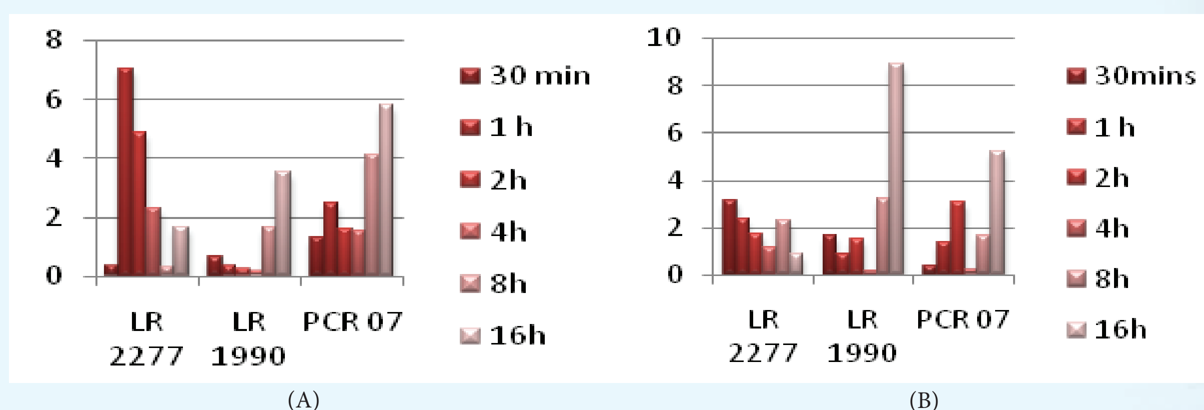


Fig. 4: The expression pattern of three putative R genes when challenge inoculated with *Phytophthora capsici* 05-06 (A) and 98-93 (B).

c. Direct shoot organogenesis and plantlet regeneration from leaf explants of *P. colubrinum*

A regeneration protocol via direct shoot bud formation was standardized using greenhouse grown leaf explants of *P. colubrinum*. The basal medium composition as well as the 6-benzyladenine (BA) and 1-naphthaleneacetic acid (NAA) concentration for direct organogenesis from leaf explants was evaluated and three of the tested media was found to give plantlet regeneration by means of direct shoot bud formation. The regenerated shoot buds were elongated on full strength MS medium with same hormone concentration and rooted in hormone free half strength MS medium II (containing macro and micro nutrients at half strength). The maximum number of shoots was produced from leaf discs cultured on half strength MS medium II supplemented with 2 mg/l BA and 0.01 mg/l NAA. The plant regeneration and rooting of the plants took a total of around four months from culture initiation (Fig. 5). As an initiative for transformation, a regeneration protocol via direct shoot bud formation was also standardized using greenhouse grown leaf explants of *P. colubrinum*.

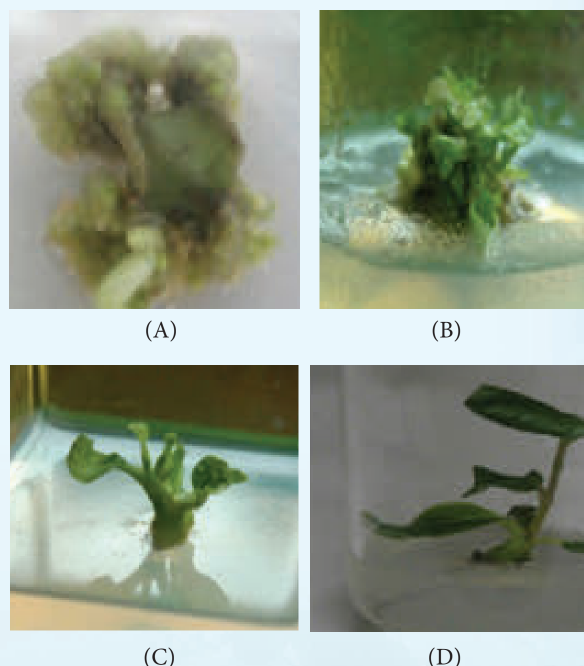


Fig. 5: *In vitro* shoot bud formation and plantlet regeneration from leaf explants of *P. colubrinum*. (a) Shoot bud induction at the cut end of leaf, (b) multiple shoot elongation, (c) single shoot transferred to rooting medium, and (d) rooted plantlet.

d. Development of leaf protein map for resistant black pepper variety 'IISR Shakthi'

A rapid method for total protein extraction with no poly phenol contamination was developed followed by highly reproducible 2-D electrophoresis. Protein profiles were generated with both PH 3-11 and 4-7 IPG strips. Image analysis with Image master platinum 6.0 yielded good quality protein spots starting from saliency value at 10 onwards. With the fixed saliency of 1000, 15 spots were excised, subjected to in-gel digestion followed by LC-MS/MS Analysis. Mass spectral data were analyzed using MASCOT 2.4 on Proteome Discoverer 1.4. Several proteins got identified with tremble database with molecular weight and PI. Rec A like NTPase protein - novel transmembrane protein, chaperons (heat shock proteins), sugar kinase, actin, ATP synthase beta subunit, subunits of rubisco activase, subunits of ribo kinase, Epo X hydrolase like protein, thiamine thialose synthase, subcellular oxygen evolving proteins and manganese stabilizing proteins representing all kinds of protein groups were located. 2-D proteomics coupled with mass spectrometry yielded many black pepper proteins with various biological significance (Fig. 6). With the lack of whole genome sequence for black pepper, the identified proteins forms functional information in this crop for the first time and also ensures an excellent experimental procedure for studying black pepper – *Phytophthora* interaction with the proteomics platform.

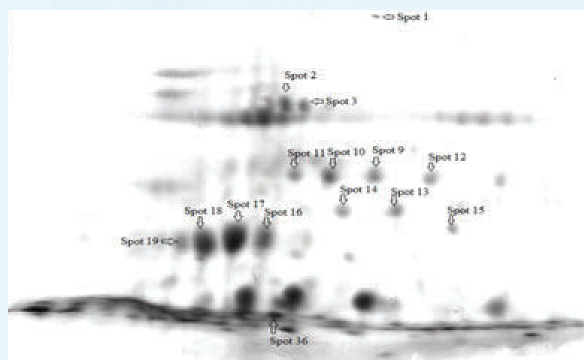


Fig. 6: nano LC-MS LTQ-Orbitrap showing proteins from IISR Shakti.

d. Identification of defense related genes in black pepper using cDNA library

To develop an understanding of the genes that are associated in defense mechanism against foot rot disease caused by *P. capsici* in black pepper, a full length cDNA library was generated. Total RNA was isolated from a moderately resistant variety (IISR Shakthi) at 1, 2, 4, 8, 16, 24, 48 and 72 h after inoculation (hai) with *P. capsici* (05-06) and just before inoculation (0 hai). cDNA library generated consists of more than 1000 clones having insert ranging from 250 to 3 Kb size (Fig. 7).

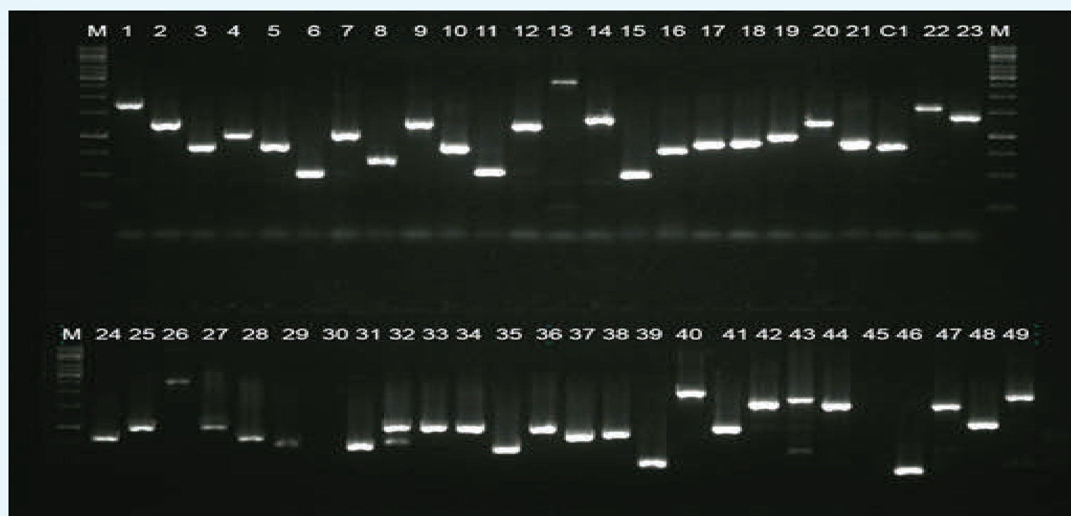


Fig. 7: Representative cDNA clones containing inserts ranging from 250 bp to 3 Kb size

e. Characterization of *R. similis* effector genes

High quality genomic DNA and total RNA were isolated from *R. similis*. Specific primers were designed for detecting functional genes such as acetyl cholinesterase, transthyretin, actin, endoglucanase, glutathione s-transferase and FMRF- like peptides from *R. similis*. For this, cDNA have been amplified from the total RNA and PCR parameters are being standardized for amplifying and cloning the above genes.

4. HOST RESISTANCE

a. Screening of hybrids and mapping populations for *Phytophthora* resistance

Thirty eight open pollinated progenies of IISR Shakthi along with the parent IISR Shakthi and Subhakara (check) were screened by leaf and stem inoculations. The experiment was done with three replications using the virulent isolate 05-06, maintained in National

Repository of *Phytophthora*, IISR, Kozhikode. Among the 38 progenies screened, IISR Shakthi OP 116 was found to be the most susceptible. IISR Shakthi OP 34, 46 and 188 were found to take up leaf infection but tolerate stem inoculation. The progeny IISR Shakthi OP 48 was found to be tolerant to leaf infection with an average lesion diameter of 9 mm. Thus progenies more susceptible and more tolerant than the parent IISR Shakthi could be identified providing an evidence for the segregation of progenies for *Phytophthora* resistance. Profiling of these genotypes with molecular markers for tagging *Phytophthora* resistance genes is in progress.

b. Genotyping and phenotyping of mapping populations

Thirteen genotypes of the association mapping population were morphologically characterized based on DUS guidelines of PPV & FRA. ISSR profiling of association mapping population is in progress. Profiling using 10 primers was completed. Sixteen SSR markers developed in black pepper were tested in five genotypes (Subhakara, Sreekara, P24-O-4, IISR Shakthi and *P. colubrinum*).

c. Targeted expression analysis of defense/defense related genes

RNA was isolated from challenge inoculated IISR Shakthi (resistant) and Subhakara (susceptible) with *P. capsici* (05-06) at different time intervals (0.5, 1, 2, 4, 8, 12, 16, 24 hours post inoculation), and the analysis of expression of R genes (NBS4 and NBS5) by qRT-PCR was done. The expression pattern was different between resistant and susceptible cultivars suggesting that R genes had a distinct pattern of expression and plays a critical role in *P. capsici* (05-06) stress tolerance. There is an observed expression shift of R genes at various times after inoculation. The resistant cultivar showed early response when compared to susceptible. The higher level of transcript suggest that it is likely responsible for the large part of resistance in black pepper (Fig. 8).

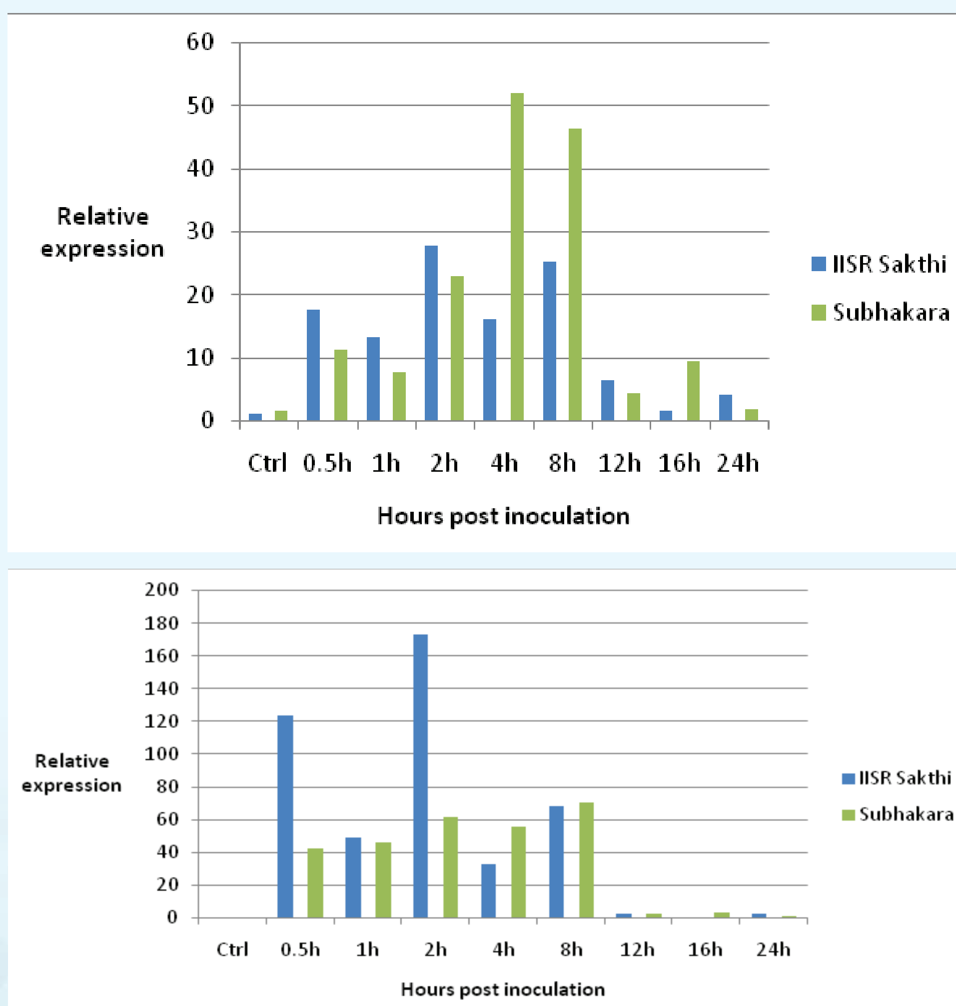


Fig. 8: Expression of R genes in resistant and suceptible black pepper lines inoculated with *Phytophthora capsici*. (*top*) CBNS4, (*bottom*) CBNS5

d. Isolation of resistance gene candidates

Isolation of resistance gene candidates from IISR Shakthi, Sreekara, Subhakara, an open pollinated progeny of IISR Shakthi (O4-P24) and two wild black pepper species *viz. P. colubrinum* (Acc. No. 392) and *P. ornatum* (Acc. No. 3362) was continued with modified degenerate primers (oligonucleotide) designed from known R-genes. PCR amplification resulted in the production of 500 bp amplicon in all the plant materials. A total of 550 colonies were obtained upon transformation. Of this 50 colonies were sequenced. The sequence data showed identity to disease resistance genes available in GenBank, supported by low e-values. The level of identity of these sequences to RGAs isolated from other plant species ranged from 40-51% and 78-99% similarity to other *Piper* RGA sequences (Fig. 9 & 10).

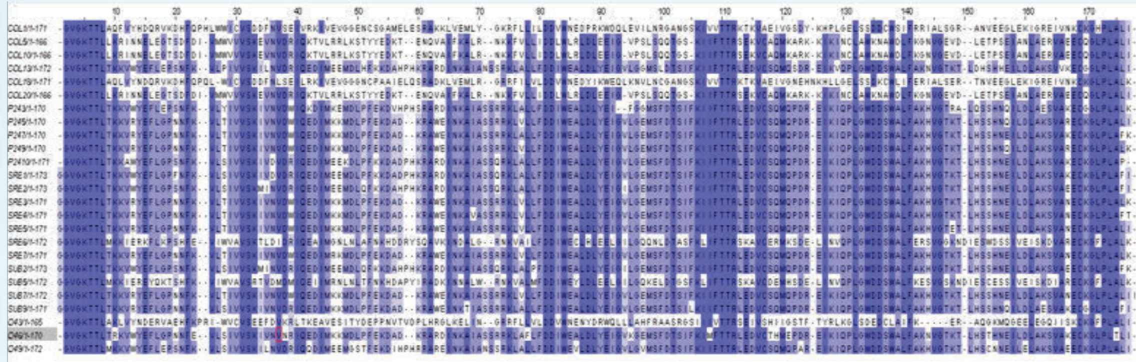


Fig. 9: The NBS analogs of *Piper nigrum* and *P. colubrinum* so far identified were found to be non-TIR-NBS-LRR subfamily with highly conserved tryptophan(W) as the last residue of kinase-2 domain.

5. DISEASE MANAGEMENT

a. Evaluation of *Trichoderma* isolates

Geographically different isolates of *Trichoderma* were evaluated for the third consecutive year against *P. capsici* under pot culture conditions for growth promotion and disease suppression. Among the 15 isolates evaluated, PhytoFuRa10 was highly promising with 82.96% disease control (with less than 10% disease incidence) followed by PhytoFuRa 8 and PhytoFuRa15 with 65.54 and 63.38% disease control, respectively, when compared to control having a disease incidence of 85.55%. In the pooled analysis, PhytoFuRa-10 showed the least disease incidence (20%) followed by PhytoFuRa-12 and PhytoFuRa-15 (33% each), while the control showed 91% disease incidence. Highest growth promotion was observed by the isolate PhytoFuRa-3 followed by PhytoFuRa-14 and highest biomass production was shown by the isolate PhytoFuRa-10.

b. *In vitro* effect of endophytic fungal metabolites on *R. similis*

Nine short listed endophytic fungal isolates which are effective against *P. capsici* were used for *in vitro* assay (metabolites) against *R. similis*. The maximum percentage mortality (60%) was observed by the isolate BPEF-73 (*Daldinia eschscholzii*), followed by 40% mortality by BPEF-75 (*Fusarium* sp.) and three isolates

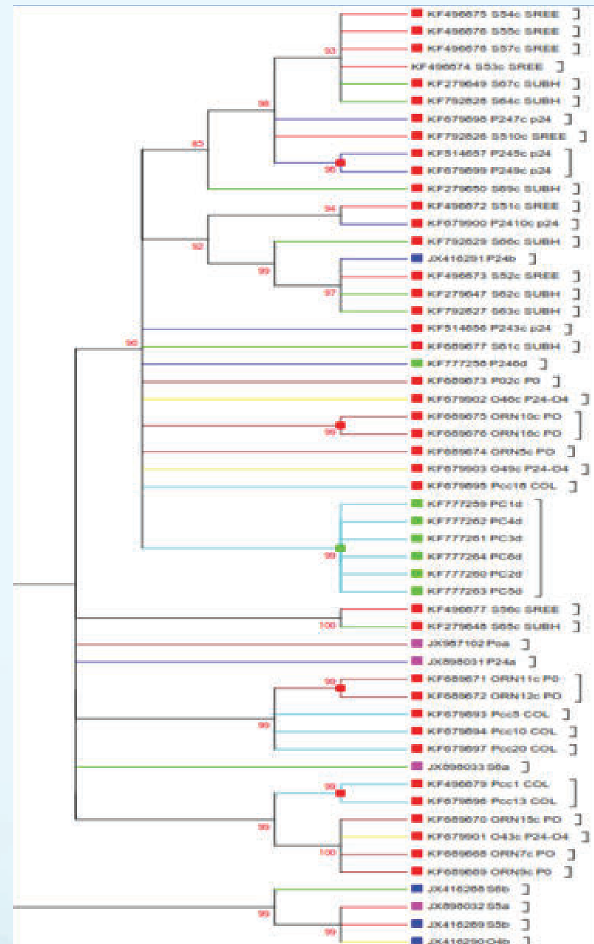


Fig. 10: Average distance tree showing percentage identity of nucleotides between *Piper* RGA sequences

BPEF-11(*Diaporthe* sp.), BPEF-81(*Ceriporia lacerata*) and BPEF-83 (*Phomopsis* sp.) showed 20% mortality. Other four isolates tested didn't show any effect on *R. similis*.

6. GENOMICS AND BIOINFORMATICS

a. Whole genome sequencing of *Phytophthora* sp. (05-06)

De novo hybrid assemblies using sequence reads from two NGS platforms (Illumina and Roche/454) were made for 05-06 isolate of *Phytophthora*. The *de novo* hybrid assembly of two next-generation sequencing (NGS) technologies (Illumina and Roche/454 sequencing) yielded 63.8 Mb genome size at an N50 contig length of 4724 kb, with contig lengths ranging from 200- 42,775. The *de novo* hybrid assembly gives out 32,044 contigs and 47,280,344 bases using Newbler Assembler. A reference assembly was also conducted to compare *P. capsici* genome of Joint Genome Institute and percentage identity was 95.35% with an average read depth of 50x. Variants were detected using probabilistic variant detection algorithm in CLC Workbench after removing duplicates. There were 25,569 variants compared the reference, of which 4,113 were deletions, 8,621 were insertions and 11,826 were SNPs. On filtering the variants using filter marginal variant calls a final 6316 variants were obtained which consisted of 2134 insertions, 1152 deletions and 2743 SNPs. Structural annotation was carried out using *ab-initio* gene prediction methods and an approximate of 22,358 coding sequences and 54,485 exons were obtained. Simple sequence repeats (SSR) analysis revealed that there are 1344 SSRs out of 32,044 contig sequence analysed. Whole genome alignment and comparison with reference genome revealed 1,298,146 SNP sites. Around 917 genes are common with reference genome of *P. capsici* (JGI), and 5501 genes are unique in *P. capsici* isolate of IISR. Blast homology based functional annotation revealed the presence of various proteins important for the survival of *Phytophthora* sp. in host plants and virulence associated proteins crucial for its infection.

b. Comparative genomics using conserved domain identification and Blast2GO for whole genome of 05-06

Conserved domain search to identify the protein families present in exonic regions of whole genome sequences of *Phytophthora* sp. infecting black pepper (05-06) have been studied. Blast2GO, Fast Annotator, and GoAnna were used for classifying gene ontology and unigenes obtained. For the purposes of this study, we first merged the results, and then classified GO associations, pathway genes and unigenes directly. After identification of homology based unigenes, all the protein translations of exon sequences has been subjected to CDD finding using RPS-BLAST, to identify the domain present in the genome. There were 1440 unique domain super families present. CDD search and InterProScan identified a total of 1,57,325 domains in the whole genome.

c. Initial reconstruction of metabolic pathways from gene annotation data

The copy number, localization and comparative analysis of domains responsible for pathogenesis in IISR 05-06 and 98-93 isolates of *Phytophthora* with already published ones were attempted. As of now, around 52 effector domains related to *Phytophthora* pathogenesis have been identified and are being studied. Once the potential effectors which are of main cause to the pathogenesis will be identified, their metabolic pathway networks will be studied and analyzed to mimic their interaction in the living system.

d. Development and maintenance of genome and transcriptome databases

Sequence Repository of IISR is a database designed to store the sequence information generated under the project. Information like sequences and their related information will be stored and displayed.

The PhytoFuRa portal, genome and transcriptome databases, various online resources developed under the project were maintained with timely updates.

CENTRAL PLANTATION CROPS RESEARCH INSTITUTE, KASARAGOD

Principal Investigator:

Dr. VINAYAKA HEGDE

1. BIODIVERSITY

a. Collection of *Phytophthora* infected arecanut specimens and isolation of *Phytophthora* spp.

Phytophthora infected arecanut samples were collected from Karnataka, Kerala and Goa states during 2013 monsoon season. In total 41 isolates were purified after isolation. The pathogen was identified as *P. meadii* in areacnut and *P. palmivora* in coconut based on morphological traits (colony characters and sporangial shape) and confirmed by sequencing ITS region.

b. Morphological characterization of *P. meadii* isolates

Morphological variability study revealed significant variation of *P. meadii* isolates in colony growth rate on four types of media tested such as potato dextrose agar, carrot agar, V8 agar and arecanut extract agar media. The highest colony growth rate was observed in arecanut extract agar followed by carrot agar and V8 agar media. The degree of sporulation varied with the media, all the isolates exhibited very good and early sporulation in arecanut extract agar medium but poor sporulation in potato dextrose agar medium. The isolates exhibited considerable variation in colony morphology across four types of media tested.

All isolates except isolate P12 were heterothallic, produced caducous, ovoid to ellipsoidal shape sporangia, size varies from 34.1-41.6 µm (length) & 20.3-28.7 µm (breadth) and these were identified as *P. meadii*. P12 is homothallic, produced non caducous, ovoid and distorted shape sporangia, size (L x B) varies from 35.0-50.2 µm & 22.5- 33.3 µm and it was identified as *P. heveae*.

c. Pathogenic variability

All the isolates of *P. meadii* have produced typical symptoms of fruit rot disease on three days after inoculation and the lesion diameter ranging from 6.4 to 19.4 cm. Highest lesion diameter was produced by isolate P19 collected from Yennekala, Belthangadi Taluk of Dakshina Kannada District.

2. HOST - PATHOGEN - ENVIRONMENT INTERACTION

a. Epidemiological studies on fruit rot of arecanut

Daily temperature, relative humidity, rainfall (from May 2013- to October 2014) and fruit rot disease incidence (every week during south west monsoon season) data were collected from Kidu and Dharmasthala. Correlation of climatic factors with disease incidence showed occurrence of disease during first week of July in Kidu coinciding with higher rainfall, high relative humidity and low temperature and maximum disease severity during last week of July. In Dharmasthala, disease incidence was noticed from second week of July and maximum disease severity during first week of August.

3. HOST RESISTANCE

a. Recording of bud rot disease incidence in MGD variety

Bud rot disease incidence in seven varieties of coconut was recorded during monsoon season (2013) in an established coconut garden. Among the seven varieties, the coconut variety Malayan Green Dwarf recorded less disease incidence (24.4 %).

b. Evaluation of *Phytophthora* tolerant/resistant cocoa accession under field condition

Phytophthora tolerant/resistant cocoa grafts were planted in disease prone area at CPCRI, RC, Kidu. There was no disease incidence in this year. The studies are in progress.

4. DISEASE MANAGEMENT

a. *In vitro* evaluation of fungicides and biocontrol agents against highly virulent isolate of *P. meadii*

Among five fungicides and three locally available

bio-products tested at different concentrations (125 ppm to 3000 ppm) against highly virulent isolate under *in vitro* condition, Iprovalicarb 5.5% + Propineb 61.3% (Melody duo) and Metalaxyl 8%+ Mancozeb 64 % (Mixol 72) at 125 ppm exhibited 100% mycelial growth inhibition (Table 1). Among three *Trichoderma* spp. tested, *T. virens* exhibited highest mycelial growth inhibition of *P. meadii* up to 62.5% inhibition in simultaneous inoculation of *P. meadii* + *Trichoderma* and 35.2% in inoculation of *Trichoderma* 48 hour after inoculation of *P. meadii*.

Table 1. Screening of fungicides against *Phytophthora meadii*

Fungicide	Concentration (ppm)	Percent growth inhibition
Iprovalicarb+propineb (Melody duo)	125	100a
	250	100a
	500	100a
Metalaxyl+mancozeb (Mixol)	125	100a
	250	100a
	500	100a
Cymaxanil+mancozeb (Curzate)	125	34.8hij
	250	48.4d
	500	100a
Carbendazim+mancozeb (Companion)	125	42.0fg
	250	51.1d
	500	80.1b
	1000	100a
Potassium phosphonate (Akomin)	2000	36.1hi
	3000	39.1fgh
	4000	42.2ef
	5000	71.2c
Potassium phosphonate (Biopot)	2000	30.2iklm
	3000	33.0ijk
	4000	38.5fgh
	5000	49.1d
Biofight	2000	30.2jklm
	3000	32.0ijkl
	4000	36.4ghi
	5000	47.2de
Hexaconazole (Samarth)	125	3.4p
	250	5.5o
	500	19.0n

b. Field trials on management of fruit rot disease of arecanut

Field trial on management of fruit rot disease of arecanut was laid out in CPCRI, RC, Kidu and farmer's field at Dharmasthala with eight treatments using Iprovalicarb + Propineb (Melody duo), Metalaxyl + Mancozeb (Mixol72), Phosphorous acid (Akomin), two locally available bio-formulations (chemical content not mentioned or not registered as fungicide) being used by arecanut farmers) and Bordeaux mixture as standard check. The observation on disease incidence was recorded on alternate days. Lower disease incidence of 8.3 and 19.1% was noticed in formulation 1 sprayed plot in Kidu and Dharmasthala, respectively. This was followed by formulation 2 (14 and 20%) and Akomin (19.4 and 22%). However these treatments were on par with Bordeaux mixture (26 and 35.2% disease incidence). The disease incidence under control plot was 90 and 94.4% in Kidu and Dharmasthala, respectively.

CENTRAL POTATO RESEARCH INSTITUTE, SHIMLA

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Dr. Mohammad Alimuddin Khan
Dr. Vinay Bhardwaj
Dr. Shashi Rawat
Dr. Sundaresha S
Dr. Jagesh Tiwari

1. BIODIVERSITY

a. Collection, maintenance and DNA isolation of *Phytophthora infestans*

Large number of *P. infestans* infected potato samples were collected from different geographic locations *viz.*, Himachal Pradesh, Uttarakhand, Meghalaya, Uttar Pradesh and Karnataka. Over 150 isolates were purified on Rye Agar Media and maintained. Extraction of DNA from 250 isolates has been done by using Qiagen kit and CTAB method.

b. Genotype differentiation and fingerprinting of *P. infestans* using SSR markers

Out of the 20 published SSR markers only five markers *viz.*, Pinf SSR1, Pi04, Pinf SSR2, Pi 89, and Pi4B showed better polymorphism in the Indian population of *P. infestans*. Two alleles (229 and 245 bp) were observed with primer Pinf SSR1 in 96 isolates of *P. infestans* (Fig.11). Three alleles were observed with Primer Pi04 (Fig. 12) in 248 isolates of *P. infestans*. Among three alleles, 171 bp size loci were observed in most of the isolates.

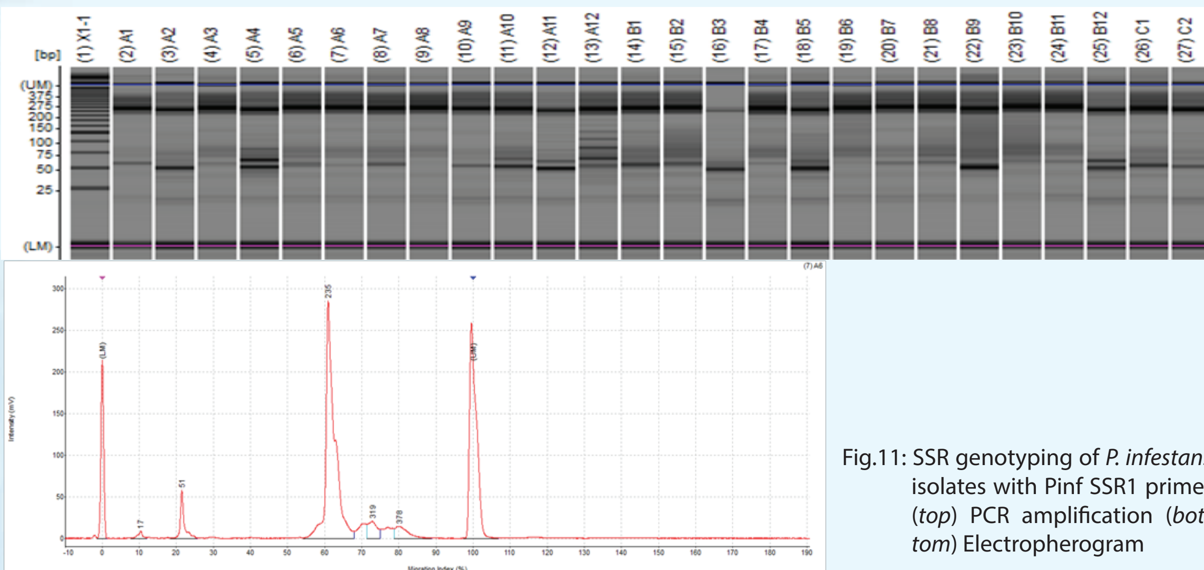

Fig.11: SSR genotyping of *P. infestans* isolates with Pinf SSR1 primer (*top*) PCR amplification (*bottom*) Electropherogram

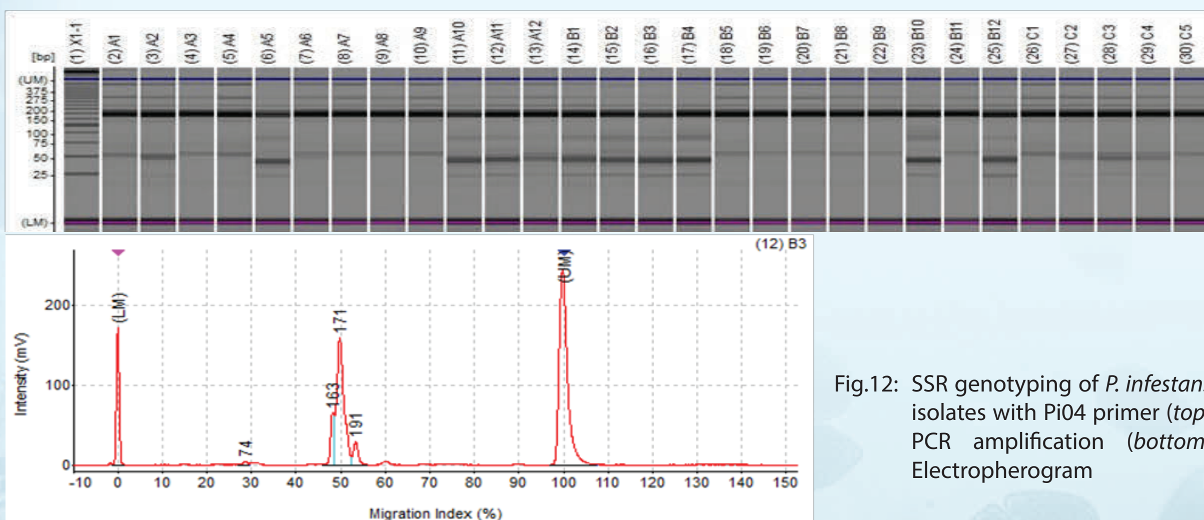

Fig.12: SSR genotyping of *P. infestans* isolates with Pi04 primer (*top*) PCR amplification (*bottom*) Electropherogram

c. Mt haplotype analysis for lineage determination

A sub set of population comprising 115 isolates collected from Himachal Pradesh, Meghalaya, Punjab, Bihar, Karnataka, Uttar Pradesh and Uttarakhand was analysed with primer F2-R2 (digested with *MspI*) and F4-R4 (cut with *EcoR1*) for mitochondrial haplotyping which revealed that all the isolates belonged to the Ia haplotype (Fig. 13). It tends to suggest that the new population which was introduced during 2002 is on the rise and has displaced the old population (Ib) in most of the regions.

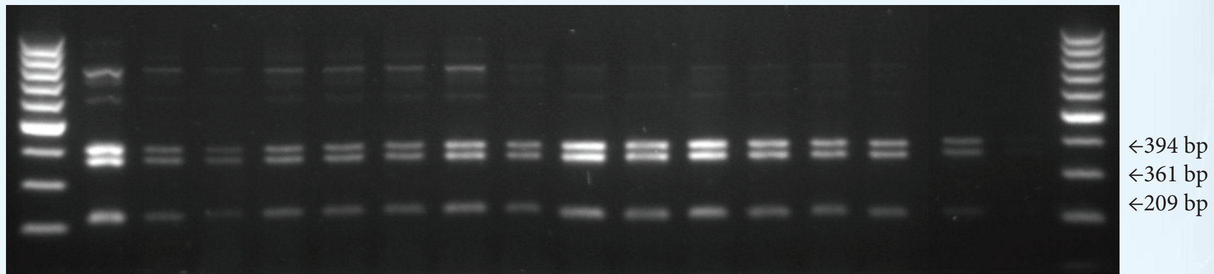

Fig. 13: Mt haplotyping with primer F4F4 & F4R4 digested with EcoR1 Lane: 1-15 *P. infestans* isolates M: 100 bp N: Negative control

2. DIAGNOSTICS

a. Production of *P. infestans* antibodies for development of lateral flow device (LFD)/ Dipstick

P. infestans cell wall based protein gene (392 bp, Fig. 14) has been isolated and cloned into pETSUMO expression vector. Positive clones were confirmed through colony PCR by using vector specific T7 promoter primer and revealed the 650 bp fragment size (Fig. 15) and confirmed the integration of the gene. Protein was extracted and purified using G-Bioscience protein extraction kit. The purified protein was detected through SDS PAGE, which revealed the ~12 KDa protein size (Fig. 16) and has been customised for developing polyclonal antibody.

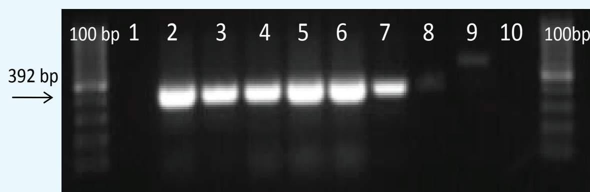

Fig. 14: Colony PCR amplification of *Phytophthora infestans* cell wall based protein gene in T/A vector. Lane 1 & 11 - 100 bp ladder, Lane 2-9= colonies

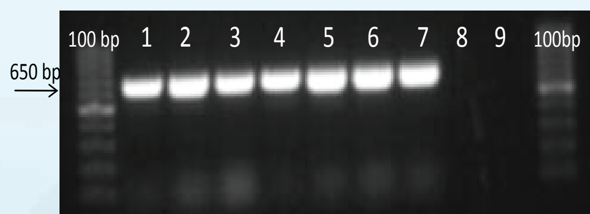

Fig. 15: Colony PCR confirming the integration of cell wall based protein gene in pETSUMO vector. Lane 1 & 10 = 100 bp marker; 2-8= colony number; 9-Blank

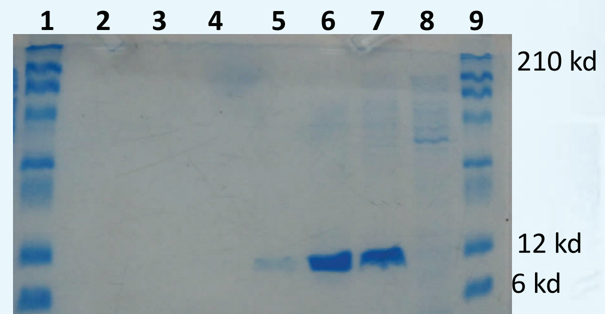

Fig.16: SDS-PAGE results of purified recombinant protein; Lane 1&9- PAGE marker, Lane 2, lane 3, lane 4, lane 5 Elution-3; lane 6-Elution-2; lane 7- Elution-1; lane 8-Uninduced.

b. Development of duplex PCR

Duplex PCR protocol was standardized for the simultaneous detection of *P. infestans* and *Alternaria solani* in a single reaction (Fig.17). Similarly, duplex PCR protocol was standardized for simultaneous detection of *P. infestans* and *Fusarium* spp. in a single reaction (Fig.18).

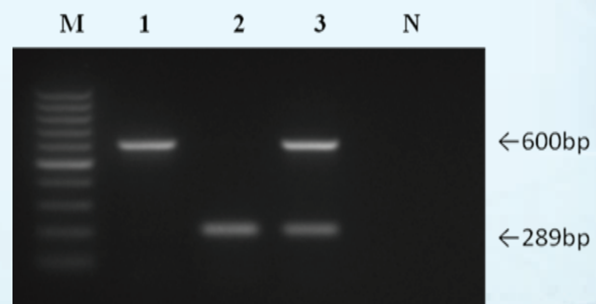

Fig.17: PCR detection of *P. infestans* and *Alternaria solani* in duplex PCR. Lane1- *P. infestans*, 2-*A. solani*, 3- combined *P. infestans* and *A. solani*, N- negative control, M- 100 bp DNA ladder

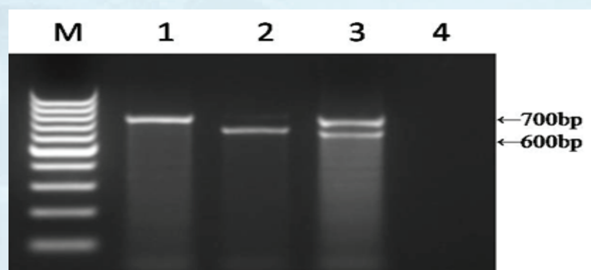

Fig.18: PCR detection of *P. infestans* and *Fusarium* spp. in duplex PCR. Lane 1- *Fusarium* spp., 2- *P. infestans* , 3- combined *P. infestans* and *Fusarium* spp., N- negative control, M- 100 bp DNA ladder

3. EPIDEMIOLOGY

a. Effect of temperature on growth of *P. infestans*

A total 47 isolates of *P. infestans* from different geographic regions were tested *in vitro* at different temperatures for their adaptability. Results revealed that the isolates could grow up to 28°C and highest mycelial growth was observed in isolates from north western hills (2.68 – 9.0 cm^2) followed by Tarai isolates.

b. Development of web based decision support system for the direct benefit of the farmers

Computer based decision support system has been developed for management of late blight based on i) decision rules for forecasting first appearance of late blight in plains during rainy and non-rainy years based on temperature, RH, and rainfall data, ii) decision rules for need based application of fungicides, and iii) regression models for yield loss assessment. All these modules have been combined to develop web based decision support system for western Uttar Pradesh.

4. HOST RESISTANCE

a. Development of AVR3-siRNA transgenic lines for late blight resistance

The Avr3a-siRNA transgenic lines developed last year and found positive through bioassay were tested for copy number analysis through southern. All the four lines were found positive for presence of NPTII gene. Three out of the four lines had single copy of transgene (Kufri Khyati 1037, Kufri Pukhraj 2137, Kufri Pukhraj 2155) and one line (Kufri Pukhraj 2086) had three copies of transgene (Fig. 19). In the present study, vector backbone pBI121 have selectable marker gene NPTII at left border, so the copy number of NPTII gene corresponds to the copy number of Avr3a gene.

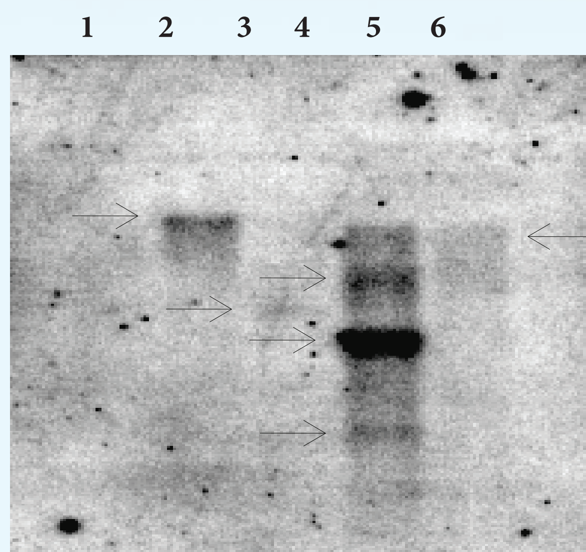

Fig. 19: DNA gel blot of control and four iIR-Avr3a RNAi lines. Lane 1- Kufri Khyati control and 6- Kufri Pukhraj control. Lane 2- Kufri Khyati 1037, Lane 3- Kufri Pukhraj 2137, Lane 5- Kufri Pukhraj 2155, Lane 4- Kufri Pukhraj 2086

b. Allele mining in *Solanum* germplasm: isolation and sequence analysis of novel NBS-LRR encoding late blight resistance gene analogues (RGAs) from wild potato species

Of the 39 *Solanum* species (49 accessions), 15 species (20 accessions) were found highly resistant (HR) to late blight after challenge inoculation with *P. infestans* (Fig. 20). The highly resistant 15 wild species were selected for further studies for PCR amplification using 53 gene-specific primers targeting late blight resistance in potato (Table 2). Of the 53 markers, 14 were successfully amplified in the samples. The prominent and single bands amplified by nine primers were used for cloning and sequencing. Finally, RGAs were cloned as a result of ~900 bp product amplified by RGA1F/R in each sample from SML01, PNT43, PLD47, TRF22, CPH62, LES29, HCB06, VER55, JAM07, PLT62, STO61, PLD80, LES34 and STO40, ~500 bp by CT88F2/R2 from LES29, ~600 bp by RB 629/638 from STO61, and ~700-bp by primer 1521/518 from HCB06. Thus, from a total of 53 primer pairs, 17 RGAs were isolated by three primers RGA1F/R, RB 629/638 and CT88F2/R2 representing the late blight resistance gene from *Solanum* species or its analogues in potato. The phylogenetic analysis based on the preliminary sequence analysis of the DNA sequences of 17 RGAs and 15 known late blight resistance protein genes from the NCBI database is shown in Fig. 21.

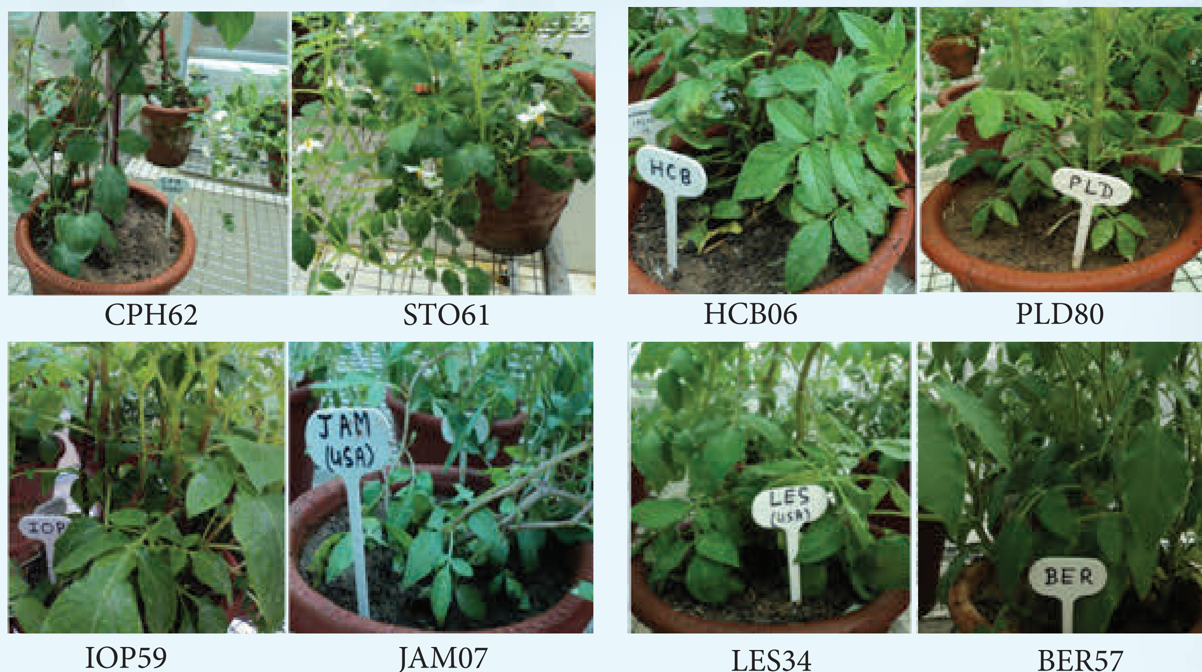

Fig. 20: Late blight resistant wild *Solanum* species (selected) screened by challenge inoculation of *P. infestans* in the controlled environmental conditions.

Table 2: Details of new resistance gene analogues (RGAs) isolated from various wild potato species

Species ID	Marker	Sequence	ORF	Maximum identity (BLASTn)
SML01	RGA1F/R	864	289-864	AY426263.1
PNT43	RGA1F/R	858	85-801	AY303171.1
PLD47	RGA1F/R	881	254-763	AY303171.1
TRF22	RGA1F/R	881	251-751	AY303171.1
CPH62	RGA1F/R	846	85-846	AY303171.1
LES29	CT88F2/R2	630	33-630	HM131813.1
LES29	RGA1F/R	889	85-789	AY303171.1
HCB06	1521/518	701	184-642	AY426259.1
HCB06	RGA1F/R	859	85-837	AY303171.1
VER55	RGA1F/R	846	85-834	AY303171.1
JAM07	RGA1F/R	886	85-822	AY303171.1
PLT62	RGA1F/R	880	120-809	AY303171.1
STO61	RGA1F/R	869	105-869	AY303171.1
STO61	RB 629/638	894	85-789	AY303171.1
PLD80	RGA1F/R	880	119-880	AY426263.1
LES34	RGA1F/R	870	243-785	AY303171.1
STO40	RGA1F/R	849	85-789	AY303171.1

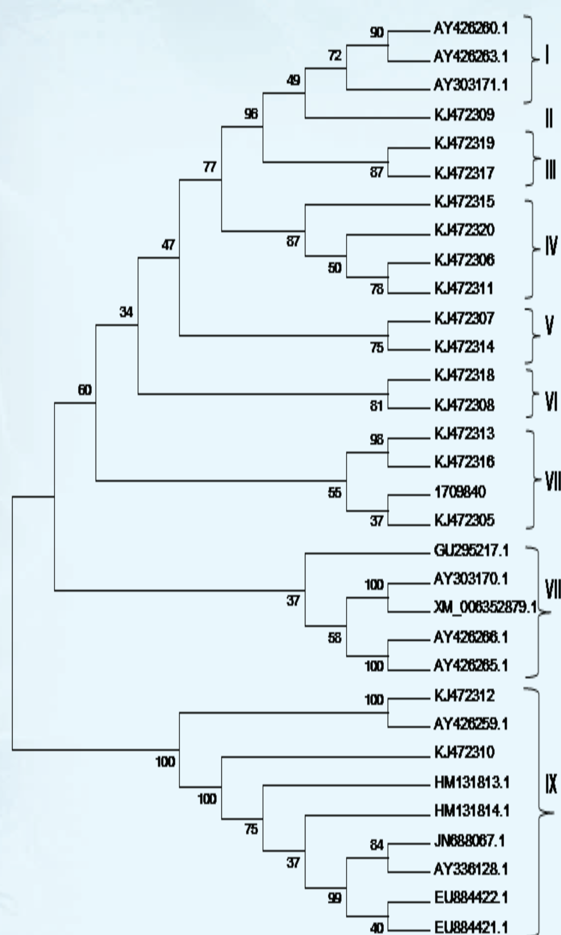

Fig. 21: Phylogenetic analysis based on the DNA sequences of 17 RGAs and 15 known late blight resistance protein genes from the NCBI database.

c. Isolation and cloning of genes from diploid mapping population for late blight resistance in potato

From the previously identified QTL (quantitative trait loci) for resistance to late blight in a diploid mapping population of 126 F1 progenies between *Solanum spegazzinii* (susceptible) × *S. chacoense* (resistant), an attempt was made to amplify the AFLP fragments flanking the QTL regions from the highly resistant mapping population. Consequently, a gene (QTL) was amplified and sequenced from the mapping population (SC-34) of about 1100 bp size (Fig. 22a). Further, to make a gene construct in the binary vector pRI201 at restriction cite of *NdeI* and *SalI*, target PCR product and vector were restricted with the enzymes (Fig. 22b). Finally, the gene construct was prepared by ligation of the restricted insert and the vector (Fig. 22c) for the transformation to the commercial potato cultivars.

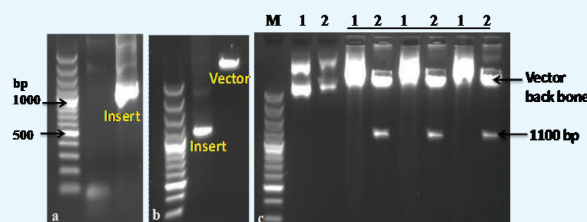

Fig. 22: Amplification of a QTL for late blight resistance from a mapping population (SC-34). a) PCR amplification of gene, b) Restriction digestion of insert and vector prior to ligation, and c) Gene construct with insert and vector checked with restriction (underligned, #1: unrestricted, #2: sample, SC-34).

d. Identification of genotypes with multiple resistance genes

Seventy-eight potato (*S. tuberosum*) accessions were screened for presence of late blight resistance genes R1 and R3a utilising PCR based markers R1AS (1400 bp), COSA (210 bp) and R3 (1380 bp). Ten potato accessions showed presence of R1 gene, while seven accessions possessed both R1 and R3a genes. About 18 clones possessing stacked R1and R3a genes were evaluated for late blight resistance through detached leaf bioassay. The resistance level in these clones ranged from resistant to highly resistant. All clones were advanced to F1C2 generation. Sixty-three accessions including 58 advanced potato hybrids (Late blight & PCN resistant) and five varieties *viz.*, Kufri Jyoti, Kufri Giriraj, Kufri Girdhari, Kufri Swarna and Kufri Neelima were screened for presence of genes conferring resistance to *Globodera* spp. using gene specific markers. H1 gene imparting resistance against Ro1 pathotype of *G. rostochiensis* was present in 37 accessions. Grp1 gene conferring resistance to *G. pallida* (TG432 marker) was present in 24 accessions. SNP marker HC_QRL was present in 44 accessions and marker SPUD 1636 associated with the Gpa5 & Gpa6 QTL was present in four breeding lines and variety, Kufri Swarna. Besides, 14 elite genotypes possessing multiple disease resistance genes with late blight (R1, R2, R3), PVY resistance (Ryadg gene) and PCN resistance (HC, H1) were identified through marker assisted selection (MAS). About 13,968 hybrid TPS were obtained in 15 successful crosses to stack multiple disease resistance genes.

5. DISEASE MANAGEMENT

a. Testing of rhamnolipid based formulation against *P. infestans*

Rhamnolipid based formulation was tested for its phytotoxicity and bioefficacy on potato plant against late blight. The result from detached leaves showed

a significant reduction in lesion area when the leaves were treated at a concentration of 0.2% (lesion area 0.06 cm^2 as compared to control 9.8 cm^2 on 5th day of inoculation). However, at concentration 0.15% and below a lesion area of 1.5 cm^2 and more was observed on 5th day of inoculation (Fig. 23). No phytotoxicity was observed with the formulation up to 0.25% concentration on whole plant.

Rhamnolipid based formulation was tested under field trials (last year) at 0.2% concentration where it showed mean disease severity of 30.92% in comparison with mancozeb (disease severity 23.33 %). On the basis of detached leaf and field trial results, mass collection of rhamnolipid was done for three multilocation trials for current season.

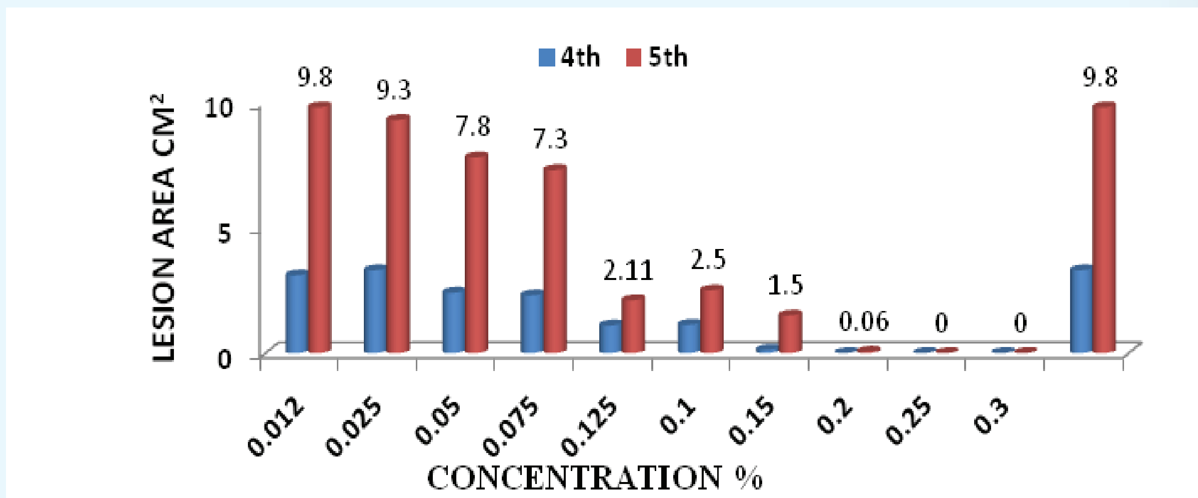

Fig. 23: Effect of rhamnolipid-based formulation on late blight lesion development on potato leaves

Rhamnolipid based formulation (0.25%) was tested under field trials at three different locations. The terminal disease severity in rhamnolipid formulation was 45% (compared to 100% in control), 47.5% (as against 92.5%) and 59.2% (as against 76.64%) at Modipuram (Table 3), Lavad, Meerut (Table 4) and Jalandhar, respectively.

Table 3. Evaluation of Rhamnolipid based formulation against late blight of potato at Modipuram

Treatment	Disease severity (%) at different dates				Disease controlled (%)	Yield (t/ha)
	23/1/14	31/1/14	5/2/2014	12/2/14		
T1 - Rhamnolipid 0.25%	1.00	18.75	22.50	45.00	55	22.32
T2 - Mancozeb-0.2%	0.75	15.50	19.75	37.50	62.5	21.01
T3 - Chlorothalonil -0.2%	0.25	13.25	14.75	32.50	67.5	21.66
T4 - Propineb-0.2%	0.88	18.75	23.50	38.75	61.25	20.65
T5 - Control	1.38	19.75	67.75	100.00		19.89
CD (0.05)				11.20		3.99

Table 4. Evaluation of Rhamnolipid based formulation against late blight of potato at Lavad

Treatment	Disease severity (%) at different dates					Disease controlled (%)	Yield (t/ha)
	4/2/14	10/2/14	17/2/2014	24/2/14	3/3/14		
T1- Rhamnolipid 0.25%	1.13	7.00	13.00	31.25	47.50	48.65	20.47
T2 - Mancozeb -0.2%	0.75	4.00	7.50	15.00	22.50	75.68	21.38
T3 - Chlorothalonil -0.2%	0.75	4.25	7.50	18.75	31.25	66.22	19.33
T4 - Propineb -0.2%	1.38	7.50	11.25	30.50	37.50	59.46	17.09
T5 - Control	1.63	11.25	26.25	77.00	92.50		16.50
CD (0.05)					11.89		4.96

6. GENOMICS

a. Silencing of transposable elements in *P. infestans* to reduce pathogen dynamism

Retrieved *P. infestans* Pol operon sequence from Broad Institute of Technology, *P. infestans* genome database to develop RNAi construct. Identified the effective region (372 bp), which generates at least 3-4 siRNA by using siRNA prediction tool software and designed the sense and antisense primer for 372 bp region to develop the RNAi construct (Fig. 24).

b. Whole genome sequencing of *Phytophthora infestans* A2 mating type

P. infestans A2 mating type whole genome sequencing with 10x coverage has been undertaken. As the data is insufficient for assembly, paired end sequencing has been initiated. For this, 3Kb paired end library has been prepared and sequencing is in progress.

ATGGATCCATATGATATGATCCTGGGCATGCCCTGGCTTGAGAAGCACGAGCCCTGGATC-
GACTGGCGAGGCAAGGCAATCGGTGCCAGCCGCCCTGCGCAGTCCGACAGAGCATTG-
GTGAGTCATGTTCCCACCTCTGTCAGGACCCGGGGCGCCCGCGAGGGCTGCCAGGG-
TACCAAAGCATCCGGCAGATGCTTGGGAGTCGTCGACGTATATGACGACTCCGAGGAT-
GTTTTGATGGTGGCCGCGCCCAGAAGAGGCGCCGGCCAAGTGGGTAACTTAGGTCCA-
CAGGCGGGTAACTTAGTTCCGCAGACAGCTGCAGCTGTCATGAACACTGAGAACAGCGT-
GTTCAGAGTGGGTAACAGAGTTCCACTCACGGTCGCCCAGACCATAACTGAAGAGGAA-
GACGTCGGATACGCTTCCTGTGTGGGTAACACAGTGCCACACGCGGTCGCCCAGACCT-
TAACTGAAGAGGAAGGCGTTGAACACGCTTCCTGTGTGGGTACAACAGTACCACACGAGGC-
CACGAGTGCCTCGGATGTCGTAGCGACGACATCCCTAGACGTGGGCAGCAAAGCTCCCCGT-
CACAGAAGCCGTCGGCAAAGACGCCGCCGCCGCCTCAAGTCGGATCCGAACTTCGTTC-
CGATCGACGTCGCTTCAAGCGACTCGGAGTCCAGGGCCCGGGCTCCTCCGACAAGGCCG-
GTGGAAGAGTGCTATCACATCTTCGATAGCGAGACGGGTTTACCCGTCAAGGCATCTGGT-
GTCCACTTGGAACAGTTGCCAGAGGTTGCAGAGATACTGAACCTCGAGGAAATGACGGCT-
GAGTCTTTCCTGGCCAACCTCAAGGCTGGAGAGATCGCTGAGATGGTACTCATAAGACCT-
GAGACCACTCAGGAGGAGCTGAACTCCTCCTCTGTCTTGGACGAGAACGTCCTGGAAGA-
CATGAACAAGCAGCGTCAGGCGCGCTTGGGTTCTGAGATTCTCAAGAACCCCAAGGATC

Fig. 24: *Phytophthora infestans* gypsy-like retrotransposon GypsyPi-1a, complete sequence

CENTRAL TUBER CROPS RESEARCH INSTITUTE, THIRUVANANTHAPURAM

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Co-investigators:

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Dr. RS Misra

1. BIODIVERSITY

a. Cultural characterization of *P. colocasiae*

Colony morphology of *P. colocasiae* isolates was studied on four media *viz*., potato dextrose agar (PDA), Czapek dox Dextrose Agar (CDA), Carrot Agar (CA) and V8 agar which showed varying morphology with respect to the different media used (Fig. 25). Based on the morphological variation exhibited in PDA medium, isolates of *P. colocasiae* were classified into nine groups (Table 5). Isolates from the same field/region had similar growth patterns. There was a significant difference in the growth rate of isolates from different morphotypes (P ≤ 0.05). Isolates depicting cottony and stellate morphology had faster growth rates, while isolates with uniform concentric ring morphology were the slow growers. The remaining isolates had an intermediate growth rate. The optimum temperature for growth of *P. colocasiae* isolates was 28 to 30°C. Isolates with cottony and stellate morphology showed slight growth at 35°C, demonstrating their high temperature tolerance. No growth was observed for any of the isolates at 40°C. There were significant differences (P ≤ 0.05) in the sporangial morphology among different isolates of *P. colocasiae*. They were ovoid to ellipsoid shaped, with an average length and breadth of 32.6-52.7 µm and 18.6-32.2 µm, respectively.

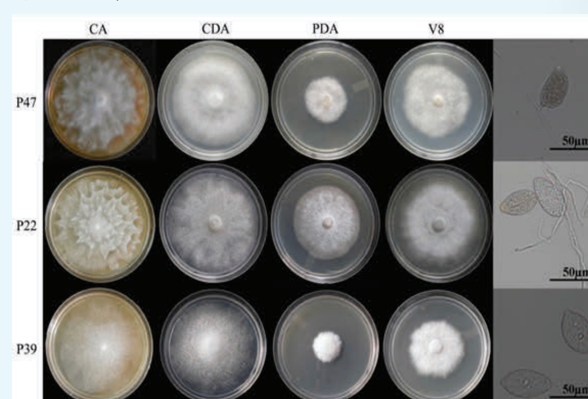

Fig. 25: Colony morphology of *Phytophthora colocasiae* on different media along with their sporangia

Table 5. Culture characteristics of morphological groups of *Phytophthora colocasiae* isolates in the study

Morphology group	Colony appearance	No. of isolates	Sporangial dimensions (µm)		Growth rate (mm day $^{-1}$)	Lesion diameter 4 d.a.i.(cm)
			Length	Breadth		
Group 1	Cottony	17	37.80±0.11b	30.83±0.16e	5.66±0.03g	2.66±0.05d
Group 2	Stellate	5	41.76±0.14c	32.26±0.17f	5.73±0.03g	0.00±0.00a
Group 3	Cottony with concentric rings	4	41.80±0.11c	32.20±0.11f	5.43±0.03f	1.46±0.05b
Group 4	Plain with irregular concentric rings	8	52.60±0.23f	27.70±0.15d	5.23±0.03e	4.46±0.05i
Group 5	Irregular pattern	3	47.26±0.14d	22.43±0.14c	5.03±0.03d	2.33±0.05c
Group 6	Plain	5	49.66±0.17e	19.70±0.17b	5.10±0.00d	3.80±0.10g
Group 7	Uniform with concentric rings	4	52.76±0.14f	30.76±0.14e	4.03±0.03a	4.06±0.05h
Group 8	Uniform without pattern	3	32.76±0.12a	18.63±0.08a	4.23±0.03b	3.03±0.05f
Group 9	Flat with concentric rings	1	52.73±0.12f	30.56±0.12e	4.50±0.00c	2.83±0.05e

b. Metalaxyl sensitivity of *P. colocasiae* isolates

Responses to the fungicide metalaxyl were determined by growing the isolates on potato dextrose agar amended with Ridomil (250 mg metalaxyl ml^{-1}) suspended in sterile distilled water and added to potato dextrose agar at 0, 0.1, 1, 5, 10, and 100 µg ml^{-1}. Among the *P. colocasiae* isolates studied, 52% were classified as sensitive, 38% were moderately resistant and 10% were resistant to metalaxyl.

c. Molecular characterization based on ITS region

The ITS sequence analysis revealed 97–99 % nucleotide sequence similarity to each other and 96–99 % similarity among the isolates of *P. colocasiae* available in the GenBank Database (data not shown). The representative sequences were deposited in NCBI and the accession numbers assigned are KC505326 –KC505334. Phylogenetic analysis of *P. colocasiae* isolates clearly portrayed the variation among the isolates. The robustness of the clustering was further supported by high bootstrap values (Fig. 26).

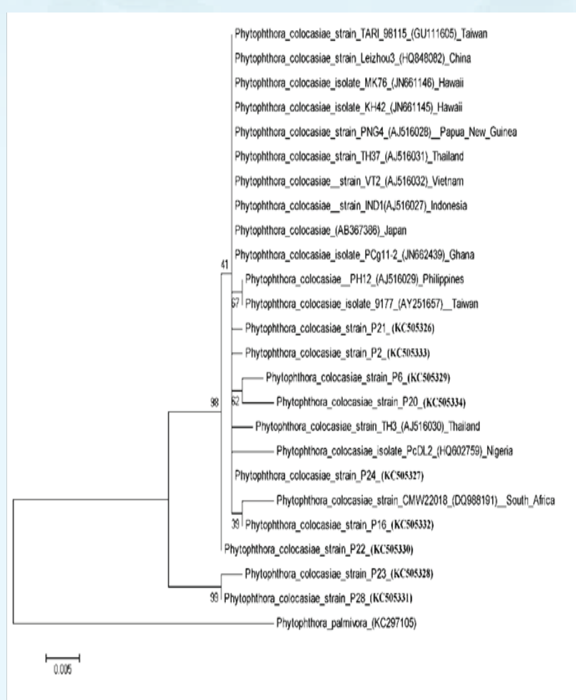

Fig. 26: Maximum Composite Likelihood phylogeny of *Phytophthora colocasiae* isolates along with other published sequences. *Phytophthora palmivora* was used as the out group. Numbers of nodes represent bootstrap values (2000 replicates)

d. Analysis of genetic diversity using start codon targeted polymorphism (ScoT) marker

Eight pre-screened primers that produced distinct, reproducible bands with high polymorphism were selected for the ScoT analysis (Table 6). Among these the highest number of amplification products (22) was obtained with the primer ScoT 1, while the lowest was with ScoT 11 (09). All the polymorphic bands were scored and a dendrogram was constructed using unweighted pair group mean algorithm. The UPGMA clustering divided the isolates into two major clusters (Fig. 27) with high bootstrap values. Cluster I formed the major group in 48 isolates while cluster II had only two isolates. The clustering of isolates in the dendrogram was not correlated with geographical origin or phenotypic characters. The cophenetic correlation coefficient between dendrogram and the original similarity matrix were significant for ScoT marker (r = 0.904).

Table 6. Analysis of genetic diversity in *Phytophthora colocasiae* using start codon targeted polymorphism (ScoT) marker

Marker	Primer code	Sequence 5´- 3´	No. of bands	No. of polymorphic bands	Mean no. of bands	Polymorphism (%)
SCoT	SCoT 1	CAACAATGGCTA CCACCA	22	22	3.5	100
	SCoT 2	CAACAATGGCTA CCACCC	17	17	5.1	100
	SCoT 11	AAGCAATGGCTA CCACCA	09	09	2.3	100
	SCoT 12	ACGACATGGCGA CCAACG	18	18	6.5	100
	SCoT 14	ACGACATGGCGA CCACGC	15	15	5.5	100
	SCoT 20	ACCATGGCTACCA CCGCG	18	18	4.4	100
	SCoT 21	ACGACATGGCGA CCCACA	12	12	4.1	100
	SCoT 61	CAACAATGGCTAC CACCG	10	10	3.5	100
		Total	121	121	35.1	800
		Average	26.89	26.89	7.80	100

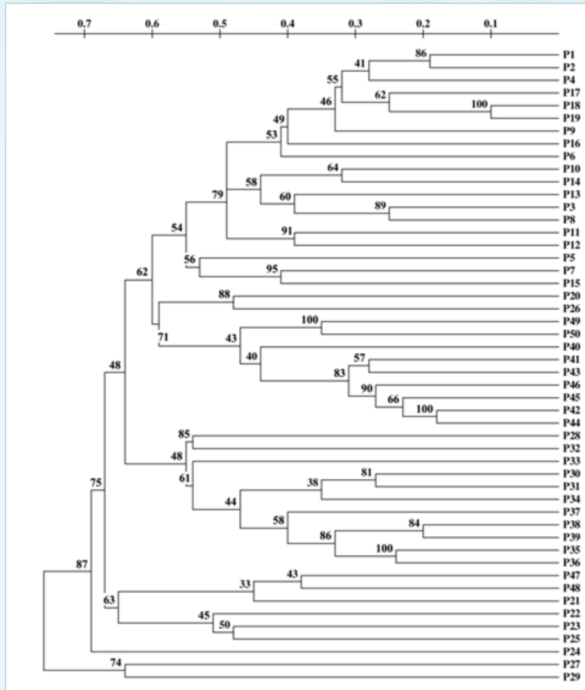

Fig. 27: UPGMA dendrogram based on SCoT markers

e. Analysis of genetic diversity of *P. colocasiae* using RAMS markers

The Random Amplified Microsatellite (RAMS) markers were used to study the diversity of thirty *P. colocasiae* isolates. Amplification was done with three sets of primers *viz.* CCA-primer, CGA-primer and GT-primer. In most cases amplifications were specific for each primer, reflecting the genetic diversity of the isolates used for the study. An example of RAMS amplification is shown Fig. 28. The data analysis is in progress.

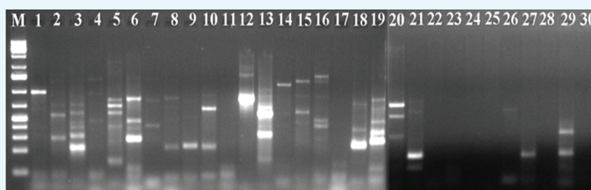

Fig. 28: Amplification profile of CCA primer with 30 isolates of *Phytophthora colocasiae*

2. DIAGNOSTICS

a. Standardization of real-time PCR assay for quantifying *P. colocasiae* from inoculum

Real-time quantitative PCR (qPCR) was standardized for the detection of *P. colocasiae*. To quantify *P. colocasiae* genomic DNA and to evaluate the amplification efficiency and the detection limits of the qPCR assay, a 10 fold dilution series of *P. colocasiae* genomic DNA (25 ng µl^{-1} initial concentration) was used to create a standard curve. The Ct values for each PCR reaction were interpolated from the standard curves to calculate the amount of *P. colocasiae* DNA in a sample. The minimum detection limit was 12.5 fg for PCSP-RL, 125 fg for PCSP-G and 1.25 pg for PCSP-T (Table 7). The melting curve analysis at the end of the cycling reactions revealed single dissociation peak at 82°C (PCSP-RL and PCSP-T) and 86°C (PCSP-G) indicating the specific binding of the designed primers (Fig. 29).

Table 7. Detection limits of the primer pairs in real-time PCR for quantifying *P. colocasiae*

	PCSP-RL	PCSP-T	PCSP-G
25 ng µl^{-1}	15.93 (0.25)	18.10 (0.73)	15.86 (0.07)
2.5 ng µl^{-1}	18.62 (0.22)	18.51 (0.28)	18.39 (0.45)
0.25 ng µl^{-1}	21.45 (0.37)	21.82 (0.23)	21.22 (0.18)
25 pg µl^{-1}	24.18 (0.21)	20.56 (0.07)	21.79 (2.68)
2.5 pg µl^{-1}	27.36 (0.32)	28.69 (0.58)	28.21 (0.52)
0.25 pg µl^{-1}	29.81 (0.34)	30.34 (0.53)	30.13 (0.84)
25 fg µl^{-1}	31.79 (0.20)		32.15 (0.92)
2.5 fg µl^{-1}	31.58 (0.83)		

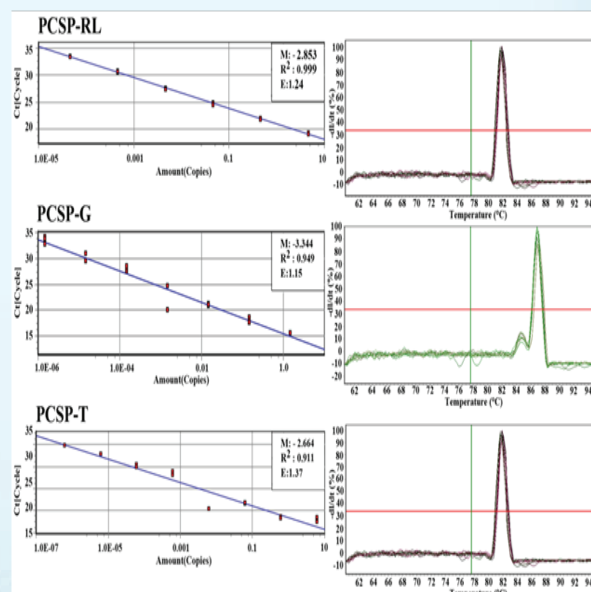

Fig. 29: Standard curves and melt curve analysis of the real-time PCR assay for quantifying *P. colocasiae*

This method was further used for detecting *P. colocasiae* from naturally and artificially infected leaf, tuber and soil. The earliest time point at which *P. colocasiae* could be detected in artificially infested samples was 18 h and 15 h post-inoculation in conventional and real-time PCR, respectively. The method was also compared with conventional PCR and routine isolation methods.

3. HOST RESISTANCE

a. Grouping of accessions based on pathogenicity assay

A total of 100 taro accessions maintained by the CTCRI were screened for their resistance against *P. colocasiae*. The accessions showed varying levels of resistance to *P. colocasiae.*

4. MANAGEMENT

a. Field evaluation of the effective biocontrol agent

The efficacy of potent *Trichoderma* strain TR7 in controlling leaf blight was evaluated underfield conditions. There were twelve treatments for the study. The results of the study showed significant difference in the biometric parameters of the plants whereas other parameters such as yield and disease incidence were not significantly different. Tuber treatment with *Trichoderma* showed significant differences in plant height and number of leaves which was on par with other *Trichoderma* treatments.

b. Pot trial evaluation of *Trichoderma* cultures from different centres

A pot trial experiment was initiated with all PhytoFuRa *Trichoderma* cultures for evaluating their efficacy in controlling leaf blight disease. The initial response shows that there is a significant difference in the biometric parameters of the plant treated with different *Trichoderma* spp. (Fig. 30).

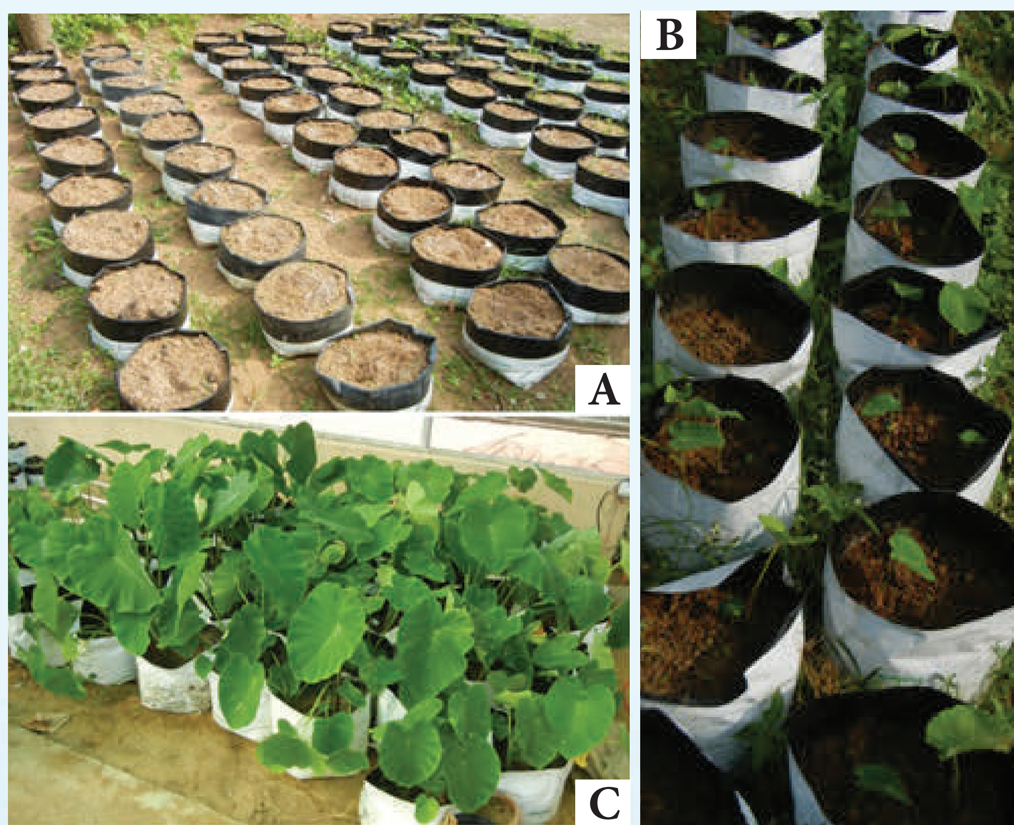

Fig. 30: Different stages of pot trial using PhytoFuRa *Trichoderma* cultures (A) Initial stage after planting of corms (B) growth after one month of planting (C) growth after three months of planting.

DIRECTORATE OF OILSEEDS RESEARCH, HYDERABAD

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1. BIODIVERSITY

a. Isolation and characterization of *Phytophthora* isolates from castor

A total of 20 isolates of *Phytophthora* from different geographical areas were isolated from safflower and castor infected fields. Morphological characterization and ITS sequencing of the *Phytophthora* revealed that the species associated is *Phytophthora nicotianae*.

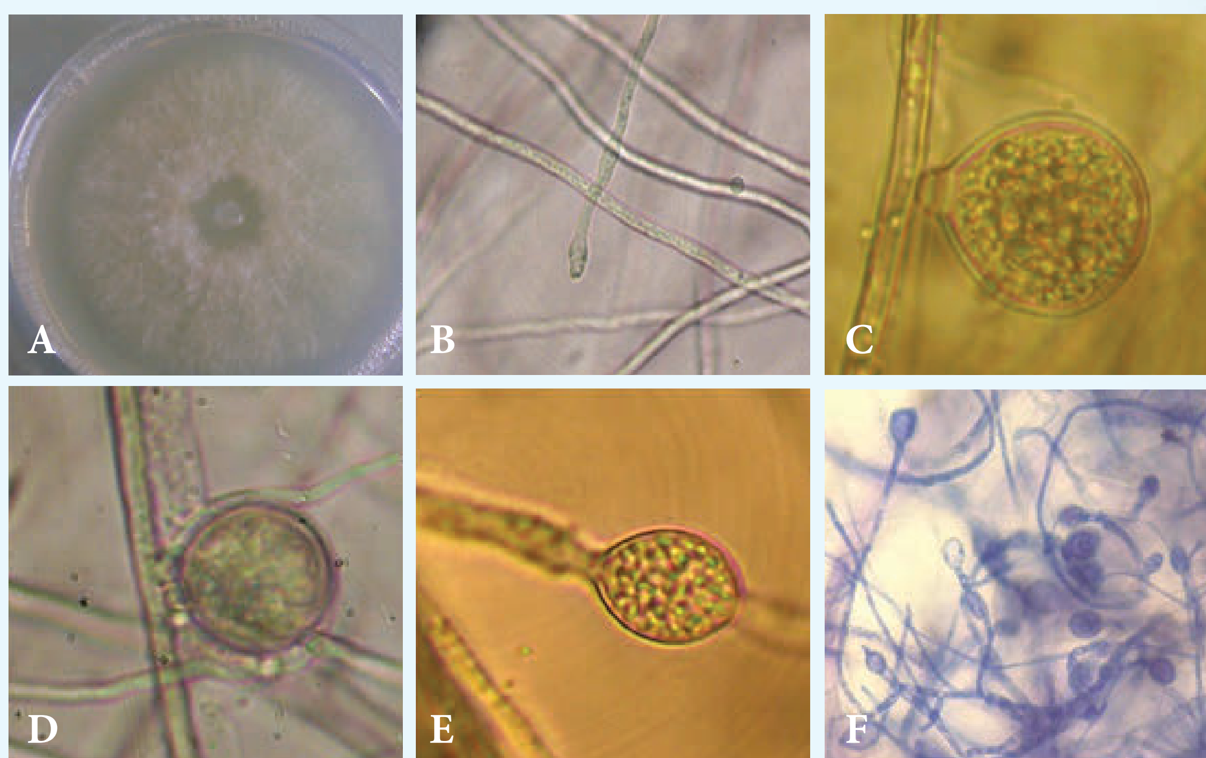

Fig. 31: Morphological characterization of *P. nicotianae*. (A) Dense rosette spreading aerial mycelium, (B) coenocytic hyphae of up to 7-10 nm in diameter, (C&G) ovoid terminal sporangia with prominent papilla, (D) intercalary sporangium, (E) chlamydospore. Bar=10 µm, and (F) multiple sporangia

b. Pathogenic variability in *P. nicotianae*

Nine *P. nicotianae* isolates were tested for their pathogenic variability on susceptible cultivar of castor and safflower. The isolates Phy-3 collected from Andhra Pradesh and Phy-9 collected from Kerala were highly virulent.

2. HOST RESISTANCE

a. Standardization of screening technique against *P. nicotianae*

Among different screening techniques (germination towel technique, soil/sand cup method, agar bit inoculation on detached leaves or on leaves of potted plants, zoospore inoculation method), agar bit inoculation method was found most effective compared to the other methods as leaf blight symptoms appeared within 24-48 h after inoculation. In this method, leaves were inoculated with pathogen on the reverse side of the leaf using a disc of agar (5 mm) colonized with *Phytophthora*. Agar bit was covered with small wet cotton and sealed with cellophane and inoculated plants were maintained in glasshouse with temperature and humidity controls.

b. Reaction of castor cultivars to *Phytophthora nicotianae*

A total of 25 castor cultivars and 21 different safflower breeding lines and popular cultivars were screened against *Phytophthora* leaf blight by agar bit inoculation method using 45 days old seedlings. Castor genotypes *viz.*, GCH 4, GCH 7 & 3216 R were resistant and cultivars GCH 2, GCH 6, GAUCH 1, TMU 5a and PCH 222 were moderately resistant to *Phytophthora* leaf blight (Fig. 32).

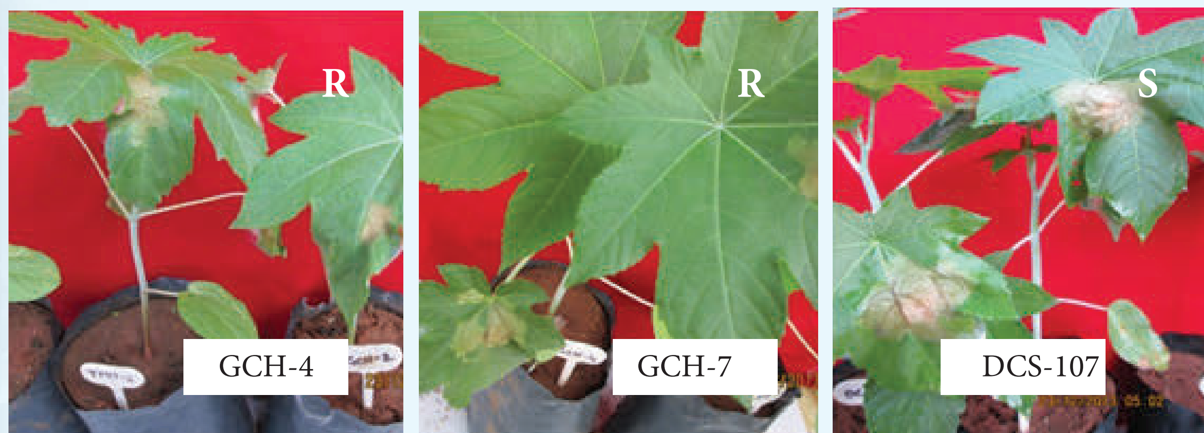

Fig. 32: Reaction of castor cultivars to *Phytophthora nicotianae*. A. GCH-4 (resistant), B. GCH-7 (resistant) and C. DCS-107 (susceptible)

c. Reaction of safflower cultivars/breeding lines to *Phytophthora nicotianae*

In safflower, three wilt resistant breeding lines and one cultivar (W-05-2028, W-2026, W-2037-9 and Bhima) have shown less than 20% disease severity (Fig. 33) and all other cultivars have shown > 40% disease severity.

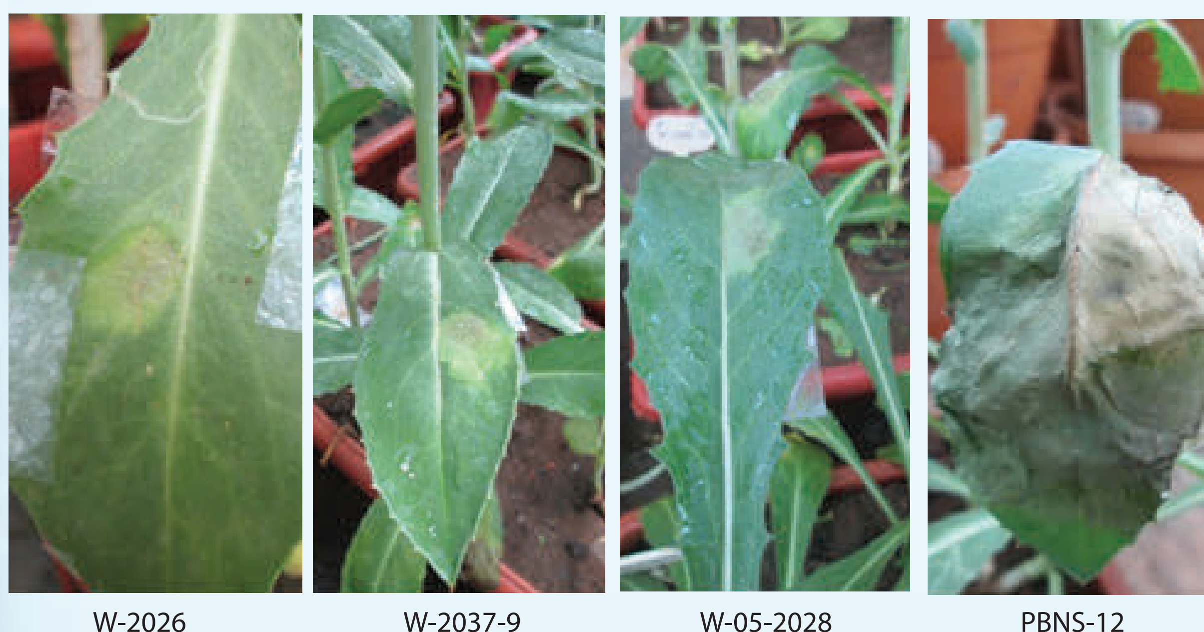

Fig. 33: Reaction of safflower cultivars to *Phytophthora nicotianae*

3. DISEASE MANAGEMENT

a. Screening and selection of potential biocontrol agents against *Phytophthora* seedling blight

Six *Trichoderma* isolates and one *Pseudomonas fluorescens* Pf2 were screened against *Phytophthora* seedling blight of castor by agar bit inoculation on detached leaves. Among the six *Trichoderma* isolates, high disease reduction (61.5%) was obtained with *Trichoderma asperellum* TaDOR673 treatment (Fig. 34). Two isolates *viz.*, *T. harzianum* Th4d and *T. asperellum* TaDOR 7316 were the next best treatments with 55.6% disease reduction.

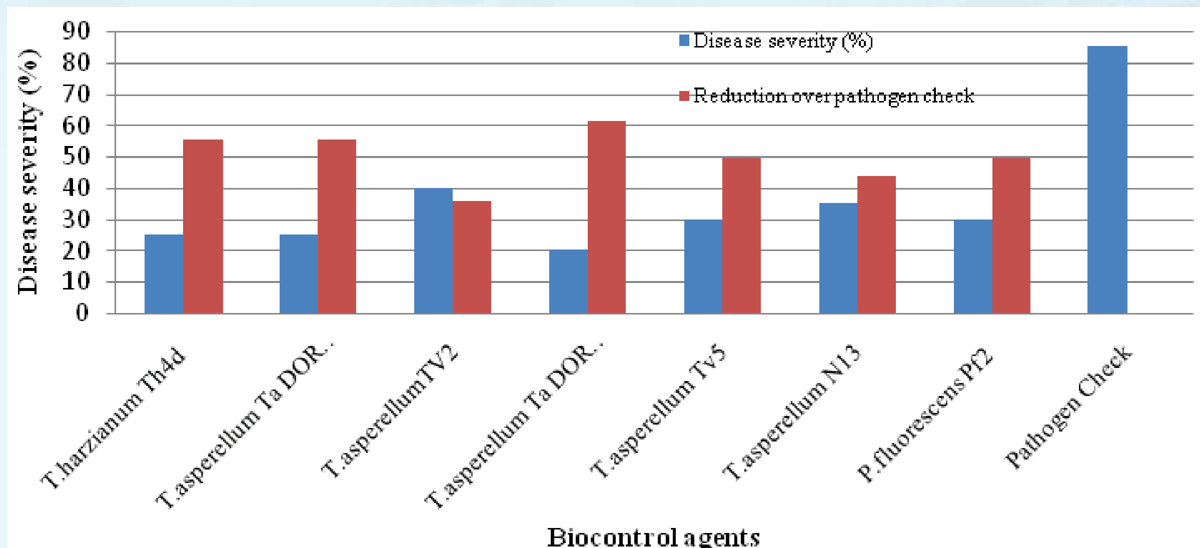

Fig. 34: Screening of *Trichoderma* isolates and *Pseudomonas fluorescens* against *Phytophthora* seedling blight in castor

b. Screening of PhytoFuRa *Trichoderma isolates* against *Phytophthora* leaf blight of castor

Trichoderma isolates were tested for their efficacy using the 'detached leaf technique'. Among the 15 PhytoFuRa coded *Trichoderma* isolates, isolate PhytoFuRa-13 was found to be the most effective as it recorded significantly less disease severity of *Phytophthora* leaf blight (5%) and 79.1% disease reduction. The other two effective isolates were PhytoFuRa 9 and 12 recording about 20% disease severities. The other best isolates were PhytoFuRa- 1, 3, 6 and 7 the treatment of which has recorded a disease reduction of 55.6% (Fig. 35).

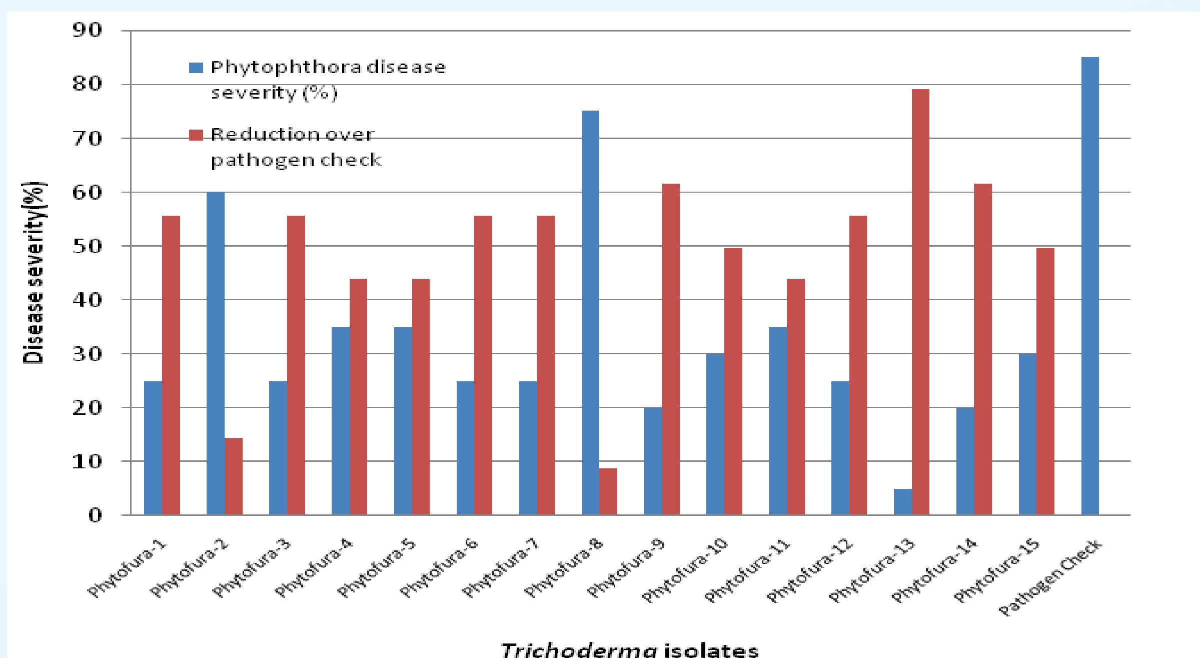

Fig. 35: Efficacy of 15 PhytoFuRa *Trichoderma* isolates against *Phytophthora* leaf blight of castor

c. Management of *Phytophthora* damping off and seedling blight of castor by chemical and biological agents under field conditions

Phytophthora disease incidence was significantly low in captan at 2g/kg, metalaxyl + mancozeb 0.2% and *Trichoderma harzianum* Th4d SC at 1 ml/kg treatments recording a disease incidence of 16.2, 18.6 and 20.8% whereas the pathogen check recorded 60.8% (Fig. 36).

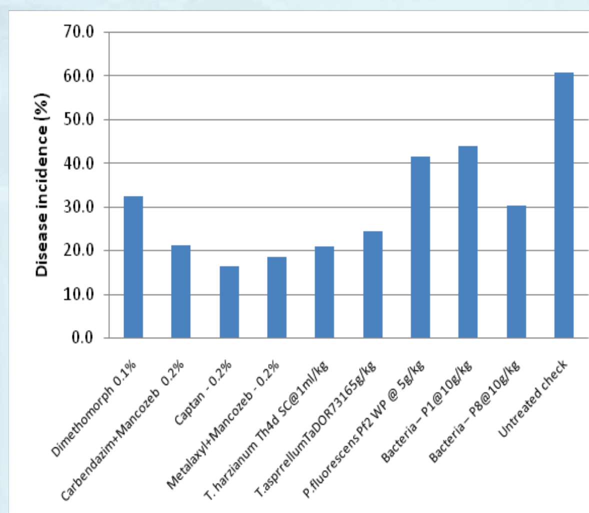

Fig. 36: Management of *Phytophthora* damping off and seedling blight in castor

d. Screening of different chemical and biological agents against *Phytophthora* seedling blight in safflower

Different chemical and biological agents were screened against *Phytophthora* in safflower by following a rapid *in vivo* testing. Among the chemical and biological agents seed treatment captan (0.2%) and dimethomorph (0.1%) showed least disease severity (23 & 29%) against *P. nicotianae* seedling blight. *T. asperellum* (TaDOR7316) 5g/kg, *P. fluorescens* and bacteria P1 10g/kg treatments showed disease severity 34.5, 35.5 and 38.5% compared to the pathogen check (61%) .

e. Evaluation of chemicals and biological agents against *Phytophthora* damping off and seedling blight of safflower

Field evaluation of the treatments used in *in vivo* screening showed that *Phytophthora* disease incidence was significantly low in captan@ 2 g/kg, carbendazim + mancozeb 0.2% and *Trichoderma harzianum* Th4d SC@ 1 ml/kg treatments recording disease incidence of 13.7, 15.1 and 19.1% (Table 8) whereas the pathogen check recorded a disease incidence of 56.5% .

Table 8. Effect of seed treatment with chemical and biological agents on *Phytophthora* damping off and seedling blight in safflower

Treatment	Disease severity (%)
Dimethomorph 0.1%	26.8
Carbendazim+Mancozeb (SAAF) 0.2%	15.1
Captan -0.2%	13.7
Metalaxyle+Mancozeb - 0.2%	19.7
Trichoderma harzianum Th4d SC@1ml/kg	19.1
Trichoderma harzianum Th4d SC@2ml/kg	21.3
Pseudomonas fluorescens Pf2 WP @ 5g/kg	38.8
Bacteria – P1	40.2
Bacteria – P8	28.1
Check (untreated check)	56.5
CD (=0.05)	3.69
CV%	7.71

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1. BIODIVERSITY

a. Survey of apple orchards and incidence of collar rot disease

Periodical survey of different apple orchards located in districts of Chamba, Sirmour and Kinnaur indicated that incidence of collar rot disease (*Phytophthora cactorum*) varied between 2.7-19.7, 1.3-22.4 and 0.3-9.2%, respectively. Maximum incidence of the disease was observed in apple orchard at Jattota (Shalooni Block) and Baragarh (Tisha Block) of Chamba District, Leonana in Sirmour District and Lippa in Kinnaur District of Himachal Pradesh.

b. Isolation of *Phytophthora* from soil samples and selection of virulent isolates

Ten different *P. cactorum* isolates were isolated from the soil/ diseased bark samples collected from Chamba, Sirmour and Kinnaur apple growing areas. Morphology studies showed that maximum size of sporangium and mycelium width were recorded in the isolate PC3 with whitish floppy radiate colony followed by 2, 5, 7 and 9, respectively. Isolate 1 and 6 produced creamish white colonies with slightly smaller sporangia. The size of oospore in all the isolates varied between 22-25 µm.

c. Virulence studies

Virulence of above 10 isolates were tested on susceptible rootstock MM106 by excised twig method and data obtained indicated that all the isolates produced different sized lesions. Isolate PC3 exhibited maximum lesion size (64.6 mm) followed by isolate 7, 9, 2 and 5, respectively. On the basis of lesion size (>50 mm) five were designated as highly virulent, two were virulent, two moderately virulent and one was less virulent. In addition to it, virulent strain produced dark brown lesions whereas less virulent produced light brown lesions. It was further observed that the isolates having larger sized mycelium and sporangia were more virulent.

2. HOST-PATHOGEN-ENVIRONMENT INTERACTION

a. Disease progression studies

Data on disease incidence were recorded periodically under nursery conditions (sick plot) during this year also. The disease first appeared in the last week of March when the mean soil temperature (minimum and maximum) ranged between 7.9-9.3°C and 19.0-21.8°C, respectively, and soil moisture up to 5 cm depth was 66.2-69.1% accompanied with frequent rains (2-3 rainy days/week). Similarly, the mean minimum and maximum soil temperature at 10 cm depth varied between 6.4-8.1°C and 17.0-18.0°C with the soil moisture varying between 72.6-78.3%. Further, the results obtained indicated (Fig. 37) that the disease increased with greater speed with the occurrence of mean soil temperature ranging between 18.3-28.4°C and soil moisture varying between 65.1-82.3% up to 10 cm depth during April 26th –22nd August 2013.

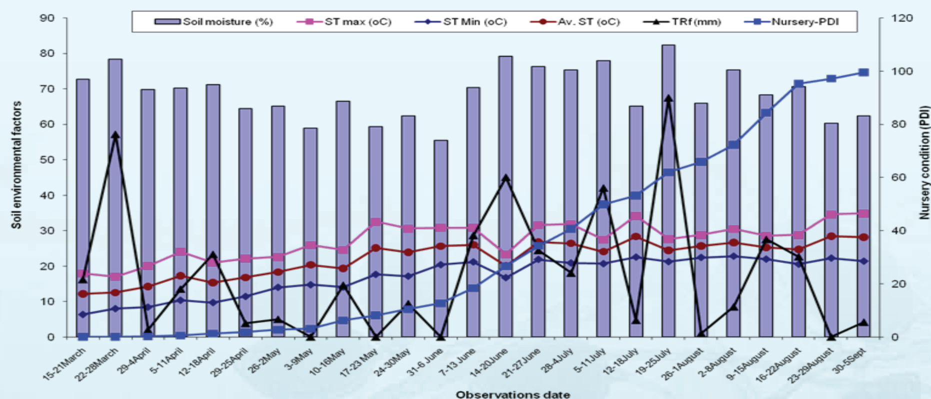

Fig. 37: Effect of soil environment at 10 cm depth on the initiation of collar rot disease in apple under nursery conditions.

In orchards, the disease was first noticed on 22 March with the occurrence of mean minimum and maximum soil temperature ranging between 7.8-8.6°C and 21.8 -23°C and soil moisture 64.3-66.2% at 5 cm depth accompanied with frequent rains (1-3 rainy days). Similarly, mean minimum and maximum soil temperature (up to 10 cm depth) varied between 6.4-8.0°C and 17.9-19.7°C while soil moisture was between 60.1-64.3%. The disease increased with greater speed when the mean soil temperature was between 18.3-22.7°C and soil moisture at 10 cm was between 58.9-82.3%. On further analysis it was observed that the disease incidence was positively correlated with rainfall, soil moisture and minimum soil temperature and was negatively correlated with soil temperature at 5cm.

In nursery condition

Y1 5 cm = -74.37 + 1542.2X1+1549.0X2-3086X3 -0.3680X4 +0.5902X5 (R2 =0.6775)

Y110 cm = -103.46+299.01X1+304.38X2-598.42X3-0.1725X4 + 0.8324X5 (R2=0.6007)

Y1 20 cm = -438.19+0.9567X1+3.0559X2-1.0752X4+ 5.8147X5 + (R2= 0.6913)

In orchard condition

Y2 5 cm = -7.850+2.007X1+1.009X2+0.3487X4-0.8094X5 (R2=0.6492)

Y2 10 cm = 20.038+342.56X1+341.16X2-681.11X3 + 0.30125X4 + -0.9602X5 (R2 =0.5003)

Y3 20 cm = 57.164+0.1719X1+2.934X2 +0.1723X4-1.454X5 (R2=0.4956)

Where X1 = Rainfall (mm)

X2 = Min Soil temperature °C

X3 = Max soil temperature °C

X4 = Soil moisture (%)

Y1 = Disease level under nursery conditions,

Y2 = Lesion size at collar region under orchard conditions.

3. HOST RESISTANCE

a. Evaluation of apple seedlings for resistance under nursery conditions

The seeds of 26 pollinizer cultivars were collected, stratified and sown in pots containing sterilized soil in a nursery. The procedure adopted was same as in previous year. The least seedling mortality was observed with *Malus floribunda* followed by Bray Burn, Star Crimson, Winter Delicious, Gloster and Golden Delicious.

b. Adaptive trial on resistant rootstocks

In the adaptive trials laid out last year at Poojan and Sainj in Kullu District, Gihiri and Chhatri in Mandi District and Seema and Dalgaon in Shimla District, the resistant rootstocks (M9 and MM111) are performing well, whereas in the susceptible rootstocks (MM106) there was a mortality of 50-60%. In addition, these resistant rootstocks have survived in the sick soil under nursery conditions at Nauni. During the current year (February 2014), 40 grafted plants of moderately resistant (MM111, M7), resistant (M9) and susceptible (MM106) lines were distributed for plantatin in hot spot areas at Ruhanda, Ruhmani in Mandi District, Summa, Sainj and Poojan in Kullu District and Dalgaon in Shimla District.

c. Development of molecular markers for screening host resistance

No unique bands were detected when 19 cultivars and seven rootstocks of apple with different levels of *Phytophthora* resistance were screened with 18 RAPD primers. On screening with 38 different ISSR primers, only two primers namely HB11 and UBC 848 gave the unique band in the resistant rootstock M9 (Fig. 38). Sequencing of eluted band is in progress.

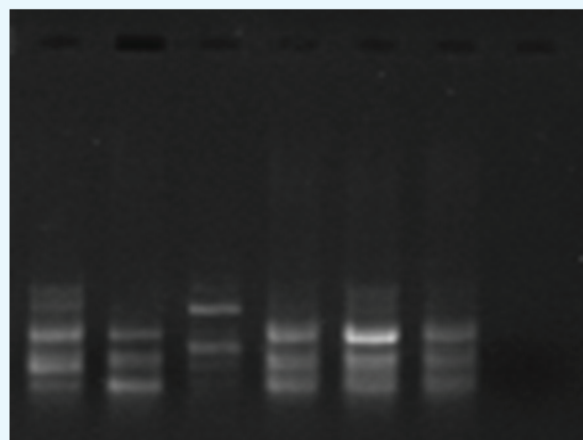

Fig. 38: Banding pattern in apple rootstocks with IISR primer HB11 (sequence 5'- GTG TGT GTG TGT CC -3')

4. DISEASE MANAGEMENT

a. Evaluation of *in vitro* effective antagonists under nursery conditions

Fungal (*Trichoderma harzianum*-5. *T. viride* -4, *T. virens* -2 and *Penicillium funiculosum*) and bacterial antagonists (*Bacillus* sp. -4 and *Pseudomonass* p. -3)were evaluated under field conditions in the nursery. Fifteen apple seedlings were planted in each plot of 1m^2 and each treatment was replicated three times. Data on

disease mortality was recorded periodically and the disease severity was noted by following 0-5 disease rating scale in the month of December 2013 by uprooting the plants. The results showed that addition of BCAs, 20 days prior to planting of apple seedlings, was highly effective. Among different BCAs, *T. harzianum* -5 was highly effective followed by *T. hamatum* -2, *T. viride*- 4 and *Bacillus* sp. - 4, respectively.

Table 9. Evaluation of biocontrol agents against collar rot disease in apple under field conditions

Treatment*	Disease control (%)			
	20 days before	10 days before	0 days**	10 days after
Trichoderma harzianum -5 (TH5)	78.6	67.1	58.2	43.4
Trichoderma hamatum -2 (THM-2)	77.3	64.3	54.2	41.5
Trichoderma viride - 4 (TV4)	76.6	63.5	53.1	41.8
T. virens -2 (TVS-2)	73.6	61.7	52.1	40.2
Penicillium funiculosum	72.2	60.1	51.3	39.2
Bacillus sp.- 4 (BS 4)	75.5	70.2	64.1	52.1
Pseudomonas sp. – 3 (Ps -3)	73.1	67.3	60.2	49.8
Control	1.4	0.3	1.2	0.8
CD at 5% level	2.61	2.34	1.96	2.54

*Fungal antagonists in talc based culture (2.5 x 10^6 cfu/g) were applied @ 50 g/plot of 1 m^2 and bran based culture (2.0 x 10^3) @ 150 g/plot of 1 m^2. Bacterial antagonists in coconut coir based culture (9.1 x 10^9 cfu/g) were applied @ 150 g/plot of 1 m^2 and talc based culture (9.1 x 10^9 cfu/g) @ 50 g/plot of 1 m^2.

** target pathogens were added simultaneously.

b. Evaluation of number of applications of effective BCAs of collar rot in apple seedlings under nursery conditions

Promising BCAs of the above experiment were added 20 days prior to planting with repeated doses at monthly intervals. For this, 15 apple seedlings were planted in each plot of 1 m^2 and each treatment was replicated three times. Data on disease mortality and disease severity were recorded as mentioned above. Increased disease control was obtained as number of applications of BCAs increased (Table 10). Maximum disease control (>97%) was recorded in the treatment wherein four consecutive applications were made.

Table 10. Effect of frequency of BCA applications on collar rot disease of apple

Treatment*	Disease control (%) in different application of BCAs			
	Single	Two	Three	four
Trichoderma harzianum -5 (TH5)	78.6	88.6	96.6	98.8
Trichoderma hamatum-2 (THM-2)	77.3	89.1	95.0	97.9
Trichoderma viride -4 (TV4)	76.6	87.2	96.4	98.2
T. virens -2 (TVS-2)	73.6	82.4	94.2	97.0
Penicillium funiculosum	72.2	79.2	90.8	96.4
Bacillus sp.- 4 (BS 4)	75.8	84.2	94.4	98.6
Pseudomonas sp. -3 (Ps -3)	73.2	81.8	94.2	97.2
Control	1.4	0.3	1.2	0.8
CD at 5% level	2.41	2.14	1.78	1.27

c. *In vitro* evaluation of novel fungicides

Screening of the ten novel fungicides and four commonly used fungicides was repeated for the second consecutive year. The results revealed that soil drenching with Curzate (0.3%) was highly effective both under nursery (96.6 PDC) and orchard conditions (increased shoot length 36.2 cm against 5.7 cm in untreated control), followed by Cabrio Top (0.25%), Sectin (0.25%), Ridomil Gold (0.3%) and Matco (0.3%), respectively. Fungicide namely Melody Duo (0.25%) was the next best fungicide followed by Ergon 500SC (0.15%), Infinito (0.25%) and Amistar (0.15%). Other fungicides *viz*., Polyram, Indofil M-45, Kocide and Blitox were comparatively less effective both under nursery and orchard conditions.

d. Studies on compatibility of fungicide and botanicals with effective BCAs

Compatibility of effective fungicides (Curzate M, Cabrio top, Matco) and botanicals (*Murraya koeningi*, *Eucalyptus* leaves and mustard cake) was evaluated with three most effective BCAs *viz*., *T. harzianum* - 5, *T. hamatum*-2 and *Bacillus* sp. (BS4) under *in vitro* conditions. The results indicated that all the fungicides and botanicals were compatible with the above BCAs.

e. Studies on compatibility of BCAs and fungicides in controlling collar rot under pot and nursery conditions

Under pot conditions: Studies indicated that pre-inoculation treatments with either BCAs or Curzate exhibited more disease control in comparison to their post inoculation applications (Table 11). Separate addition of TH5 and *Bacillus* sp.-4 at 7 days prior, simultaneously (0 days), 7 days and 12 days after inoculation with target pathogen provided, 80.4, 70.3, 55.2, 43.1 and 82.5, 71.8, 57.2 48.0% disease control, respectively.

Table 11. Effect of combined application of biocontrol agents and effective fungicides against collar rot (*P. cactorum*) disease in apple

Treatment*	Disease control (%) after 45 days of inoculation			
	7 days before	0 day**	7 days after	12 days after
Trichoderma harzianum-5 (TH5 5) @2% (bran culture) + 0.2% (talc based culture)	80.4	70.3	55.2	43.1
Bacillus sp.-4 (BS-4) @ 2% (coconut coir culture) 0.2% (talc based culture)	82.5	71.8	57.2	48.0
Curzate @ 0.25%	81.2	73.3	61.1	46.3
Curzate @ 0.3%	93.5	82.2	71.7	56.5
TH5+ Curzate@0.25%	90.1	83.6	68.1	52.4
TH5+ Curzate @0.3%	96.7	89.1	75.2	68.2
BS4+ Curzate @0.25%	96.8	86.1	80.0	63.5
BS4+ Curzate @0.3%	98.6	92.6	88.8	72.8
TH5+BS4+ Curzate @0.25%	98.9	93.1	89.2	73.4
TH5 BS4+ Curzate @0.3%	100.0	98.2	89.5	74.1
TH5 + BS4	91.6	82.5	72.6	60.1
Control	1.5	0.6	1.3	1.6
CD at 5% level	2.13	2.87	2.52	3.11

* TH5 in talc based culture = 2.5 x 10^6 cfu/g and in bran based culture = 2.0 x 10^3 cfu/g, BS4 in coconut coir based culture = 6.4 x 10^9 cfu/g and in talc based culture = 8.5 x 10^9cfu/g

** target pathogen and treatments were added simultaneously

Addition of Curzate at higher concentration (0.3%) gave enhanced disease control (> 12-21%) than addition at 0.25%. Further combined application of TH5 and Curzate at both the concentrations provided less disease control. Therefore it may be concluded that application of TH5 or BS-4 combined with Curzate at 0.3 % provided higher disease control in comparison to their separate application. Almost similar results were obtained when the experiment was repeated with *Trichoderma hamatum* -2 (THM2) and *Bacillus* sp.-11 (BS11).

Under nursery conditions: Under nursery conditions almost similar results were obtained with slightly lower disease control. Combined application of TH5, BS4 and Curzate (0.3%) at pre-planting stage was highly effective to control collar rot infection and provided almost complete control.

Similar studies were undertaken to find out compatibility of Cabrio top (0.15, 0.2%) with other effective biocontrol agents *T. hamatum*-2 (THM2) and *Bacillus* sp.-11 (BS-11) under nursery conditions and results obtained are given in Table 12 which indicated that the combined effect of BCAs with Cabrio top (0.2%) provided 100.0, 94.4, 81.9 and 74.3 % disease control when applied 20, 10 before 0 (simultaneously) and 10 days after inoculation. The pre-inoculation treatments proved more effective than post infection treatments. Further the combined application of HHM-2 with Cabrio Top exhibited slightly better disease control in comparison to bacterial antagonists BS11.

Table 12. Effect of combined application of biocontrol agents and effective fungicides against collar rot (*P. cactorum*) disease in apple under field conditions in the nursery

Treatment*	Disease control (%)			
	20 days before	10 days before	0 days**	10 days after
Trichoderma hamatum 2 (THM2) @2% (bran culture) + 0.2% (talc based culture)	76.3	65.7	54.8	40.5
Bacillus sp.-11 (BS-11) @2% (coconut coir culture) 0.2% (talc based culture)	75.1	71.5	60.8	50.3
Cabrio Top @0.15%	70.2	62.3	50.2	47.5
Cabrio Top @0.2%	74.5	68.1	58.3	44.3
THM2+ Cabrio Top @0.15%	82.5	78.6	62.9	52.9
THM2+ Cabrio Top @0.2%	89.2	82.3	67.6	56.7
BS11+ Cabrio Top @0.15%	81.5	76.1	63.5	52.4
BS11+ Cabrio Top @0.2%	88.7	83.4	68.8	59.7
THM2+BS11+ Cabrio Top @0.15%	88.6	78.6	70.1	61.2
THM2 BS11+ Cabrio Top @0.2%	100.0	94.4	81.9	74.3
THM2+ BS11	82.2	75.8	67.8	58.4
Control	0.6	1.4	0.8	1.4
CD at 5% level	2.52	2.71	3.12	2.26

* THM2 in talc based culture = 2.5 x 10^6 cfu/g and in bran based culture = 2.0 x 10^3 cfu/g, BS11 in coconut coir based culture = 6.4 x 10^9 cfu/g and in talc based culture = 8.5 x 10^9 cfu/g

** target pathogen and treatments were added simultaneously

f. Development of integrated disease management (IDM) strategy

During the period under report, different highly effective bio-control agents (BCAs) compatible with fungicides, various bio-resources, bio-fumigation with mustard plants, fungicides, soil solarization (nursery conditions) and approach grating (orchard condition) and cow urine decoction were further evaluated in different combinations under pot, nursery (sick plot) and orchard conditions to develop an integrated disease management (IDM) strategy in apple.

Under pot conditions pre-inoculation treatments with BCAs, fungicides and amendments exhibited enhanced disease control in comparison to their post-inoculation applications. Among the different treatments, combined applications of BCAs (TH5/ THM2 + BS11/BS4/ KB6) and fungicides provided almost complete control (>95%), when applied seven days prior to inoculation. Combining fungicide application with bio-resources/ biofumigation with mustard plant was also effective, whereas different combinations of bio-resources alone were comparatively less effective. Addition of cow urine decoction (7.5% @ 5 l /1m^2) provided 88.2, 82.5, 70.2 and 60.2 P DC, respectively, when added 7 days before, simultaneously and 7 and 12 days after inoculation of the target pathogen.

Under nursery conditions (sick plot) pre-plant treatments were more effective in providing the higher disease control. Further it was observed that combination of BCAs (THM2/TH5+BS11/BS4) or amendments (Mustard cakes +Murraya leaf) or BCAs + mustard cake with Curzate or Cabrio Top or Ridomil Gold were individually most effective and provided almost complete control of disease when applied 20 days prior to planting of seedlings. Combined applications of TH5+BS11+Mustard cakes+ Curzate (0.3%) followed by THM2+BS4+ mustard cake+ Ridomil Gold (0.3%), TH5 +BS11+ Curzate (0.25%)/ Cabrio Top (0.2%), THM + BS4 + Mustard plant fumigation + Ridomil Gold (0.3%), Mustard cake+ Melia seed + Curzate (0.3%), Mustard plant fumigation + TH5+ Curzate (0.3%) were

also highly effective and provided disease control between 91.0-100%.

On combining the above management inputs with soil solarisation, it was observed that treating solarised beds with different bioagents/bioresources 20 days prior to planting proved better but was on par with 10 days prior, simultaneous and 10 days after treatments. The combination of BCAs (TH5+BS11, THM2+BS11, TH5+BS4, THM2+Bs4) with soil solarization provided complete control. Similarly, combined application of soil amendments (mustard cakes, leaves of *Murraya koenig*i, seeds of *M. azedarach* @ 200 g/m^2) or bio-fumigation with mustard plants (mulching whole plant @ 2 kg/ m^2) with soil solarization provided 97.5, 96.2, 98.2 and 100% control, respectively. Planting of root stock MM111 in solarised plots exhibited almost complete (>96.4%) control.

Under orchards conditions addition of cow urine decoction thrice @7.5% (10 l/tree) during March, June and August accompanied with approach grafting and two applications of Curzate (0.3%) during April and August was most effective and it increased the shoot growth to 42.6 cm against 6.8 cm in untreated plants. It also provided the maximum recovery of the lesion to the tune of 38.8%. Next best treatment was combined application of red soil (10 cm thick layer in plant basin), cow urine decoction (thrice @7.5% (10 l/tree) during March, June and August and approach grafting (40.1 cm shoot growth and lesion size recovery 38.2%).

ICAR RESEARCH COMPLEX FOR NEH REGION, UMIAM

Principal Investigator:

DR. AMRITA BANERJEE

Co-investigator:

Dr. G T Behere

1. BIODIVERSITY

a. Molecular detection of *Phytophthora* sp. isolated from citrus

Out of the 13 *Phytophthora* isolates from citrus maintained in laboratory, seven were identified as *P. citrophthora* by PCR using species-specific primers (Fig. 39).

Fig. 39: Morphological and molecular detection of *Phytophthora* sp isolated from citrus in Meghalaya

2. DISEASE MANAGEMENT

a. Morphological and molecular identification of seven *Trichoderma* species from North-East India

Potential *Trichoderma* isolates collected from different parts of North-east India and maintained in the laboratory were identified and characterized by morphological (Fig. 40) and molecular means. Seven isolates which varied much in their morphology were further identified through PCR using primer pair ITS1 and ITS4. These isolates showed identity with seven different *Trichoderma* species *viz., T. asperellum, T. harzianum, T. koningiopsis, T. brevicompactum, T. longibrachiatum, T. virens*, and *T. saturnisporum* (Fig. 41). The morphological grouping of *Trichoderma* isolates based on conidiophore branching was identical with the molecular grouping (sequence identity and phylogeny). Thus the ITS1 region could serve as useful and reliable marker for species identification in the genus *Trichoderma*. The sequences were deposited in GenBank with accession numbers KC874892 -98.

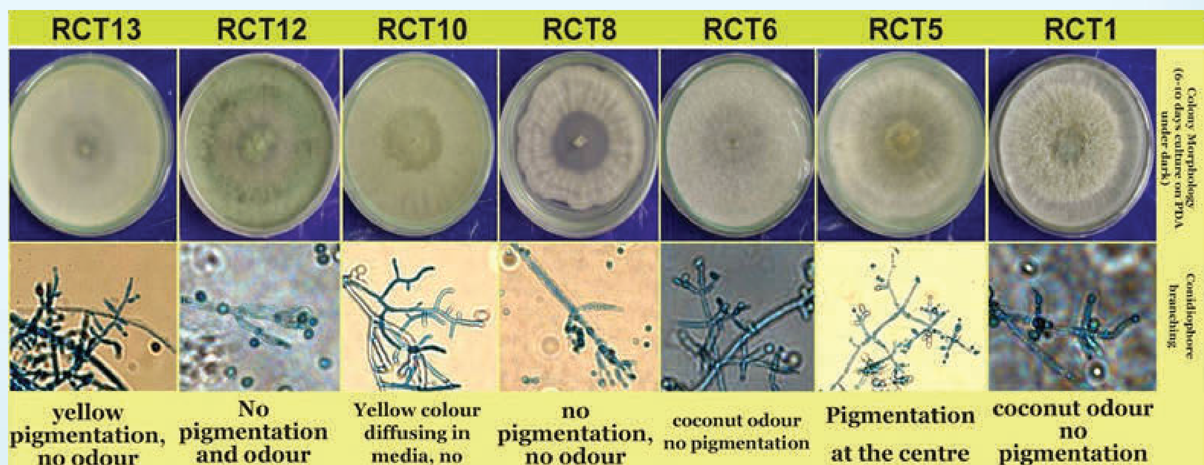

Fig. 40: Morphol ogical features of *Trichoderma* species isolated from North-East India

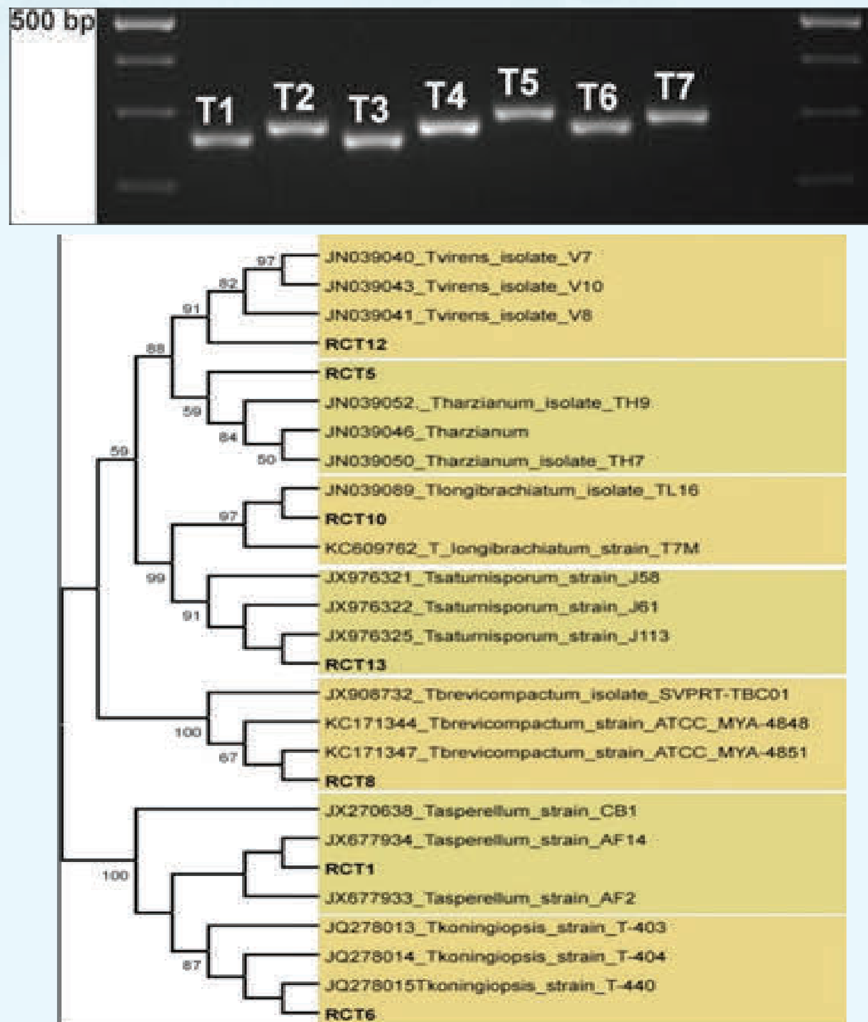

Fig. 41: Molecular characterization of *Trichoderma* strains isolated from North-East India. (*Top*) PCR amplification of ITS1 region of *Trichoderma* species using ITS1/ITS2 primer Lane L-100 bp ladder, T1 =RCT1, T2 = RCT5, T3 = RCT6, T4 = RCT8, T5 = RCT10, T6 = RCT12, T7 = RCT13 and (*bottom*) Phylogenetic grouping of *Trichoderma* species on the basis of ITS1 sequence data.

NATIONAL RESEARCH CENTRE (NRC) FOR CITRUS, NAGPUR

Principal Investigator:
DR. A K DAS

Co-investigator:
Dr. I P Singh

1. BIODIVERSITY

a. Collection and conservation of *Phytophthora* isolates infecting citrus

Surveyed citrus orchards in Vidarbha region of Maharashtra, Tripura, Nagaland, Manipur and Mizoram states of NEH region, part of AP and soil, root, leaf, fruit and water samples were collected for isolating *Phytophthora* spp. A total of 28 *Phytophthora* spp. isolates (18 isolates of *P. nicotianae*, 6 isolates of *P. palmivora*, and 4 isolates of *P. insolita*) were isolated and purified.

b. Morphological characterization

Colony morphology of 20 isolates was studied. The colony of *P. nicotianae* isolates showed dense cottony mycelium to cottony aerial mycelium with no specific pattern growth. All *P. palmivora* isolates showed stellate pattern with uniform margin on V8 agar whereas less defined petalloid pattern with irregular margin was observed on PDA. The *P. insolita* isolates showed chrysanthemum/ floral pattern type colony on V8 agar while pettaloid pattern was observed on PDA. The growth rate varied from 5.87 – 13.06 mm/ day in V8 agar and 4.18 – 8.43 mm/day in PDA for above isolates.

Sporangial morphology of 24 isolates was studied. In *P. nicotianae* isolates sporangia vary from ovoid to globose with prominent papillae and non-caducous. In case of *P. palmivora*, sporangia are variable in shape, mostly ovoid to globose, ellipsoid, limoniform with prominent papillae and caducous. Sporangiophore shows sympodial and simple sympodial branching. All the isolates were found to be A1 mating type. All *P. insolita* isolates were found homothallic.

c. Characterization of *Phytophthora lacustris* (NRCPh -112)

Phytophthora lacustris (formerly *P. taxon salixsoil*) was isolated (isolate NRCPh112) from rhizosphere soil of a citrus orchard in Sriganganagar, Rajasthan. In liquid culture sporangia (Fig. 42) were non-caducous, non-papillate, ovoid to obpyriform, with an average (15 sporangia) length x breadth of 43.7 x 34 µm and an l/b ratio of 1.28. Internal and external proliferation of sporangia and hyphal swellings were observed while chlamydospores were not produced. Isolate NRCPh112 induced selfing of an A1 isolate of *P. nicotianae* when paired on carrot agar thus functioning as 'silent A2' mating type. Colony morphology at 25±1°C was petaloid or chrysanthemum-like on V8 juice agar (Fig. 42A) whereas uniform wooly colonies were formed on potato dextrose agar (PDA). The average daily growth rate at 25±1°C on V8A, PDA and corn meal agar (CMA) was 7.9, 5.9 and 7.1 mm per day, respectively. On CMA optimum and maximum temperature for growth were around 25°C and 37°C, respectively. *P. lacustris* has similar morphology as *P. gonapodyides* but was identified by sequence analysis of the ITS (JQ424900), β-tubulin (JQ520346) and translation elongation factor 1 α (JQ520347) gene regions. The ITS sequence of isolate NRCPh112 showed 99% similarity with *P. lacustris* isolate ICMP16270 from New Zealand (GenBank code JF804803).

Fig. 42: Morphology of *Phytophthora lacustris*. A. Rosaceous pattern on V8 juice agar. B. Sporangium. C. Oogonium with amphigynous anthredium.

d. Mefenoxam (metalaxyl) sensitivity

Fungicide mefenoxam sensitivity was determined by growing the isolates on corn meal agar (CMA) amended with mefenoxam-mancozeb. There was no moderate isolate (M) that exhibited growth on media amended with 5 µg ml^{-1} greater than 40% of that on non-amended media. There was no resistant isolate (R) that exhibited growth on media amended with 50 µg ml^{-1} greater than 40% of that on non-amended media (rather complete inhibition of growth on media amended with 50 µg ml^{-1} of mefenoxam-MZ was observed). All the 34 (NRCPh 121 – NRCPh 154) isolates, were found sensitive.

e. Molecular diversity analysis

ITS-RFLP analysis of the PCR products of all the isolates under study revealed a difference in their restriction pattern. *MspI* digestions revealed three bands of 404, 390 (seen as one broad band of 400 bp) and 120 bp in *P. nicotianae* isolates whereas two clear bands of 508 and 389 bp were observed in *P. palmivora* isolates (Fig. 43). Correspondingly, *AluI* digestion revealed three bands of 745, 117 and 52 bp in P. *nicotianae* isolates, while restriction fragments of 501, 160, 157 and 42 bp were noticed in *P. palmivora* isolates. In case of *P. citrophthora* isolate (NRCPh-147), *MspI* digestions revealed 3 bands of 371, 295 and 226 bp (Fig. 43). This also confirms our previous non-molecular morphological assessment.

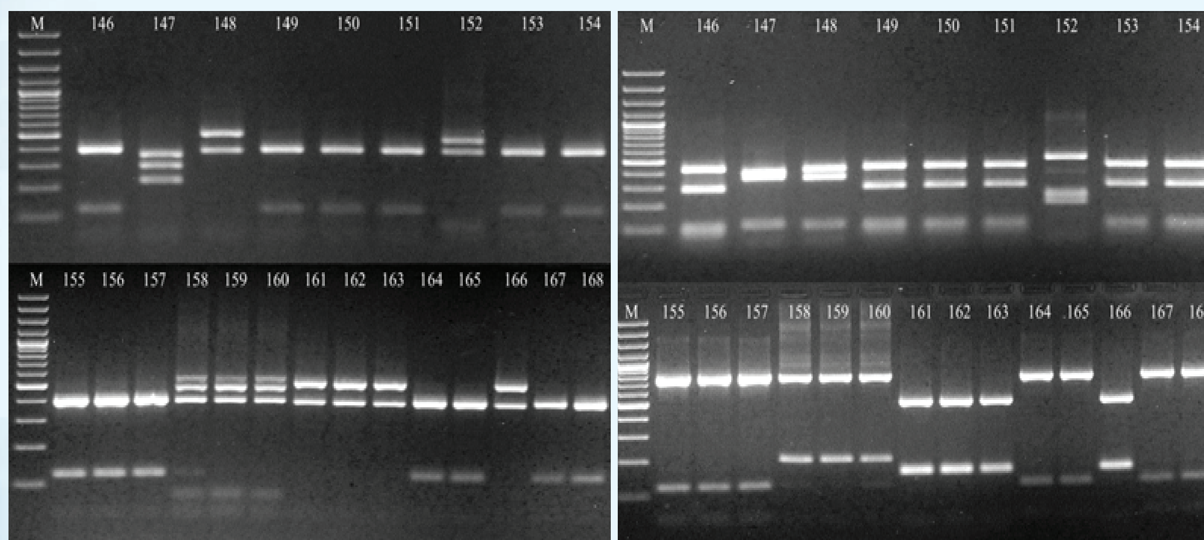

Fig. 43: RFLP profile of *Phytophthora* isolates obtained after digestion of ITS4/ITS6 amplification products. (Left) with *Msp I* and (right) *Alu I*. Lane 146-168: corresponding isolate nos. M. 100 bp marker.

f. Multi-locus sequence typing

To study the diversity of *P. nicotianae* isolates a multi-locus sequence typing (MLST) approach was carried out. In this study a total 18 *P. nicotianae* isolates (Table 13) across different citrus growing regions in India were studied. Three genomic loci *i.e.* internal transcribed spacer (ITS) region, β-tubulin gene and elongation factor-alpha (EF-1) were amplified by using specific primers and were sequenced. The sequencing data was assembled and submitted to NCBI Genbank (as mentioned in Table 13). Altogether, a total of 121 sequences (ITS region, β-tubulin, elongation factor-1α, cytochrome oxidase-1 and cytochrome oxidase-2 genes) of *Phytophthora* isolates were submitted in the GenBank database.

Phylogenetic trees were constructed using MEGA 5.01 for all the individual loci (Fig. 44 a, b, c) and sequences obtained by concatenation of all three loci (Fig. 44 d). Evolutionary history was inferred using the Neighbor-Joining (NJ) method. The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number of differences method and are in the units of the number of base differences per sequence. All positions containing gaps and missing data were eliminated. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The MLST analysis showed that there is considerable intra-specific diversity within *P. nicotianae* isolates collected from different citrus growing regions of the country.

Table 13. Isolates of *Phytophthora nicotianae* examined in MLST study

Isolate No.	GenBank accession No.			Place of collection	Host
	ITS	β-tubulin	EF-1		
NRCPh-18	JF792531	JN203066	JN257115	Mohpa, Vidarbha	Nagpur mandarin
NRCPh-56	JF792539	JN203068	JN257116	Redni, Baramati, Pune	Mosambi
NRCPh-58	JF792540	JN203069	JN257117	Mehlanwali, Hoshiarpur, Punjab	Kinnow
NRCPh-61	JF792541	KF384190	KF515637	Bichholi, Hoshiarpur, Punjab	Kinnow
NRCPh-66	JF792542	JN203070	JN257118	Loni, Varud	Nagpur mandarin
NRCPh-70	JX965375	KC984194	KC984198	Mordongre, Pandurna, M.P.	Nagpur mandarin
NRCPh-71	JX965376	JN203071	JN257119	Mordongre, Pandurna, M.P.	Nagpur mandarin
NRCPh-76	JX965377	KF384191	KF515638	Maywadi dapuri, Varud	Nagpur mandarin
NRCPh-81	JX965379	KC984195	KC984199	Mehlanwali, Hoshiarpur, Punjab	Kinnow
NRCPh-89	JN559843	KC984196	KC984200	Nalgonda, A.P	Satgudi sweet orange
NRCPh-98	JN559846	KC984197	KC984201	Mazithar Govt. Nursery	Sikkim Mandarin
NRCPh-99	JX965380	JN807439	KC984202	Mazithar Govt Nursery	Sikkim Mandarin
NRCPh-104	KF010300	KF384192	KF515639	Cherupalli, nalgonda, A.P	Rangpur lime
NRCPh-109	JQ308813	KF384193	KF515640	Bhilwara Jhalawar, Rajasthan	Nagpur mandarin
NRCPh-111	JQ308814	KF384194	KF515641	Nahardi, Nahar district, Rajasthan	Nagpur mandarin
NRCPh-120	KJ143627	KF384196	KF515643	Periyakulam, Tamilnadu	Acid Lime
NRCPh-121	KJ143628	KF384197	KF515644	Periyakulam, Tamilnadu	Acid Lime
NRCPh-123	KF010301	KF384198	KF515645	Ankelamma Gudur, Kadapa district, A.P	Sathgudi sweet orange

a b c d

Fig. 44: Phylogentic analysis of *Phytophthora nicotianae* isolates using (a) ITS region, (b) partial sequences of β-tubulin, (c) Elongation factor-1α (Ef-1α), (d) concatenated tree for the 3 loci.

2. DIAGNOSTICS

a. Multiplex PCR for simultaneous detection of *P. nicotianae* and *P. palmivora*

Primers were designed specific for *P. nicotianae* (Pn1/ Pn2) and *P. palmivora* (Pal1s / Pal2a) based on *Ypt1* gene and ITS region, respectively. We standardized the protocol using pure culture DNA of the individual species as well as DNA mixture of the two species. Results indicated that multiplex PCR with primer pairs Pn1/Pn2 and Pal1s / Pal2a amplified two amplicons of size~ 400 bp and ~650 bp in case of mixed infection of *P. nicotianae* and *P. palmivora*. In case of single infection, only the respective specific PCR products were observed (Fig. 45).

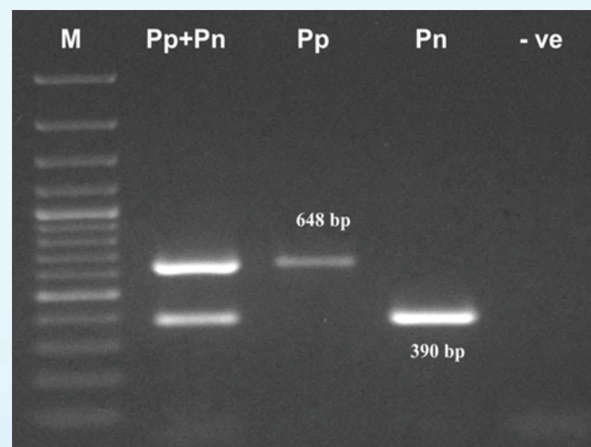

Fig. 45: Multiplex PCR for simultaneous detection of *P. nicotiane* and *P. palmivora* using specific primer pair Pn1/Pn2 and Pal1s/Pal2a. M: 100 bp ladder.

b. Real-Time qPCR (TaqMan) detection of *P. nicotianae*

DNA was extracted from fungal mycelia, soil, root and water samples using commercially available kits. Primers and probes (Taqman) were designed from the ITS region of *P. nicotianae* as reported earlier (P.nicF:5′ -GAA CAA TGC AAC TTA TTG GAC GTT T- 3′, P.nicR:5′ –AAC CGA AGC TGC CAC CCT AC– 3′, Pn.Pro: 5′ –/56-FAM/TTC ACC AGT CCA TCA CGC CAC AGC/6TAMARA/– 3′). This study developed a sensitive and effective method to detect specifically *P. nicotianae* in infected root, water and soil samples (Table 14). Analysis of more tissue samples, soil and water for the presence of *P. nicotianae* is in progress.

Table 14. Ct values for different environmental samples analyzed through Real-time PCR method

Sample	Source/Plant type	Ct value	Quantity
Root-1	Nagpur mandarin	30.02	0.0262569
Root-2	Nagpur mandarin	30.43	0.0200969
Water-1	Ambazari lake	30.80	0.0158411
Water-2	NRCC pond	32.40	0.0056952
Soil-1	Nagpur mandarin	28.04	0.0969075
Soil-2	Galgal	29.74	0.0315883
+ve control	*P. nicotianae*	18.63	48.0047
Non-template control	Nuclease free water	undetermined	0

3. HOST RESISTANCE

a. Screening of citrus rootstocks to evaluate resistance against *Phytophthora* spp.

Eleven rootstock seedlings (mostly rough lemon and Rangpur lime selections) were raised in polybags in glasshouse conditions for screening against *Phytophthora*. These rootstock seedlings were inoculated with *P. nicotianae* (chlamydospores and freshly released zoospores). After six weeks of inoculation, the seedlings were uprooted by carefully removing the pot mix with minimum disturbance to roots. Observations related to root rot and *Phytophthora* population were recorded. Results indicated that all the rootstocks were susceptible to highly susceptible towards *P. nicotianae* root rot infection. The rootstock Galgal (*C. pseudolimon*) was also found susceptible. Six hybrid rootstock (NRCC-1 to NRCC-6) seedlings were also raised in polybags in glasshouse conditions for screening against *Phytophthora* root rot.

b. Identification of molecular markers associated with *Phytophthora* root rot (PRR) tolerance/ resistance in citrus

Previously a total of 20 decamer oligonucleotides of arbitrary sequences were tested for PCR amplification in genomic DNA isolated from 13 selected citrus rootstocks/ cultivars (tolerant and susceptible). The primer OPA 8 was observed to produce a polymorphic band of ~250 bp only in tolerant rootstock cultivars. For converting the RAPD marker into a SCAR, that particular DNA band was eluted, cloned in TA vector and sequenced. Blast analysis showed that it was a part of *Poncirus trifoliata* CTV resistance gene locus. This contig reportedly contains several putative disease-resistance genes similar to the rice Xa21 gene, the tomato Cf-2 gene, and the *Arabidopsis thaliana* RPS2 gene.

To develop SCAR primer specific to *Phytophthora* resistant rootstock in citrus, this sequence was subjected to online primer designing tool Primer3 and a standalone primer designing software FastPCR. Total two different SCAR primer pairs (SC1-SC2 & SC3-SC4) were designed and validated by PCR amplification with standardization of primer concentration and annealing temperature. Out of which SC1 (Forward): 5′ – GGGGTTTTGTCGAACCACAT – 3′ and SC2 (Reverse): 5′ – GCTAAGCAACCCAAGAGGGT– 3′ worked suitably in this study (Fig. 46). This SCAR marker can be used for marker assisted selection (MAS) in citrus rootstock breeding programme aiming at the development of cultivars which are resistant/tolerant to *Phytophthora* root rot, particularly when other traits are also being evaluated.

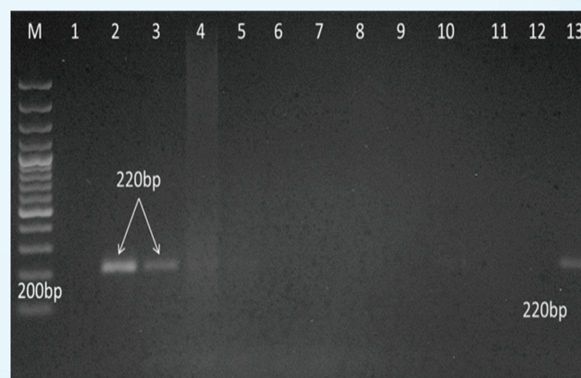

Fig. 46: PCR products obtained with primer pair SC1/ SC2 and different DNA extracts. Lanes :1 –Sour Orange, 2 –Pomeroy trifoliate, 3 – SFS, 4 – Rangpur lime (Chettalli), 5 – Rough Lemon (Rahuri), 6 – Alemow, 7 – Nagpur mandarin, 8 – Cleopatra mandarin, 9 – Sweet orange, 10 – Rangpur lime, 11- Rough lemon, 12- *Citrus volkamarina*, 13- Swingle citrumello, M- 100 bp ladder

In citrus rootstock breeding programme, following crosses (Smooth Flat Sevilli (SFS) X Rangpur lime, Alemow X Rangpur lime, Adajamir X Kagzi Niboo and *C. pectinefera* X *Pomeroy trifoliate*) were made for disease resistance (root rot) using these citrus rootstock species.

4. DISEASE MANAGEMENT

a. Search for novel bioagents and testing promising bioagents against *Phytophthora* spp

The fungal biocontrol bacterial bioagents collected earlier were maintained for studying their antagonistic activities against *Phytophthora* spp. infecting citrus. Two fungal bioagents, NRCfBA -29 and NRCfBA -44 were identified as potential BCA based on *in vitro* and *in vivo* tests.

b. Effect of pH and temperature

Two most promising isolates of *T. harzianum*, NRCfBA -29 and NRCfBA -44 were studied for their growth under different pH and temperature conditions. It was observed that both the strains could grow in a wide range of pH from 4-8. Optimum temperature for growth was found to be 25-30°C.

c. Effect of different fungicides

The tolerance of these two *T. harzianum* isolates towards fosetyl Al, mefenoxam + mancozeb, carbendazim and copper oxychloride (at 100, 500, 1000 and 2000 ppm concentrations) was tested using poisoned food technique. The results revealed that at selected concentrations of fungicides, fosetyl Al, mefenoxam + mancozeb, and copper oxychloride were compatible to some extent, though the compatibility decreased with an increase in the concentration of fungicides. Whereas, carbendazim even at 5 ppm was found to be 100% inhibitory and hence could be considered as incompatible with strains, NRCfBA-29 and NRCfBA-44.

d. Development of a talc-based formulation product of *T. harizanium*

Based on the performance in earlier *in vitro* and *in vivo* tests, two best isolates of *T. harizanium*, NRCfBA-29 and NRCfBA-44 were selected for formulation development and subsequent field evaluation. A mycelial disc (6 mm diameter) was inoculated in 100 ml PDB. Conidiation was counted after seven days and the mycelial mat along with conidia from PDB were mixed thoroughly with autoclaved talcum powder pre-treated with 0.5% CMC (0.5 g CMC dissolved in 100 ml water mixed with 1 kg talcum powder). The mix was air-dried in a laminar flow hood and the colony forming units were counted on PDA amended with 100 mg/L rose Bengal, after serial dilution. The initial count of *Trichoderma* was found 1.5 - 3 x 10^7 cfu/ g of formulation. Further evaluation of these products under nursery as well as field conditions is in progress.

Phytofura

Fusarium

CENTRAL INSTITUTE FOR SUBTROPICAL HORTICULTURE, LUCKNOW

Principal Investigator:

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Dr. M MuthuKumar

1. *FUSARIUM* BIODIVERSITY

a. REP, BOX and ERIC-PCR for *Fusarium oxysporum* f.sp. *psidii*

To understand the phylogenetic relationships of F. *oxysporum* f. sp. *psidii* isolates, the REP-PCR, BOX-PCR and ERIC-PCR were evaluated and the results showed the usefulness of REP, BOX and ERIC-PCRs genomic fingerprinting as a complementary or alternate strategy to RAPD analysis (Fig. 47). The major advantages were simplicity, the universality of PCR primers, tolerance to a wider range of DNA concentrations in generating reproducible results and amenability to database analysis. In the present study both monomorphic and polymorphic banding patterns were obtained at 42°C annealing temperature but the standard annealing temperatures (53°C for BOX and 52°C for ERIC-PCR) described in literature when tested did not result in the amplification of DNA bands. The results of the study imply that BOX, ERIC and BOX-PCR are suitable, inexpensive, fast, reproducible and discriminatory DNA typing tools for *F. oxysporum* f. sp. *psidii* isolates.

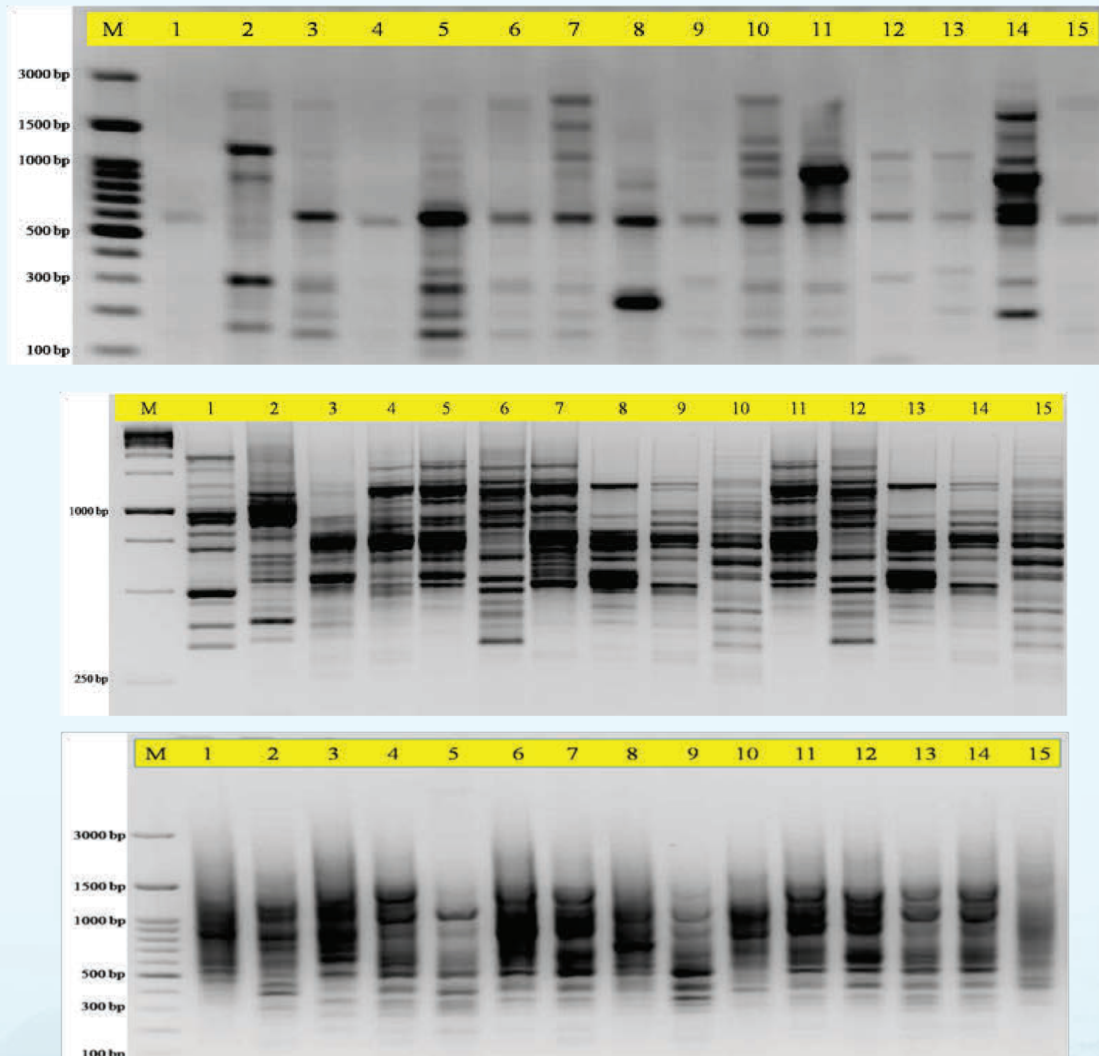

Fig. 47: Genetic diversity in *F. oxysporum* f. sp. *psidii* isolates as evidenced by PCR fingerprint patterns using (*Top*) Rep1 and Rep 2 primers, (*Middle*) BOXA-1R primer and (*Bottom*) ERIC1R and ERIC 2 primers. Lane 1-5: *F. oxysporum* f. sp. *psidii* isolates, Lane M: DNA ladder (Genoid).

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1. DISEASE MANAGEMENT

a. Management of *Fusarium* wilt in Safflower

Five fungicides and biological agents on *Fusarium* wilt in safflower under field conditions. Treatments with Captan (0.2%) and *T. harzianum* (Th4d SC) @ 2 ml/kg were found to be the most effective (Fig. 48 & 49) as they recorded significantly low incidence of *Fusarium* wilt (13.8 & 16.5%) whereas control plots recorded high incidence of wilt (33.0%). Highest seed yield (2350 kg/ha) was recorded in captan @ 0.2% followed by *Trichoderma harzianum* (Th4d) (2230 kg/ha) treatment compared to pathogen check (1290 kg/ha).

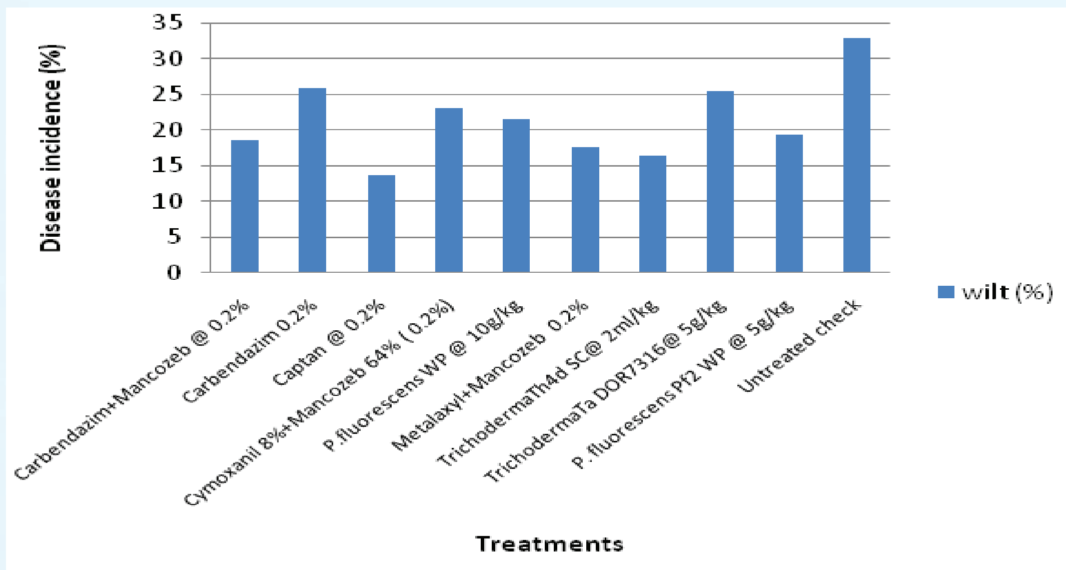

Fig. 48: Effect of fungicides and biological agents on *Fusarium* wilt.

Captan @ 0.2% *T. harzianum* Th4d SC @ 2 ml/kg Untreated check

Fig. 49: Effect of captan and *Trichoderma* treatments on disease incidence

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1. BIODIVERSITY

a. IGS- RFLP analysis

The intergenic spacer region (IGS) of 22 isolates of *Fusarium oxysporum* f. sp. *ciceris* (Foc) was amplified using primers set CNL12/CNS1 (F1: 5'- CTGAACGC-CTCTAAGTCAG-3' R1:5'- GAGACAAGCATATGAC-TACTG-3') for IGS-RFLP pattern analysis. The amplified PCR products for each of the isolates were digested with 11 different restriction enzymes, namely, *EcoRI, HindIII, AvaI, RseI (Ms1I), Bpu10I, Alw26I (BsmAI), AluI, HphI, MboI, BfuCI* and *MspI*. Restriction digests were size-fractioned by electrophoresis. Presence (1) or absence (0) of DNA bands was recorded and binary metrices were analyzed by NTSYS – PC (Version 2.0; Exeter Biological Software, Setauket, NY). Jaccard's coefficients were used to portray a dendrogram by using SHAN clustering programme by unweighted pair group method with arithmetic average (UPGMA). The complete IGS region of 22 isolates of Foc amplified with primers CNL12 and CNS1 produced ≈ 2600 bp of fragment.

Among eleven restriction enzymes, two enzymes, namely, *AluI* and *Bpu10I* produced a monomorphic banding pattern, therefore could not be used for diversity analysis. *HindIII* had only one restriction site in the amplified IGS region. Enzymes *MslI, BfuCI* and *MboI* did not produce significant banding patterns, hence could not be used for further analysis. Five restriction enzymes, namely, *EcoRI, AvaI, BsmAI, HphI,* and *MspI* were found to be suitable in resolving the haplotype among the 22 isolates of Foc.

According to restriction patterns generated by these five enzymes, five RFLP groups were easily defined. The isolates from Karnataka (Foc 126 and Foc 148; race 1), Chhattisgarh (Foc 161; race 6), Madhya Pradesh (Foc 155; race 6) and Maharashtra (Foc 124; race 7) showed similar fingerprinting pattern by using each five enzymes. Therefore they are considered to be identical in IGS sequences. The isolate Foc 118 from Andhra Pradesh representing race 1 and Foc 123 from Gujarat representing race 7 showed identical restriction patterns from individual restriction enzymes. The five groups of isolates based on restriction patterns generated from enzymes *BsmAI* and *MspI* were corresponding with the groups generated from phylogenetic analysis of partial IGS sequences of these isolates. The first group had two isolates Foc 118 and Foc 123 from Andhra Pradesh and Gujarat, respectively. The second group had 13 isolates from different place of origin representing different races (Foc 143, Foc 53, Foc 108, Foc 122, Foc 41, Foc 33, Foc 45, Foc 93, Foc 4, Foc 68, Foc 119, Foc 130 and Foc 179). The third group had five isolates (Foc 161, Foc 126, Foc 148, Foc 155 and Foc 124), whereas, the fourth and fifth groups had only one isolate each. The enzymes *BsmAI* and *HphI* showed similar restriction patterns and considered suitable for distinguishing race 4 representative isolates (Foc 53 and Foc 108 from Delhi and Foc 41 and Foc 33 from Haryana) of the pathogen from others. The UPGMA analysis of the restriction patterns obtained by these five enzymes used for the study clustered 22 isolates of the pathogen into five major groups at 40% genetic similarity (Fig. 50).

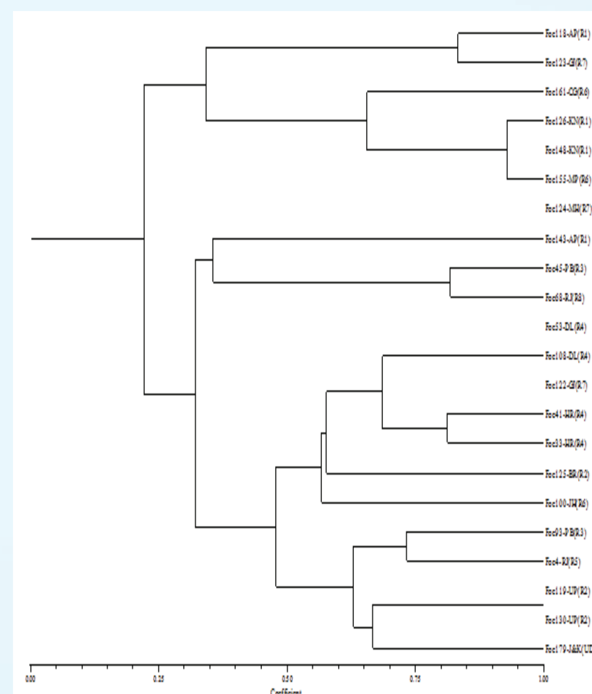

Fig. 50: Dendrogram derived from combined IGS-RFLP data of *Fusarium oxysporum* f.sp. *ciceris* with restriction enzymes, namely, *EcoRI*, *AvaI*, *BsmAI, HphI* and *MspI* by unweighted paired group method with arithmetic average analysis (UPGMA). The bottom scale is the percentage of Jaccard's similarity coefficient.

Sequence analysis of 28S nuclear large rDNA region

The 28S nuclear ribosomal DNA gene was used to determine the genetic diversity of 71 isolates of *Fusarium oxysporum* f. sp. *ciceris* (Foc) originating from 13 states of India representing different races of the pathogen. The 28S region was amplified by using a set of universal primers, namely, NL1 and NL4 which produced 605 bp fragments in all the isolates of the pathogen.

2. DIAGNOSTICS

a. IGS region based Foc specific marker

The primer pair CNL12 (5′-CTGAACGCCTCTAAGTCAG-3′) and U-49:67 (5′-AATACAAGCACGCCGACAC-3′) were used to amplify the IGS region of 22 isolates of Foc. The specificity and sensitivity of the designed marker was estimated using conventional as well real time PCR. The partial IGS gene sequence of Foc isolate (Foc 41) used to design the marker was submitted to GenBank at NCBI with accession number KF061178.

Annealing temperature for the marker PS15 F1 and R1 was determined using gradient PCR. To verify the specificity of the marker, PCR was performed. The specificity of the primer was tested against 14 Foc isolates, leaves of healthy and infected plant samples, several other important soil and seed borne fungal pathogens of chickpea, namely, *F. solani, F. udum, R. solani, R. bataticola* and *S. sclerotiorum*. To determine the sensitivity of the markers, the conventional and real time PCR assay was performed using serially diluted standard of genomic Foc DNA. Different dilutions of genomic DNA of Foc inoculated chickpea plants were used as unknown samples. The genomic DNA extracted from different parts of the artificially inoculated chickpea plant samples, namely, root, lower and upper half parts of the stem collected at 3, 5, 7, 10, 15, 25 and 45 days post inoculation was used for specific amplification with PS15 F1 and R1. Un-inoculated chickpea plant sample was used as negative control.

The primer PS15 F1 and R1 successfully amplified the genomic DNA obtained from 14 isolates of Foc representing 13 state and eight races of the pathogen as well as Foc inoculated chickpea plants and gave the product size of 197 bp (Fig. 51). Furthermore, no amplification was found in un-inoculated chickpea plant sample and other species of soil borne plant pathogens, namely, *F. solani, F. udum, R. solani, R. bataticola* and *S. sclerotiorum*. The marker was able to detect the minimum quantity of Foc DNA at 0.5 ng, while in inoculated plant sample, the detection level was 1 ng. Real time PCR assay was found more sensitive and able to amplify 0.01 pg of Foc DNA and 0.1 ng of Foc inoculated plant DNA. Standard curve showed significant correlations (R2- 0.993) between DNA concentrations and CT values obtained with a slope of -2.358 (Fig. 51).

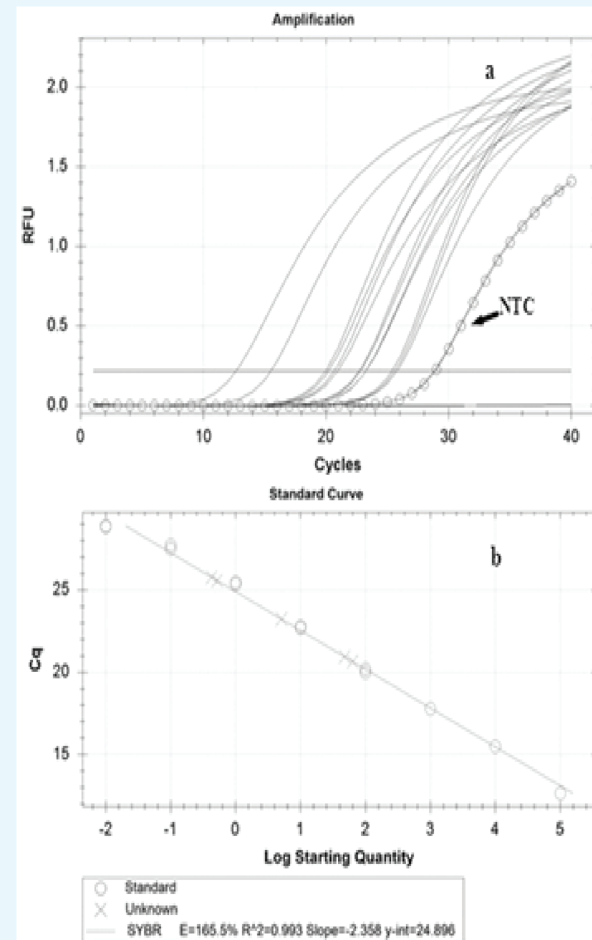

Fig. 51(a): Amplification plot generated by marker PS15 F1 and R1 (b): Standard curve analysis with marker PS15 F1 and R1. × - unknown (Foc inoculated chickpea plant), - standard (Foc genomic DNA).

The specificity of the marker was also confirmed by presence of single melting curve at 85°C. The marker set, PS15 F1 and R1, was found to be accurate in detecting Foc in root, lower and upper half part of the stem parts by amplification of the specific band of 197 bp. Furthermore, the marker was capable to detect Foc infection at an early stage (3 days post inoculation) and the intensity of the bands increased in subsequent days after inoculation as the development of the disease increased in the plants. Thicknesses of the bands were found to be more in the plant samples obtained at 45 days post inoculation.

3. HOST PATHOGEN INTERACTION

a. Differential expression pattern in the defense related genes

Differential expression pattern in the defense related genes of Foc infected resistant and susceptible chickpea plants was analyzed to determine the up regulation and down regulation process in respective genes. Highly susceptible chickpea variety JG 62 and resistant variety GPF 2 were selected for defense gene analysis using real time PCR. Chickpea seedlings at the stage of 2-3 leaves were drenched with salicylic acid, jasmonic acid and *Trichoderma* prior to Foc infection (10^6 spores/ml) separately and in combinations. Un-inoculated plant samples were used as a control. The plant samples obtained from eight different treatments (T1- Foc, T2- un-inoculated (without Foc), T3-Salicylic acid + Foc, T4- Salicylic acid, T5-Jasmonic acid + Foc, T6-Jasmonic acid , T7- *T. harzianum* + Foc, T8- *T. harzianum*) combinations for each chickpea variety were used for RNA isolation and cDNA preparation at 1-4 days after inoculation (dai). The assessment of gene expression ratio between treated and untreated susceptible (JG 62) and resistant (GPF 2) cultivars of chickpea was performed by using conventional PCR and real time PCR.

The results of the expression analysis showed the presence of expected amplicon size for all the genes. In both the cultivar at 1 dai, uninoculated plant sample showed more expression of PR protein as compared to inoculated plant sample. The expression was higher at 1 and 2 dai in resistant variety whereas at 3 and 4 dai its expression was reduced and susceptible variety showed more expression as compared to resistant variety.

The presence of Foc alone and with jasmonic acid unregulated the expression in susceptible cultivar at 3 dai. At 4 dai its expression was higher in susceptible cultivar inoculated with Foc alone and with salicylic acid and in *T. harzianum* treated plants also (Fig. 52). The expression of protein kinase was higher in resistant variety as compared to susceptible. The relative expression of protein kinase was down-regulated in susceptible variety JG 62 in response to salicylic acid in presence of Foc and in resistant variety GPF2 with salicylic acid alone at 1 dai whereas, up-regulated in the rest of the treatments as compared to the control. The expression was the highest in *T. harzianum* alone and with Foc at 1 dai and subsequently it was reduced. The application of jasmonic and salicylic acid also enhanced the expression both in resistant and susceptible varieties at 2 and 3 dai (Fig. 52).

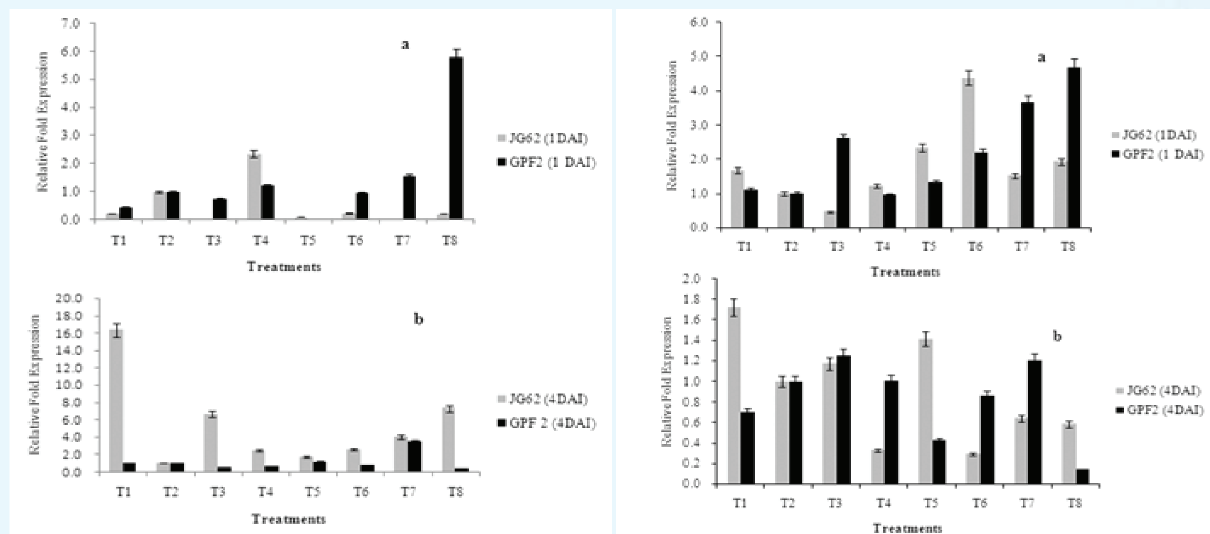

Fig. 52: Differential expression pattern in the defense related genes of Foc infected resistant and susceptible chickpea plants. (*Left*) PR 10 gene and (*Right*) Protein kinase gene

Salicylic acid and jasmonic acid alone and in presence of Foc showed relatively higher expression in both the varieties to over the control at 4 dai. The expression ratio of the acidic glucanase gene was higher in resistant variety at 1, 2 and 4 dai. The relative expression was down regulated with all the treatments in JG 62 at 1 dai with the highest in salicylic acid in presence of Foc as compared to the control, whereas, it was up-regulated with all the treatments in resistant variety at 1 dai and *T. harzianum* showed the highest up-regulation as compared to control at 1 and 4 dai. Salicylic acid treated plants showed the highest up-regulation at 2 dai in resistant cultivar whereas, in 3 dai, susceptible variety treated with jasmonic acid in presence of Foc showed the highest expression. All the treatments were again down regulated in susceptible variety at 4 dai and salicylic acid in presence of Foc showed the highest down regulation as compared to control. The resistant variety inoculated with Foc, salicylic acid with Foc and *T. harzianum* with Foc showed the up regulation. The expression of glucosidase was higher in susceptible variety as compared to resistant variety. The expression of glucosidase was up-regulated in susceptible variety in response to Foc,

salicylic acid alone and along with Foc, jasmonic acid and *T. harzianum* as compared to the control, whereas, it was down-regulated in treatments including jasmonic acid with Foc and *T. harzianum* with Foc at 1 dai. In resistant variety the expression was the highest in jasmonic acid with Foc at 1 dai. Resistant variety inoculated with Foc showed the highest expression, whereas, *T. harzianum* with Foc followed by salicylic acid showed the highest expression in the susceptible variety at 2 dai. Susceptible variety inoculated with Foc showed the highest up-regulation followed by jasmonic acid alone and with Foc at 3 dai. The resistant variety showed the up-regulation in all the treatments over the control at 4 dai. The plant samples of susceptible variety treated with jasmonic acid followed by *T. harzianum* in presence of Foc showed the highest expression whereas, salicylic acid treated resistant variety showed the highest expression at 4 dai. Expression levels of cysteine proteinase gene was higher in resistant variety at 1 and 4 dai whereas, at 2 and 3 dai, susceptible variety showed higher expression as compared to control. The relative expression of cysteine proteinase at 1 dai in both varieties gave mix response against different treatments. The highest up-regulation was in *T. harzianum* treated resistant variety followed by jasmonic acid treated susceptible variety. Susceptible variety treated with *T. harzianum* with Foc showed the highest expression at 2 dai. Susceptible variety treated with salicylic acid followed by jasmonic acid alone and with Foc showed the highest expression at 3 dai. Resistant variety treated with jasmonic acid with Foc followed by salicylic acid with Foc showed the highest expression at 4 dai.

b. Differential expression pattern of MAP kinase gene family

Mitogen-activated signal transduction pathways play an important role in development of defense levels in plants. Highly susceptible chickpea variety JG 62 and resistant variety GPF 2 were selected for mitogen-activated protein kinases (MAPKs) gene analysis. The expression analysis were estimated under the influence of eight different treatments as T1- Foc, T2- un-inoculated (without Foc), T3- Salicylic acid + Foc, T4- Salicylic acid, T5-Jasmonic acid + Foc, T6-Jasmonic acid, T7- *T. harzianum* + Foc, T8- *T. harzianum*. Un-inoculated plant samples were used as a control. Total RNA was isolated at different time intervals (1st, 2nd, 3rd, and 4th day after inoculation) and subjected to cDNA synthesis for conventional and real-time PCR. After PCR amplification a 229 bp band was detected in all plant samples.

Real-time PCR clearly showed that MAP kinase gene was differentially expressed at different time intervals in both treated and untreated plant samples. The expression analysis indicated that initially Foc infection down-regulated MAP kinase gene in both susceptible and resistant varieties and subsequently it was up-regulated only in susceptible variety at 2 dai and continued up to 4 dai. At 4 dai, it was slightly up-regulated in resistant variety also. Considering all the treatments, the resistant variety showed up-regulation at 1 dai and 4 dai over the susceptible variety, whereas, at 2 dai and 3 dai susceptible variety were more expressive over the resistant variety. At 1 dai, the expression was the highest in *Trichoderma harzianum* treated plants followed by salicylic acid in the resistant variety. At 2 dai, the highest expression was in *T. harzianum* + Foc in susceptible variety. At 3 dai, the highest expression was in jasmonic acid + Foc followed by Foc, salicylic acid and jasmonic acid in the susceptible variety, whereas, the highest expression was in the resistant variety treated with jasmonic acid + Foc (Fig. 53).

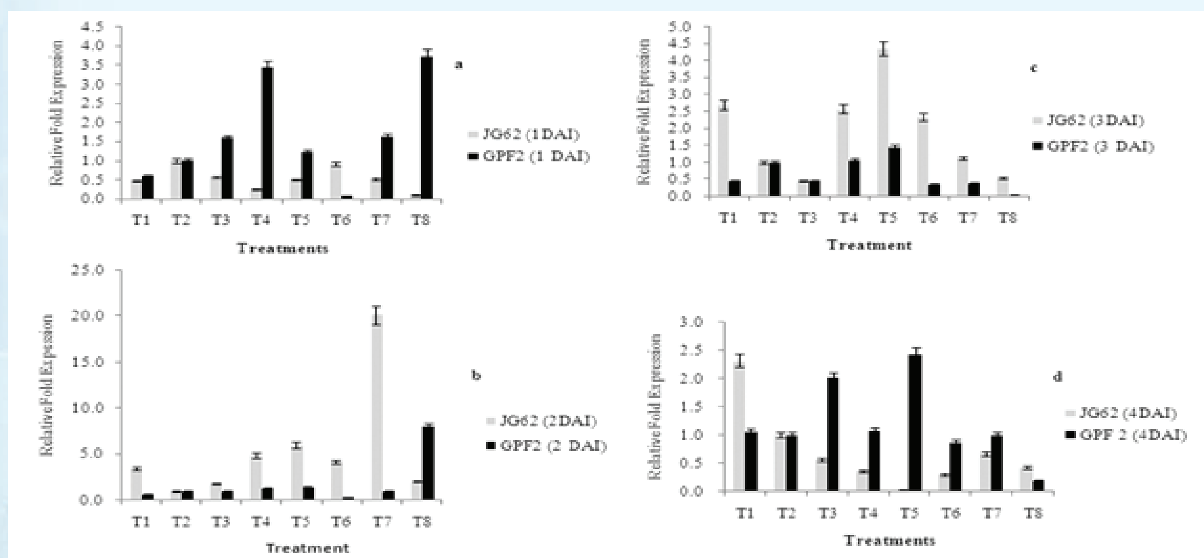

Fig. 53: Differential expression profiling of MAP KK- F1R1 gene of Foc infected resistant and susceptible chickpea plants. (a-1 DAI, b- 2DAI, c-3 DAI and d-4DAI)

c. Evaluation of a new set of chickpea differentials against isolates of the pathogen

Virulence of 11 isolates of the pathogen obtained from different sick field of the country (7 states) was tested on a new set of 10 differential cultivars of chickpea, namely, C104, JG74, CPS1, BG212, WR315, KWR108, GPF2, DCP92-3, Chaffa and JG62 in a net house during the winter seasons of 2013-2014. Based on the resistant reactions, cultivars were identified to differentiate the races of the pathogen. The results clearly indicated that all the isolates were grouped into seven races, namely, race 1 (Karnataka), race 2 (Bihar), race 3 (Punjab), race 4 (Maharashtra), race 5 (Rajasthan), race 7 (Gujarat) and race 9-new race (Jammu and Kashmir).

4. DISEASE MANAGEMENT

a. Induction of systemic resistance in chickpea in response to *Trichoderma harzianum* against *F. oxysporum* f. sp. *ciceris*

The expression of β-1,3 glucanase and polyphenol oxidase (PPO) genes in response to Pusa 5SD (*T. harzianum*) in chickpea varieties resistant (WR 315) and susceptible (JG 62) to wilt caused by *Fusarium oxysporum* f. sp. *ciceris* (Foc) was analysed under four different treatment combinations, namely, Pusa 5SD alone and with Foc, Foc alone and un-inoculated control. The expressions of genes were analyzed by conventional as well as real time PCR assay using cDNA of leaf and root samples of the plant separately. Real-time PCR was performed and the primer set specific to polyphenol oxidase gene gave a 156 bp band. The highest expression was found in the susceptible variety leaves samples treated with *Trichoderma* + Foc. In the root samples of resistant variety, the expression was down regulated as compared to the control. Except, root of susceptible variety, all treatments showed down regulation of PPO in response to Foc inoculation (Fig. 54). Amplification of cDNA using specific primer set produced a 152 bp band showed expression of β-1,3 glucanase gene transcripts in plant samples. The highest up-regulation of β-1,3 glucanase gene was found in the root samples of resistant variety inoculated with Foc followed by root samples of resistant variety inoculated with *Trichoderma*. In case of susceptible variety, the leaves showed higher up-regulation as compared to the root samples (Fig. 55).

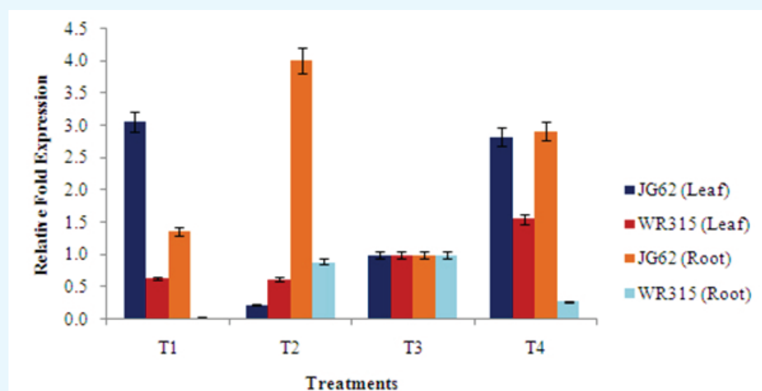

Fig. 54: Expression profiling of polyphenol oxidase gene from leaf and root samples of susceptible chickpea variety JG 62 and resistant variety WR315 at 5 days post inoculation in five treatments (T1-Foc+*Trichoderma*, T2 -Foc inoculated, T3-Un-inoculated and T4 -*Trichoderma*)

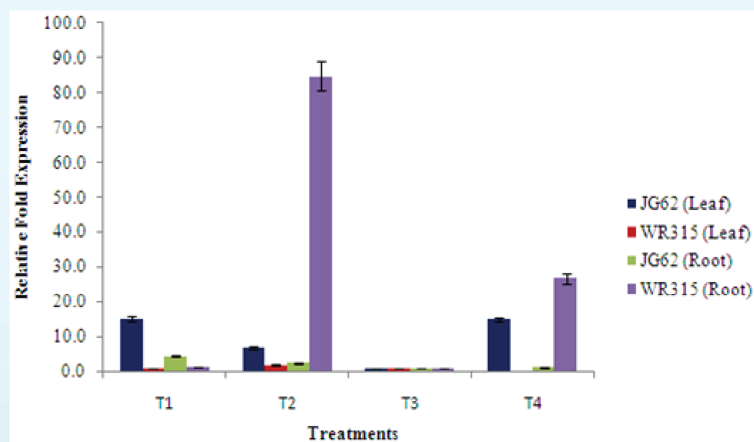

Fig. 55: Expression profiling of β-1,3 glucanase gene from leaf and root samples of susceptible chickpea variety JG 62 and resistant variety WR315 at 5 days post inoculation in four treatments (T1-Foc+*Trichoderma*, T2 -Foc inoculated, T3-Un-inoculated and T4 -*Trichoderma*)

b. Integrated management in field

The field experiment was conducted in a randomized block design with seven treatments in three replications in a sick field (with Foc) at IARI, New Delhi, India. The treatments consisted of Pusa 5SD (*T. harzianum*), talc formulation of *P. fluorescens* (Pf-80), Vitavax power, Pusa 5SD + *P. fluorescens*, Pusa 5SD + *P. fluorescens* + carboxin + thiram 37.5% (Vitavax power™), the most commonly recommended seed treatment consisting of a mixture of carbendazim (Bavistin™) +tetramethyl thiuram disulphide (Thiram™), and control (untreated seeds). Wilt incidence was recorded at 20 days intervals up to the maturity of the crop and total wilted plants per plot were presented. Grain yield was measured after the harvesting of the crop. The results of field experiments (Table 15) conducted in a sick field infested with Foc showed that the seed treatments significantly ($p<0.05$) enhanced the seed germination and grain yield of chickpea and reduced the wilt incidence as against those of the control during both the years of experimentation. A combination of Pusa 5SD (*T. harzianum*) + carboxin + Pf 80 provided significantly higher seed germination and grain yield compared to those of other treatments during both the years of experimentation as well as in mean data. The lowest wilt incidence was also recorded in this treatment and did not differ statistically from that of carboxin + TMTD during 2010-11. The next effective treatment in order of rank was carboxin + TMTD for enhancing the seed germination and grain yield, and reducing the wilt incidence followed by carbendazim + TMTD for seed germination and Pusa 5SD + Pf 80 for reducing the wilt incidence and enhancing the grain yield. However, the seed germination recorded in carbendazim + TMTD did not differ significantly with that of Pusa 5SD + Pf 80.

Table 15. Effect of seed treatments on seed germination, wilt incidence and grain yield of chickpea cultivar Pusa 362 under wilt sick field condition during 2010-11 and 2011-12

Treatment	Seed germination (%)			Wilt incidence (%)			Grain yield (Kg ha^{-1})		
	2010-11	2011-12	Mean	2010-11	2011-12	Mean	2010-11	2011-12	Mean
Pusa 5SD (*T. harzianum*)	77 (61.3)ef	89.6 (71.2)d	83.3 (66.3)d	28.8 (32.5)d	30.0 (33.2)d	29.4 (32.9)c	712.9de	972.2c	842.6d
Pseudomanas fluorescens 80 (Pf 80)	76.7 (61.1)fc	80.2 (63.6) e	78.5 (62.4)e	33.1 (35.1)e	39.0 (38.6)f	36.1 (36.9)d	648.2f	944.4cd	796.3e
Carboxin + TMTD (Vitavax power™)	82.8 (65.5)bc	95.8 (78.2) b	89.3 (71.9)b	18.5 (25.5)b	22.2 (28.1)b	20.4 (26.8)a	861.1b	1097.2b	979.2b
Pusa 5SD + Pf 80	79.2 (62.9)def	90.4 (71.9) cd	84.8 (67.4)cd	23.9 (29.3)c	25.2 (30.1)c	24.6 (29.7)b	740.7cd	1055.3b	898.0c
Pusa 5SD + Vitavax power™ + Pf 80	86.7 (68.6)a	97.5 (81.1)a	92.1 (74.9)a	16.3 (23.8)ab	20.0 (26.6)a	18.2 (25.2)a	898.1a	1305.5a	1101.8a
Carbendazim (Bavistin™) + TMTD (Thiram™)	80.3 (63.7)cde	92.9 (74.5) c	86.6 (69.1)c	36.9 (37.4)f	35.0 (36.3)e	36.0 (36.9)d	694.4e	958.3cd	826.4d
Control (untreated seeds)	68.9 (56.1)g	75.0 (60.0)f	72.0 (58.1)f	52.3 (46.3)g	55.4 (48.1)g	53.9 (47.2)e	500g	694.4e	597.2f

The figures in parentheses are transformed angular values.
The values within a column with different letters are significantly different at 5% level by using Fisher's least significance difference test.

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1. BIODIVERSITY

a. Pigeon pea wilt

Fifty isolates of *Fusarium udum* representing different states of India were subjected to their pathogenic potential on pigeonpea wilt differentials. Based on reaction of differential genotypes, 33 isolates were categorised in to seven variants. Seventeen isolates however could not be grouped in to any of the seven variants. Most of the isolates (13) resembled variant 1, whereas 8 and 6 isolates resembled variant 2 and 3, respectively. Three isolates resembled variant 4 whereas one isolate each belonged to variant 5, 6 and 7, respectively. Results indicate vast pathogenic diversity in isolates of wilt pathogen, *Fusarium udum*.

b. Chickpea wilt

Fifty nine isolates of *F. oxysporum* f.sp. *ciceri* from different chickpea growing areas of the country were tested for their pathogenic potential on 14 differential genotypes of chickpea. Disease reaction of differential genotypes indicated presence of four races (race 2, race 3, race 4 and race 5). Majority of the isolates (42) resembled race 2 of *F. oxysporum* f.sp. *ciceri*.

2. DIAGNOSTICS

a. Development of diagnostic marker for the identification of *Fusarium* sp.

In order to develop the species specific diagnostic markers for races and variants, SRAP-RGA and RAPD markers were employed for genome wide scanning in reference set of six races and five variants. For the molecular analysis, 26 SRAP-RGA coupled markers combinations were screened and to unique, reproducible bands specific to races (1 and 5) and variants (1, 3 and 5) were found and successfully sequenced. Based on the sequence information obtained from the sequence data, seven primer pairs specific to race and variant were designed and validated in reference set. Based on the result, only one primer specific to race 5 was able to reproduce exactly the same as in reference set. The sequence synteny was also confirmed by using NCBI-BLAST analysis. A schematic diagram for development of diagnostic marker through SRAP-RGA was shown in Fig. 56.

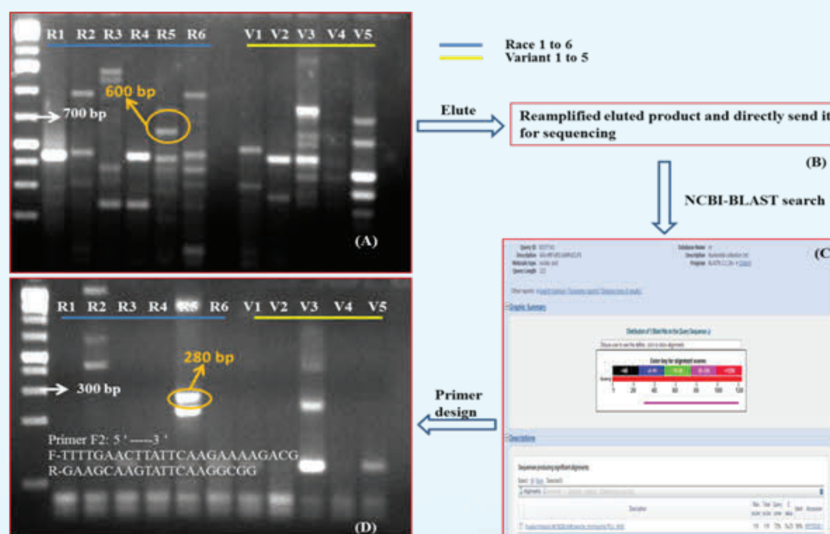

Fig. 56: A schematic diagram for development of Race-5 specific diagnostic marker.

In the same fashion, degenerate primer *viz*., random amplified polymorphic DNA (RAPD) markers were also been deployed in order to develop appropriate diagnostic markers to identify *F. udum* and *F. oxysporum* f.sp. *ciceri* in reference set of six races and five variants identified in IIPR, Kanpur. Total 39 RAPD markers were screened, where 10 markers gave thirteen unique bands for the particular race as shown in Table 16.

The unique amplicon amplified by the RAPD primers (Fig. 57A) was excised and purified using the QIAquick Gel extraction kit (Qiagen, Maryland, USA) from 0.8% agarose gel and cloned into pTZ57R/Tvector. The recombinants were screened through blue/white selection in LB/Ampicillin/X-gal/IPTG plate and positive recombinant plasmid was isolated from each overnight grown colony and presence of insert was confirmed by restriction digestion using XbaI and SmaI restriction enzymes (Fig. 57B). Two of the recombinants gave positive result of restriction digestion and were sequenced by outsourcing using vector specific universal promoter primer (M13). These sequence will be further used for diagnostic primer designing.

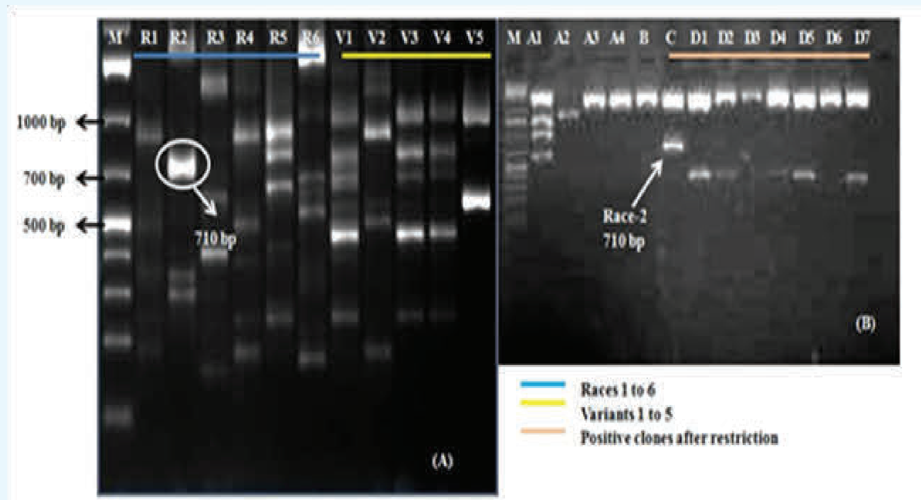

Fig. 57: Gel photograph (A) showing unique band for race 2 and Gel photograph (B) showing restriction pattern of positive clones plasmid. [M: 1 kb plus ladder]

Table 16. List of reproducible unique bands derived from RAPD primers

Primer name	Specific organism	Size (bp)
OPO-02	Race-6 and Variant-5	390 and 2000
OPO-07	Race-6	880
OPO-08	Race-3	710
OPP-10	Race-6 and Variant-5	250 and 1200
OPP-13	Race-3 and Variant-5	230 and 320
OPP-14	Race-4	500
OPO-04	Race-4	320
OPL-02	Variant-5	680
OPP-09	Race-2	250
OPL-01	Race-3	450

** Same insert but different colonies

b. Sequence variation in Internal Transcriber Spacer region of *Fusarium udum* and *Fusarium oxysporum* f.sp. *ciceri*

To classify the *Fusarium* species, ITS marker system was amplified with ITS fragments (680-800 bp) of 34 isolates representing *F. udum* as well as *F. oxysporum* f.sp. *ciceri* and sequenced these regions by outsourcing (Bangalore Genei, India). The nucleotide sequences were subjected to BLAST analysis (http: //www. ncbi. nih.gov/index.html) to find out the synteny between the species. A phylogenetic tree was reconstructed following UPGMA algorithm using the MEGA 5.2 software with 100 bootstrap replicates (Fig. 58).

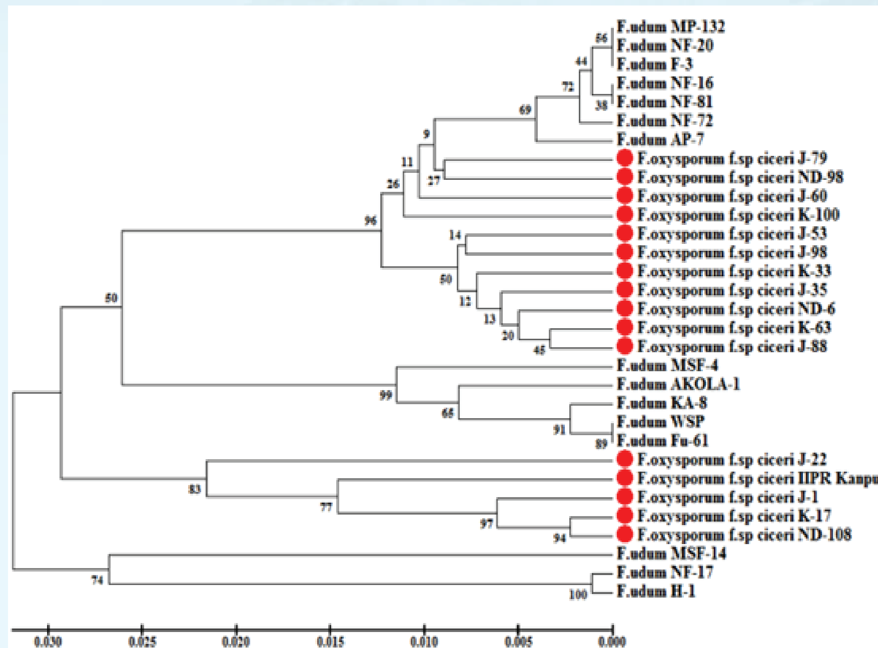

Fig. 58: Phylogenetic tree based on ITS sequences of *Fusarium udum* and *Fusarium oxysporum* f.sp. *ciceri* infecting pigeonpea and chickpea

c. Development of the genomic resources in *Fusarium* sp.

Sixty two partial sequences of internal transcriber spacer (ITS) region and translation elongation factor (TEF-1 alpha) of *F. udum*, and *F. oxysporum* f.sp. *ciceri*, causal agent for wilt disease in pigeonpea and chickpea, respectively have been submitted to NCBI GenBank using BankIt tool. These coding sequences were obtained by PCR amplification of genomic DNA of different of *F. udum* and *F. oxysporum* f.sp. *ciceri* isolates with ITS and TEF-1α primer. GenBank accession numbers allotted are alpha Elongation Factor 1 of *F. udum* - KJ720613 to KJ720630 (18 sequences), alpha Elongation Factor 1 of *F. oxysporum* f.sp. *ciceri* - KJ720631 to KJ720636 (6 sequences), ITS region of *F. oxysporum* f.sp. *ciceri* - KJ720575 to KJ720591, KJ720611& KJ720612 (19 sequences), ITS region of *F. udum* - KJ720592 to KJ720610 (19 sequences).

3. HOST RESISTANCE

Three hundred ninety eight entries of pigeonpea and 24 lines of chickpea were screened against wilt. In pigeon pea IPA 383B was highly resistant while another seven lines were resistant (Table 17). Of the 24 lines of chickpea known for their resistance to wilt were further screened in the wilt sick field. Five of them were moderately resistant while the rest of them are resistant.

Table 17. Reaction of pigeon pea and chickpea lines to wilt diseases

Lines	Reaction to wilt
Pigeon pea	
IPA 383B	Highly resistant reaction
GPS-33, BSMR 853, PI397430, Sel, ICP89048, ICP 93012, AWR 74/15 and GPS 30	Resistant
DPPA85-3, DPPA85-13, IPA 38, ICP 7200, IPA2012-2, ICP 9174, ICP 8859, ICP 8858, C11, ICP 8863, PH 1063, IPA 16 F, MAL 19, ICP 8859 and BSMR 843	Moderately resistant
MA 3, MAL 13, BDN 1, BDN2, MAL 13 and Bahar	Susceptible reaction.
Chickpea	
IPC 2005 – 24,IPC 2005 – 54, IPC 2005 – 19,IPC 2005 – 62, IPC 2005 – 18, IPC 2005 – 35, IPC 2005 – 64, IPC 2005 – 46, IPC 2005 – 45,IPC 2005 – 15, IPC 2005 – 34, IPC 2005 – 37, IPC 2005 – 44, IPC 2005 – 3, IPC 2005-41A, IPC 2005 – 41B, IPC 2005 – 30,IPC 2005 – 41A	Resistant
IPC 2005 – 59, IPC 2004 – 34, IPC 2005 – 27, IPC 2005 – 8, IPC 2005 – 26	Moderately resistant

Table 18. Reaction of chickpea lines to six races of *F. oxysporum* f.sp. *ciceri*

Lines	Reaction to chickpea wilt
IPC2004-3 and IPC2004-8	Resistant against all the six races
IPC2005-19, IPC2005-30, IPC2005-41A, IPC2005-41B, IPC2005-52, IPC2007-51, IPC2005-54	Resistant against all the five races
IPC2005-24, IPC2005-27, IPC2005-45, IPC2005-64, IPC2007-04, IPC2010-128, I PC2010-215, IPC2010-78, KGD1253	Resistant against all the four races
IPC2005-44, IPC2005-59, IPC2005-62, IPCK491, IPC2005-43, IPC2005-35, IPC2005-34	Resistant against all the three races
IPC2009-187, IPC2005-18, IPC2009-66	Resistant against all the two races
IPC2005-15 and IPC2007-50	Resistance to only one race

Thirty five chickpea lines were screened against six races of *F. oxysporum* f.sp. *ciceri* and the results are given in Table 18.

4. DISEASE MANAGEMENT

Fifteen isolates of *Trichoderma* supplied by the Project Coordinator, PhytoFuRa were evaluated for their efficiency as bio control agent against chickpea wilt under pot conditions. Six isolates T2, T3, T4, T5, T9 and T12 reduced the wilt by 32-39%. Two isolates T7 and T10 appeared to be least effective as the reduction in wilt was less than 10% whereas seven isolates T1, T6, T8, T11, T13, T14 and T15 reduced wilt around 25%.

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1. BIODIVERSITY

a. Characterization of *Fusarium* isolates based on chitin synthase gene

Chitin synthase gene, which is responsible for virulence in *Fusarium*, was amplified in 19 isolates of *F. solani* and 22 isolates of *F. oxysporum* with specific primers CHS79-F (5-TGG GGC AAG GAT GCI TGG AAG AAG-3) CHS354-R (5-TGG AAG AAC CAT CTG TGA GAC TTG-3). In total 16 isolates of *F. solani* and seven isolates *F. oxysporum* were found positive and yielded 300 bp fragment of CHS gene. The amplified products of CHS gene were eluted, purified and sequenced. The deduced nucleotide sequences were annotated by using Bio-Edit software. Annotated nucleotide sequences were CLUSTAL-W aligned and subjected to BLASTn to compare the other sequences present in database. Based on chitin synthase gene sequences of *Fusarium* (tomato and chilli) and sequences present in database, a phylogenetic tree was constructed (Fig. 59).

b. Study on pathogenic variation in *Fusarium* isolates

A total of 17 isolates of *F. oxysporum* f.sp. *lycopersici* (FOL) were amplified with endo polygalacturonase gene to study the pathogenic variation. Out of 17 isolates of FOL, 13 isolates showed positive with pgl (endo polygalacturonase gene) primer and yielded 1.5 kb amplicons. The amplified endo polygalacturonase gene fragments (~1.5 kb) from different isolates were purified and sent for sequencing.

2. DIAGNOSTICS

a. Multiplex PCR for identification of races in FOL isolates

For identification of race 1 in FOL isolates, universal primer (PGLUNI) and SP 13 were multiplexed in DNA isolated from different FOL isolates, six out of 17 isolates of FOL showed positive reaction indicating that these isolates belong to race I (Fig. 60).

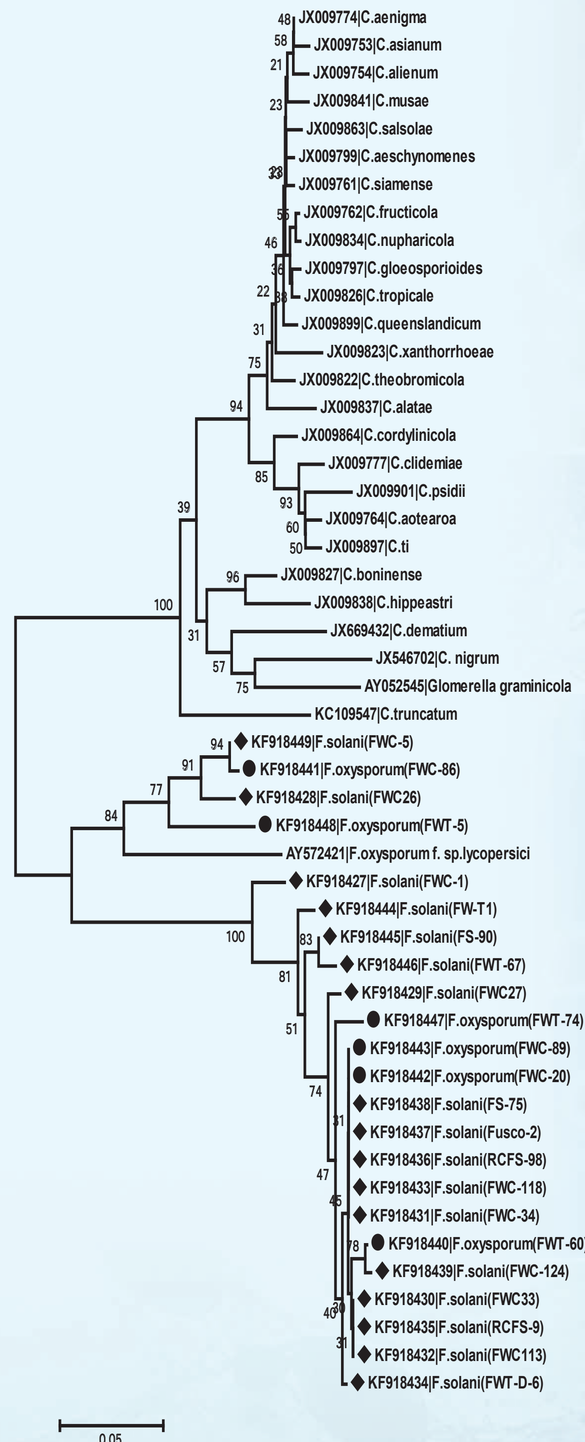


Fig. 59: A neighbour-joining phylogenetic tree obtained from the nucleotide sequences of CHS of *Fusarium* spp. and CHS of other *Fusarium* spp. in database.

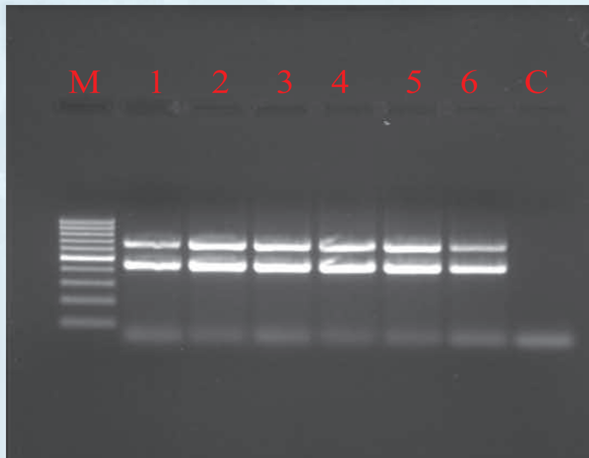


Fig. 60: Multiplexing FOL isolates with uni and sp13 primers. Upper fragment, 670 bp, representing Uni primer and lower fragment, 445 bp, representing SP13 primer. Lane 1, 100 bp DNA ladder, lane 2-7 FOL race -1 isolates and lane 8 –ve control.

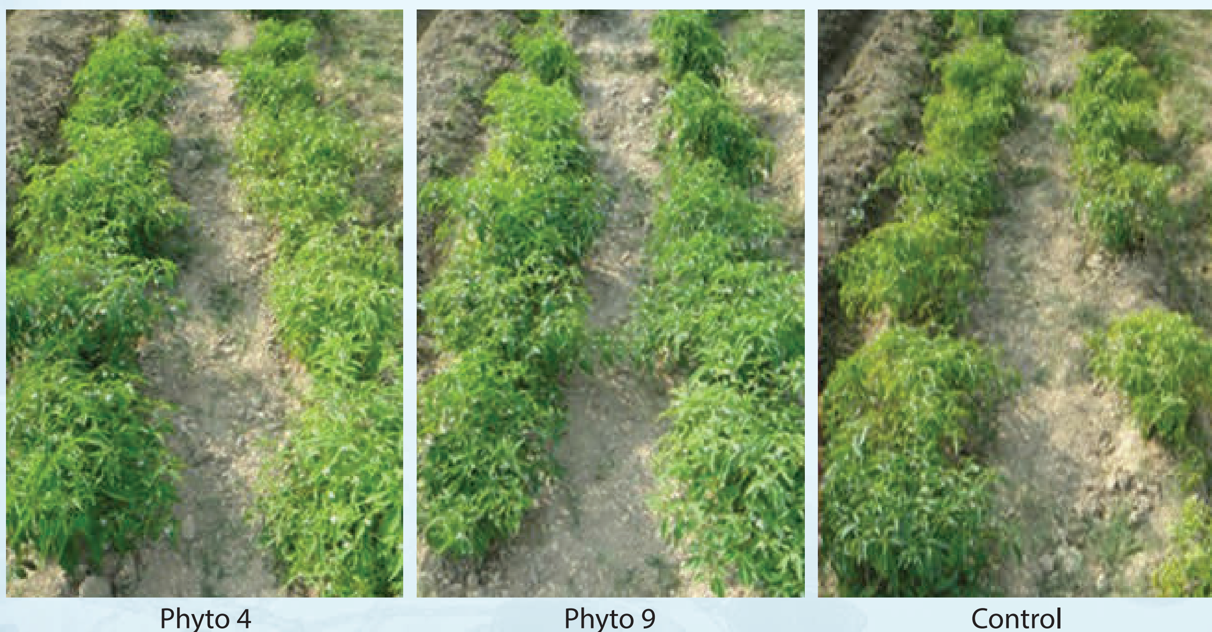
3. DISEASE MANAGEMENT

Talc based formulation of *Trichoderma* isolates (Phyto 1-15), two fungicides and botanicals (Datura and garlic extracts) were evaluated against *Fusarium* wilt of chilli under field conditions. Invariably all the treatments were effective in controlling the *Fusarium* wilt. Among them, Phyto 5 and Phyto 9 showed significantly less incidence of wilt (Table 19; Fig. 61) however, all the biocontrol agents recorded significantly high yield than the control. Similarly, all the isolates showed significant reduction of wilt incidence in tomato but Phyto 13, Phyto 3 and Phyto 1 recorded superiority in reducing the incidence as well as enhancing the yield of the crop (Table 19; Fig. 62)

Table 19. Effect of biocontrol agents, botanicals and chemicals on *Fusarium* wilt and yield of chilli and tomato under field conditions

Treatments	Chilli		Tomato	
	Wilt incidence (%)*	Yield (Q/ha)**	Wilt incidence (%)*	Yield (Q/ha)**
Phtyo-1	23.34	26.38	12.6	196.00
Phyto-2	23.02	22.36	18.8	193.60
Phyto-3	22.46	25.31	10.6	192.40
Phyto-4	16.79	28.47	18.3	180.40
Phyto-5	21.68	24.12	19.6	186.40
Phyto-6	20.65	28.87	14.0	187.73
Phyto-7	26.30	27.98	17.0	185.06
Phyto-8	25.06	23.55	14.0	180.40
Phyto-9	15.19	33.33	17.3	175.73
Phyto-10	22.74	28.33	14.6	178.73
Phyto-11	24.65	24.44	16.3	186.06
Phyto-12	21.26	30.69	13.0	182.06
Phyto-13	20.17	27.36	9.3	197.73
Phyto-14	22.66	25.34	17.6	178.40
Phyto-15	24.34	23.59	13.0	191.73
Carbendazim +mancozeb	22.94	27.33	3.6	178.73
Carbendazim	24.61	21.55	11.3	172.40
Datura extract	20.91	23.13	19.3	171.73
Garlic extract	23.76	21.50	18.6	172.06
Control	32.89	13.33	27.0	152.06
CD (5%)	4.38	7.27	5.6	8.13
CV	13.04	27.77	16.8	21.5

*Values were arc sine transformed before the analysis. Up to four harvest



Phyto 4 Phyto 9 Control

Fig. 61: Efficacy of *Trichoderma* (Phyto 4 and Phyto 9) isolates against *Fusarium* wilt in chilli under field conditions

Fig. 62: Efficacy of *Trichoderma* (Phyto 13) isolate against *Fusarium* wilt in tomato under field conditions

NATIONAL BUREAU OF AGRICULTURALLY IMPORTANT MICROORGANISMS, MAU

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1. BIODIVERSITY

a. Genetic diversity analysis by mating type sequences

PCR assay was performed to assign mating types (MAT1 and MAT2) for 20 different isolates of *F. udum* and *F. oxysporum* f. sp. *ciceri*. A single product was generated by PCR from each Fu and Foc isolates using primer pairs complementary to the alpha domain and HMG domain genes, respectively. A 320 bp portion of the alpha domain (MAT1) was obtained from isolates Fu1, Fu2, Fu3, Fu4, Fu5, Fu8, Fu11, Fu12, Fu15, Fu18, Fu19 and Fu20. Similarly, a 650 bp portion of HMG domain (MAT2) was detected in isolate Fu6, Fu7, Fu9, Fu10, Fu13, Fu14, Fu16 and Fu17. Similar trend was obtained with the isolates of Foc (Fig. 63). The presence of MAT-1 was detected in 12 isolates of FOC, while rest of the isolates showed the presence of MAT-2 gene.

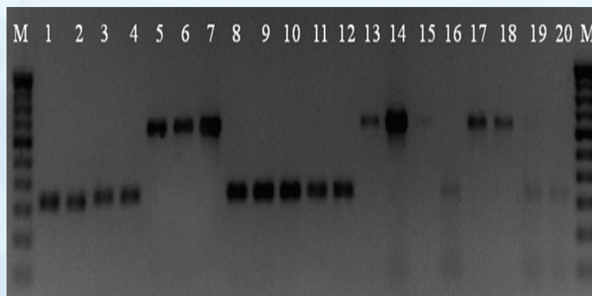

Fig. 63: PCR amplification of a MAT1 (320-bp) and MAT2 (650 bp) gene in *F. oxysporum* f. sp. *ciceri* isolates representing distinct geographical lineages. Lanes 1–20 are different Foc isolates. M is a 100-bp DNA marker.

b. Genetic diversity analysis by RAPD-PCR analysis

Among the ten RAPD primers, three primers *viz.*, OPA-2, OPA-3 and OPA-11 were chosen based on their capacity to reveal polymorphisms among isolates. These primers produced a total of 83 fragments among all the 20 isolates, giving a ratio of three polymporphic bands/primer. The size of RAPD fragments ranged from 300 to 1600 bp. RAPD analysis of genomic DNA from the pathogenic isolates revealed the presence of thirteen clusters at the arbitrary level of 50% similarity. Maximum isolates were clustered in group I (Fu1, Fu2, Fu3 and Fu4) followed by group II (Fu19 and Fu 20), III (Fu 15 and Fu 18), IV (Fu6 and Fu7) and IX (Fu 13 and Fu16). In case of Foc isolates, ten clusters at the arbitrary level of 50% similarity were obtained (Fig. 64). Maximum eight isolates were grouped in cluster III.

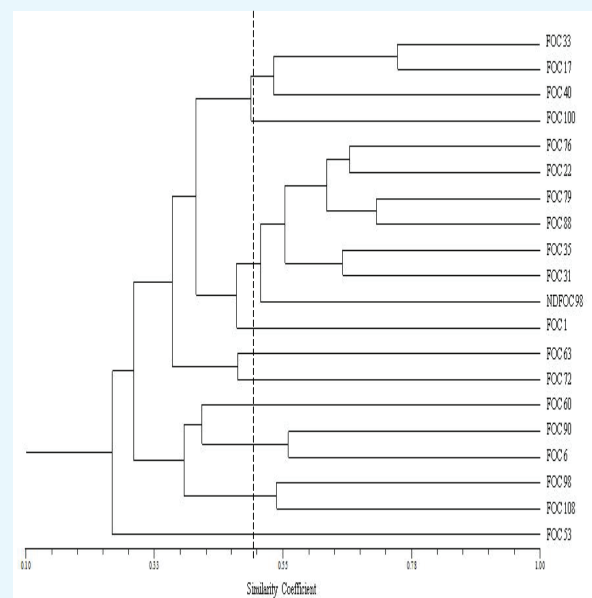

Fig. 64: Dendrogram based on banding pattern of *F. oxysporum* f. sp. *ciceri* isolates obtained from RAPD markers.

c. Genetic diversity analysis by ERIC-PCR analysis

The genetic variability among the 20 *Fusarium* isolates was assessed using ERIC-PCR and a high level of polymorphism in the banding pattern was obtained. The number of bands in the amplification profile was 188, and their size was found to vary from 150-3000 bp among these isolates. One band of approximately 500 bp amplicon size was present in all isolates. Cluster analysis based on the Jaccard's similarity coefficient (50%) showed that the isolates were divided into eight groups and giving a ratio of eight bands/isolate. Similarly, in case of Foc isolates, cluster analysis based on the the Jaccard's similarity coefficient (50%) showed that the isolates were divided into 14 clusters (Fig. 65).

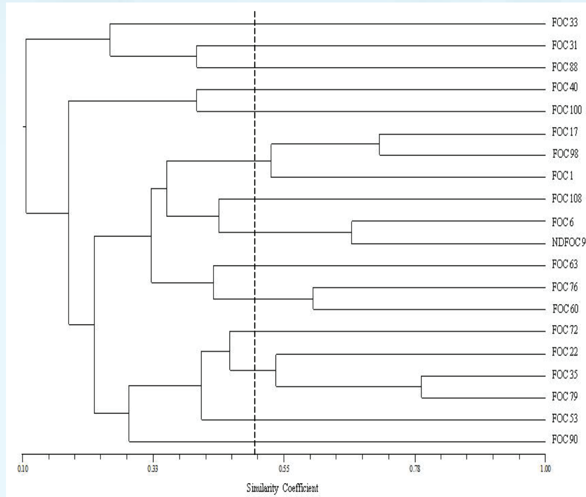

Fig. 65: UPGMA dendrogram showing genotypic diversity among the 20 Foc isolates from ERIC-PCR fingerprinting

d. Genetic diversity analysis by BOX-PCR analysis

Analysis of BOX-PCR banding pattern showed that the Fu isolates were clustered into five clusters, sharing 50-100% similarity. The banding pattern showed a total of 246 fragments in the range of 200-4000 bp, giving a ratio of five polymporphic bands/isolate. A perusal of the dendrogram revealed that the 13 isolates (Fu1, Fu2, Fu3, Fu4, Fu5, Fu7, Fu9, Fu10, Fu13, Fu14, Fu15, Fu16 and Fu17) have formed a major cluster (Cluster I), while only three (Fu18, Fu19 and Fu20) and two (Fu11 and Fu12) isolates were grouped in the third (cluster III) and fourth cluster (cluster IV), respectively. In case of Foc isolates, genotypic diversity analysis by BOX-PCR resulted in the formation of nine distinct clusters. In the cluster, a total of eight isolates were grouped.

NATIONAL RESEARCH CENTRE FOR BANANA, TIRUCHIRAPALLI

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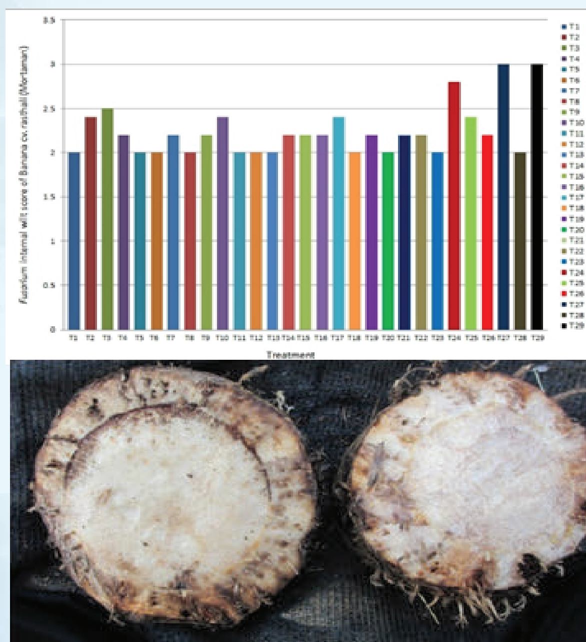
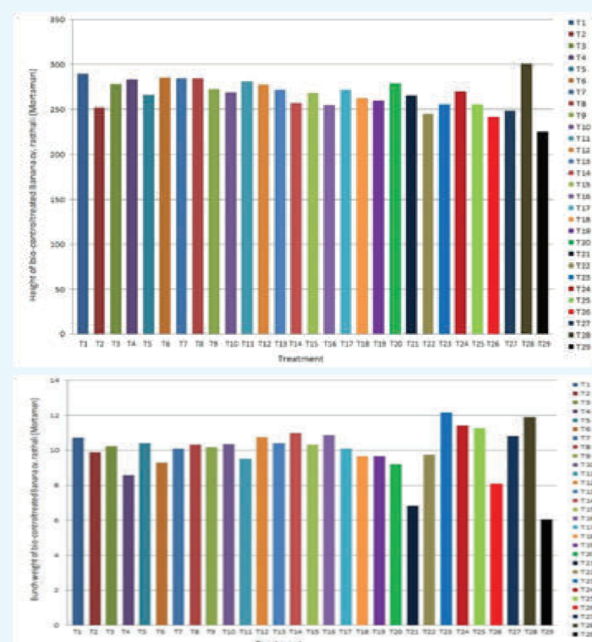
1. DISEASE MANAGEMENT

a. Field evaluation of biocontrol agents and a botanical

On cv. Rasthali (Mortaman)-AAB: Field evaluation of different effective biocontrol agents *viz*., wild endo *T. harzianum* (prr2), wild endo *P. pinophilum* (Bc2), wild endo *Penicillium* sp. (Dsr1), mutant endo *T. harzianum* (prr2), mutant endo *P. pinophilum* (Bc2) and mutant endo *Penicillium* sp. (Dsr1) individually as well as in





Treatment			
No.	Description	No.	Description
T1	Wild endo. *T. asperellum* (prr2)	T16	Endo. *Bacillus flexus* (Tvpr1) + rhizo. *Bacilluscereus* (Jrb1)
T2	Wild endo. *P. pinophilum* (Bc2)	T17	Endo. *Bacillus flexus* (Tvpr1) + rhizo. *Pseudomonas putida* (Jrb2)
T3	Wild endo. *Penicillium* sp .(Dsr1)	T18	*Trichoderma* sp. (SRT3)
T4	Mut. endo *T. asperellum* (prr2)	T19	*Trichoderma* sp. (SRT3)
T5	Mut. endo. *P. pino* (Bc2)	T20	*Trichoderma* sp. (KR2)
T6	Mut. endo. *Peni.* sp (Dsr1)	T21	*Trichoderma* sp.(KR4)
T7	Wild en. *T. asperellum.* (prr2) + Wild rhizo *T. longibr*	T22	*Trichoderma* sp. (KR8)
T8	Wild. *P. pino* (Bc2) + Wild rhizo. *T. aspe*	T23	Mut. endo. *T. asperellum* (prr2) + Mut. rhizo. *T. longibra.* + Difena
T9	Wild *Penicillium* sp. (Dsr1) + Wild rhizo *T. asperellum*	T24	Mut. endo. *P. pinophilum* (Bc2)+ Mut. rhizo. *T. aspere* + Difena
T10	Mut. endo. *T. asperellum* (prr2)+ Mut. rhizo .*T. longibrachiatum*	T25	Mut. endo. *Penicillium* sp. (Dsr1) + Mut. rhizo. *T. aspere.* + Difena
T11	Mut. endo. *P. pinophilum* (Bc2) + Mut. rhizo. *T. asperellum*	T26	Difenaconazole (0.1) % alone
T12	Mut. endo. *Penicillium* sp. (Dsr1) + Mut. rhizo. *T. asperellum*	T27	Carbendazim (0.1)% alone
T13	Endo. *P. putida* (C4r4) + rhizo. *Bacillus cereus* (Jrb1)	T28	Drenching of zimmu leaf extract
T14	Endo. *Achromobacter* sp. (Gcr1) + rhizo. *B. cereus* (Jrb5)	T29	Control (infected suckers)
T15	Endo. *hizobium* sp. (Lpr2) + rhizo. *Bacillus cereus*		

Fig. 66: Field evaluation of bio-control agents of banana cv. Andhra Rasthali (Mortaman) pre-infected with Foc. (Clockwise from top left) Internal wilt score; plant height; bunch weight; and effect of *P. pinophilum* (Bc2) + *T. asperellum* + Difenaconazole treatment on internal vascular discoloration.

different combinations was carried out for the suppression of *Fusarium* wilt disease in banana plants cv. Mortaman already infected with *Fusarium* wilt. The bioagents were given in liquid form @ 50 ml/ plant for four times at monthly intervall from the planting. Totally there were 29 treatments with five replications per treatment. Observations on plant height, girth, total number of leaves, leaf area, total number of hands, number of fingers and bunch weight, number of plants harvested and internal vascular discoloration in the corm were taken at the harvest phase.

The results showed that among the bioagents, the maximum reduction of *Fusarium* wilt disease (disease score 2 in the 1-6 disease scale) was recorded in the banana plants treated with endophytic *T. harzianum*, endo *P. pinophilum*, wild *P. pinophilum* + rhizo *T. asperellum*, endo. mut. *P. pinophilum* + rhizo. mut. *T. asperellum*, endo *P. putida*+ rhizo *Bacillus* spp., endo. mut. *Penicillium* spp. + rhizo. mut. *T. asperellum*, *Trichoderma* sp. SrT3, *Trichoderma* sp. Kr2, endo *T. harzianum* + rhizo. mut *T. longibrachiatum* + Difenoconazole (0.1%) (Fig. 6 6). Besides, the results also showed that the percentage of plants came for harvest was 100% in the above said treatments whereas in the control plants it was only 35%.

Besides, the above-mentioned bio-agents also increased the plant height (6.8 to 28.4%), girth (2.6 to 27.3%), total number of leaves (5 to 38.9%), leaf area (7.3 to 43.4%), total number of hands (up to 16.6%), number of fingers (up to 30.8%), bunch weight (41 to 101%) (Fig. 66) significantly when compared to untreated control plants.

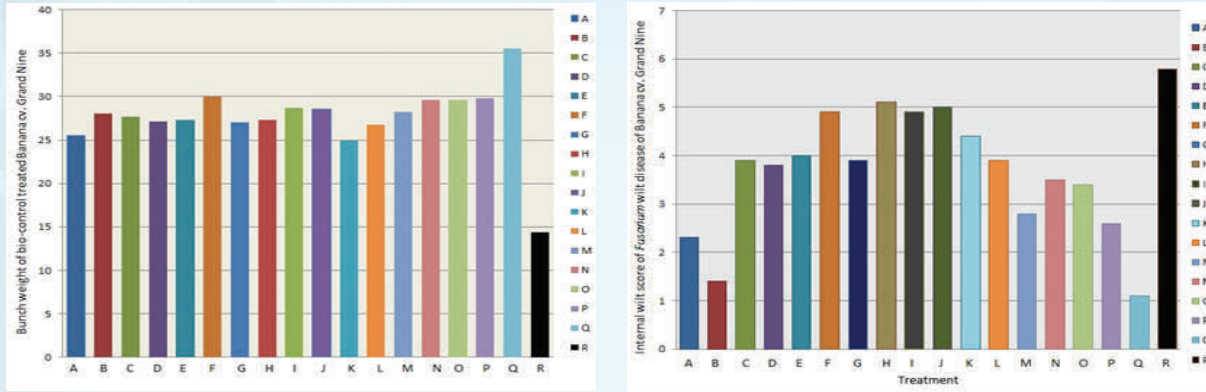
On cv. Grand Naine –AAA: A field trial conducted in a *Fusarium* wilt hot spot area at Muthalapuram in Chinnamanur taluk of Theni District to evaluate the field suppression of *Fusarium* wilt disease by the endophytic and rhizospheric fungal and bacterial isolates and a botanical. The cv. Grand Naine indicated that combined application of fungal endophytic *P. pinophilum* Bc2 + rhizospheric *T. koningii*, and liquid formulation containing endophytic *T. harzianum* Prr2 + *Bacillus flexus* Tvpr1 and single endophytic *P. pinophilum* Bc2 and Zimmu leaf extract (50% conc) in the soil have recorded maximum reduction of internal wilt disease score of 1.1, 1.2 and 1.4 and 1.5, respectively as against 5.8 in the control plants on a disease scale of 1-6 where 1 is healthy and 6 is 100% infected or dead (Fig. 67). Besides, these treatments also increased various plant growth and yield parameters such as plant height (up to 47.7%), girth (up to 49.2%), total number of leaves (up to 80.3%), leaf area (up to 95.4%) and number of hands (up to 87%), number of fingers (up to 75.4%) and bunch weight (up to 170.5%) significantly compared to control plants. Interestingly, a total of 11 different treatments recorded 100% harvest of the good bunches compared to only 35% harvest in the control plants.

2. GENOMICS

a. Transcriptomic analysis of gene expression due to the interaction of Foc pathogen and effective bio-control agents in banana

To identify the differentially expressed genes due to the interaction of Foc pathogen and effective bio-control agent in banana, Supressive Subtractive Hybridization (SSH) was carried out in cv. Grand Naine. Total RNA was isolated from the roots of Foc alone-inoculated control (Driver) and Foc + *T. harzianum* inoculated (Tester) banana plants. The total RNA concentration of Tester and Driver RNA was 103.7 µg/µl and 106.8 µg/µl, respectively. From the above said total RNA, mRNA was isolated and the concentration of Tester and Driver mRNA was 0.5 µg/µl and 0.9 µg/µl, respectively. The mRNA of these samples was immediately converted into their respective cDNAs and subjected further to SSH analysis. Finally the resultant SSH products were run in 1.8% agarose gel and the PCR products of subtracted cDNA was ranged from 250 bp to 1000 bp whereas the size of the unsubtracted cDNA was ranged from 400 bp to 1000 bp.

The subtracted cDNA was subjected to cloning using INSTA cloning kit (Fermentas) and transformed using One shot Omnimax 2T1-phage resistant cells. A total of about 808 clones were obtained and plasmid DNA was isolated from all these clones and double digested with ECORI and Pst1 enzymes for checking the presence of inserts (Fig. 68).



A	Mut.Endo. *T. asperellumI*(Prr2)	J	Endo. *Bacillus* sp. (Enbr1) + Endo. *Burkholderia* sp. (GcTcr1)
B	Endo. *P. pinophilum* (Bc2)	K	Endo. *Bacillus* sp. (Enbr1) + Endo. *Bacillus* sp. (Enbr1)
C	Mut. Endo. *Penicillium* sp. (Dsr1)	L	Endo. *Bacillus* sp. (Enbr1) + Endo. *Bacillus* sp. (GcTc2)
D	Mut. Rhi. *Trichoderma* sp.	M	Zimmu alone
E	Mut. Rhi. *T. asperellum*	N	Endo. *Pseudomonas putida* (C4r4) + Zimmu leaf extract
F	Mut. Rhi. *T. viride* (140c)	O	Endo. *Achromobacter* sp. (Gcr1) + Zimmu leaf extract
G	Endo. *Lysinibacillus* (Dsr1)+ Endo. *Bacillus* sp. (Enbr1)	P	Endo. *Bacillus flexus* (Tvpr1)+Zimmu leaf extract
H	Endo. *Lysinibacillus* (Dsr1) + Endo. *Ochrobactrum* sp. (pjr1)	Q	Drenching of liquid formulation containing *T. harzianum* (Pvr2) + *B. flexus* (Tvpr1)
I	Endo. *Burkholderia* sp. (GcTcr1) + Endo. *Ochrobactrum* sp. (pjr1)	R	Control

Fig. 67: Effect of soil drenching of liquid formulation containing combination of *Trichoderma* sp. + *Bacillus cereus* in banana cv. Grand Naine under field conditions. (Left) on the bunch yield and (Right) on the suppression of *Fusarium* wilt disease

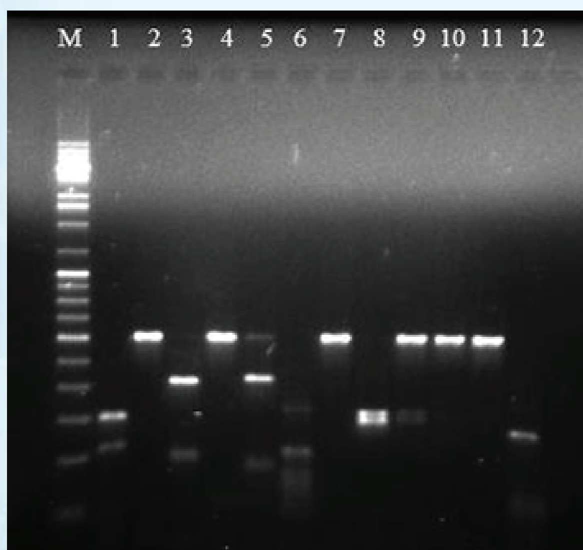


Fig. 68: Restricted products of substracted cDNA clones. M-Medium range ruler (Genei), 1-12-restricted Plasmid DNA obtained from clones of Subracted cDNA

b. Characterization of Foc pathogen affecting Grand Naine in Theni District of Tamil Nadu by VCG analysis

Recently as we have noticed the incidence of *Fusarium* wilt disease in Cavendish group of banana particularly in cv. Grand Naine which is normally infected by VCGs 01213/01216 of tropical race 4 Foc pathogen and also by VCGs 0120, 0126 etc., of subtropical race 4, the samples from different banana growing villages of Theni District were collected and the pure culture of the Foc pathogen was isolated and characterized by VCG analysis using the nit-M testers obtained from Australia. The results indicated that the Foc infecting Cavendish belong to VCG 0124 and 0125 of race 1 in Theni District of Tamil Nadu (Fig. 69). Earlier the Foc pathogen infecting Cavendish was identified as VCG 0124 and now VCG 0125 is also identified besides some unknown VCGs, which require further analysis, by molecular method.

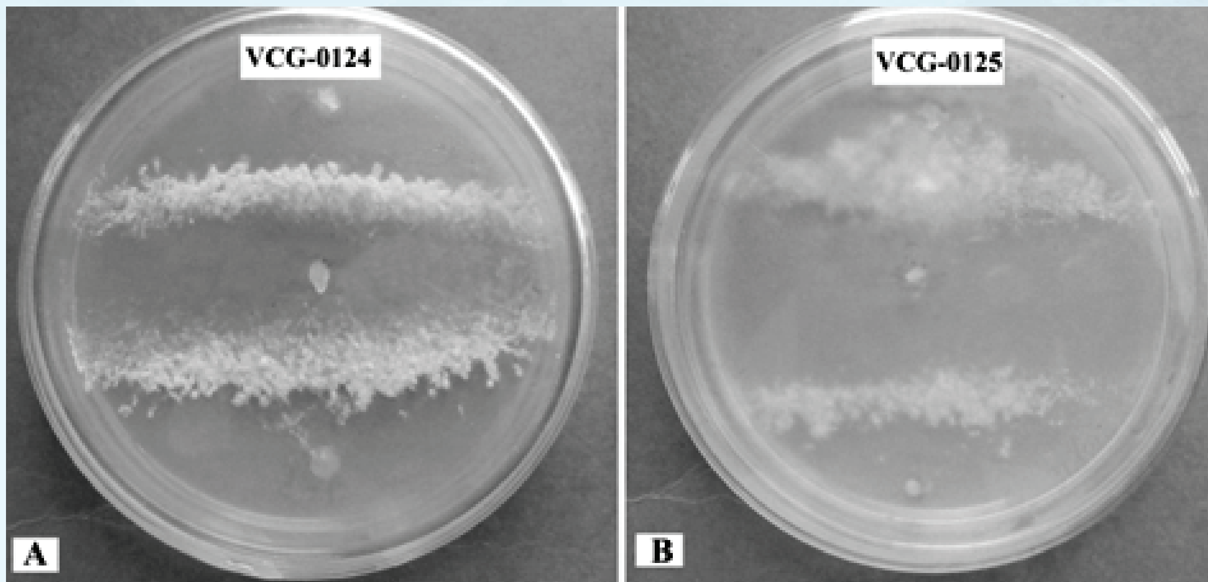
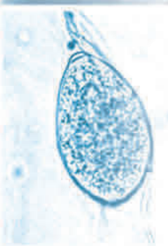


Fig. 69: VCG analysis of Foc infecting banana cv. Grand Naine in Theni District of Tamil Nadu. A. VCG-0124 and B. VCG-0125



PhytofuRa

Ralstonia

INDIAN INSTITUTE OF SPICES RESEARCH, KOZHIKODE

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1. BIO DIVERSITY

Twenty new isolates of *Ralstonia solanacearum* were collected from different ginger growing areas of Kerala and Karnataka which included two isolates from small cardamom and one from tomato. The isolates were characterized for biovar, pathogenicity and virulence. Pathogenicity of all the isolates was confirmed by inoculation on respective host plants (Table 20).

Table 20. Details of pathogenicity studies of *Raistonia solanacearum* isolates.

Isolate name	Host plant	Place of collection	Pathogenicity on ginger	Days taken for wilting	Biovar
GRs Mep 2	Ginger	Meppadi, Waynad	+	23	3
GRs Mep 3	Ginger	Meppadi, Waynad	+	8	3
GRs Mep 4	Ginger	Meppadi, Waynad	+	7	3
CaRs Mep 3	Small cardamom	Meppadi, Waynad	+	7	3
GRs Mnt 5	Ginger	Manathavadi, Waynad	+	11	3
GRs Mnt 6	Ginger	Manathavadi, Waynad	+	12	3
GRs Mnt 7	Ginger	Manathavadi, Waynad	+	11	3
GRs Idk 1	Ginger	Adimali, Idukki	+	7	3
GRs Idk 2	Ginger	Kumali, Idukki	+	17	3
GRs Spr	Ginger	Kerodi, Sakleshpur	+	9	3
TRs Klm	Tomato	Kayamkulam, Kollam	-	No wilting	3
GRs And	Ginger	Andoor, Waynad	+	11	3
GRs Pul 3	Ginger	Pulpally, Waynad	+	12	3
GRs Sik	Ginger	Sikkim	+	7	3
GRs Mnt	Ginger	Manathavadi, Waynad	+	15	3
GRs Mnt 2	Ginger	Manathavadi, Waynad	+	10	3
GRs Pkd	Ginger	Palakkad	+	14	3
GRs Tms 2	Ginger	Thamarassery, Calicut	+	17	3
GRs Tly	Ginger	Thirunelli, Waynad	+	8	3
CaRs Mep	Small cardamom	Meppadi, Waynad	+	8	3

2. HOST RESISTANCE

a. Comparison of the transcriptomes of ginger (*Zingiber officinale* Rosc.) and mango ginger (*Curcuma amada* Roxb.) in response to the bacterial wilt infection

To determine the effect of the infection by the *R. solanacearum* on gene expression in *C. amada* and *Z. officinale*, both the transcriptomes were compared. A total of 20,938 *C. amada* and 20,061 *Z. officinale* genes were expressed during infection. The differential expression analysis was performed using either RPKM or count data. Based on three fold change and FDR P value <0.005 a total 1201 genes have been identified as differentially expressed, out of which 587 genes are upregulated and 613 genes are down regulated. Further the up regulated genes were classified into functional categories related to defense response, pathways and molecular function with respect to bacterial infection. A singular enrichment analysis of GO terms also revealed that defense related GO terms are significantly enriched at $P<0.005$. Among the 54 differentially expressed transcription factors, 34 are up regulated in *C. amada* which includes WRKY, MYB, leucine zipper protein, zinc finger and GATA domain transcription factors (Table 21).

Table 21. Summary of transcription factor unigenes of *C. amada* and *Z. officinale*

Transcription factor family	Number of genes detected	Up-regulated in *C. amada*	Up-regulated in *Z. officinale*
WRKY	8	4	4
MYB	6	4	2
AP2/ERF	2	2	-
MYC	1	1	-
GRAS	1	1	8
Zinc finger	17	9	1
bHLH	1	-	1
bZIP	3	2	4
Others	15	11	16
Total	54	34	20

Genes involved in mevalonate pathway (MEP) for biosynthesis of isoprene/terpenes have been found to be upregulated substantially in *C. amada* compared to *Z. officinale* (Table 22).

Table 22. List of upregulated isoprene/terpene biosynthesis genes in *C. amada*

Protein name	Fold change	GO Functions
Fructose-bisphosphate aldolase (EC 4.1.2.13)	3901.00	GO:0006098 pentose-phosphate shunt , GO:0015976 carbon utilization
1-D-deoxyxylulose 5-phosphate synthase	10.36	GO:0016114 terpenoid biosynthetic process, GO:0006694 steroid biosynthetic process
4-diphosphocytidyl-2-C-methyl-D-erythritol kinase	34.25	GO:0006694 steroid biosynthetic process
1-deoxy-D-xylulose 5-phosphate reductoisomerase	17.50	GO:0016114 terpenoid biosynthetic process, GO:0019288 isopentenyl diphosphate biosynthetic process, mevalonate-independent pathway
4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	27.90	GO:0009862 systemic acquired resistance, salicylic acid mediated signaling pathway GO:0019288 isopentenyl diphosphate biosynthetic process, mevalonate-independent pathway GO:0009617 response to bacterium
terpene synthase activity	3.03	GO:0000287 magnesium ion binding GO:0010333 terpene synthase activity
2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	1.87	GO:0016114 terpenoid biosynthetic process
1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase	2.55	GO:0016114 terpenoid biosynthetic process GO:0019288 isopentenyl diphosphate biosynthetic process, mevalonate-independent pathway

3. DISEASE MANAGEMENT

a. Study on the non-conventional disease management strategies in ginger

A replicated trial using 20-25 g seed rhizomes with four treatments was laid out to assess single bud sprout technique of transplanting in ginger, as part of non-conventional disease management strategy in ginger. Fresh yield was on par in both single sprout transplanted and direct planting methods.

b. Evaluation of apoplastic bacteria against *R. solanacearum* infecting ginger

Bacteria (150 numbers) were isolated from the apoplastic fluid of pseudostem and leaves of ginger collected from different areas and different ginger accessions. The *in vitro* and *in planta* evaluation against *R. solanacearum* biovar 3 of ginger towards biocontrol potential showed six isolates namely, IISR GAB 24, IISR GAB 42, IISR GAB 43, IISR GAB 48 IISR GAB 107, and IISR GAB 146 as effective in suppressing bacterial wilt in ginger (Fig. 70).

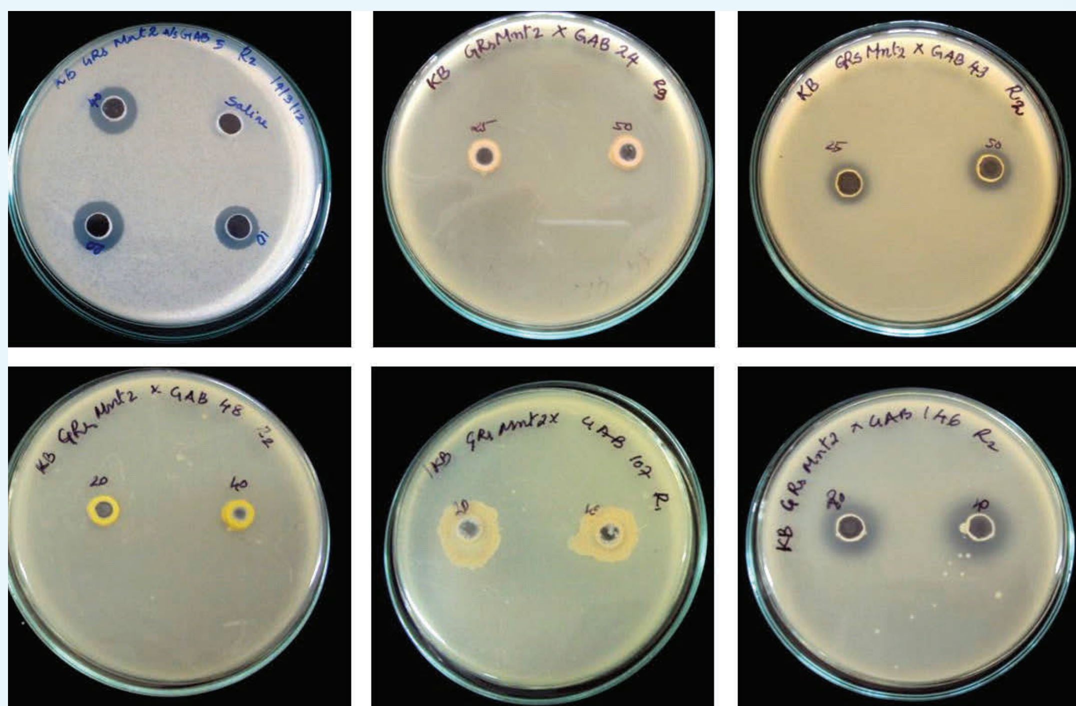

Fig. 70: Plates showing suppressive effect of apoplastic bacteria on *R. solanacearum* by agar well diffusion test.

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1. BIO DIVERSITY

One hundred and fourteen isolates of *R. solanacearum* were isolated from symptomatic plants/ tubers from Khasi hills of Meghalaya and biovar characterization and phylotyping (Table 23) were done.

Table 23. Characterization of *R. solanacearum* isolates into biovars based on the ability to utilize disaccharides and oxidize hexose alcohols

District	Number of isolates						Total
	Biovar						
	1	2	2T	3	4	5	
East Khas i hills (%)	0	20 (29.9%)	44 (65.6%)	03 (4.5%)	0	0	67
West Khasi hills (%)	0	21 (44.7%)	23 (48.9%)	03 (06.4%)	0	0	47
Total no of strains (%)	0	41 (36.0%)	67 (58.8%)	06 (5.2%)			114

Table 24. Phylotype distribution of *R. solanacearum* isolates from Meghalaya based upon DNA typing using phylotype specific multiplex–polymerase chain reaction (Pmx-PCR)

District	No. of strains					Total
	Phylo-type I	Phylotype II Subcluster/sequevar		Phylo-type III	Phylo-type IV	
		A/non-R3bv2	B/ R3bv2			
East Khasi hills (%)	03 (4.5%)	0	20 (29.8%)	0	44 (65.7%)	67
West Khasi hills (%)	03 (6.4%)	0	21 (44.7%)	0	23 (48.9%)	47
Total no. of strains (%)	06 (5.2%)	0	41 (36.0%)	0	67 (58.8%)	114

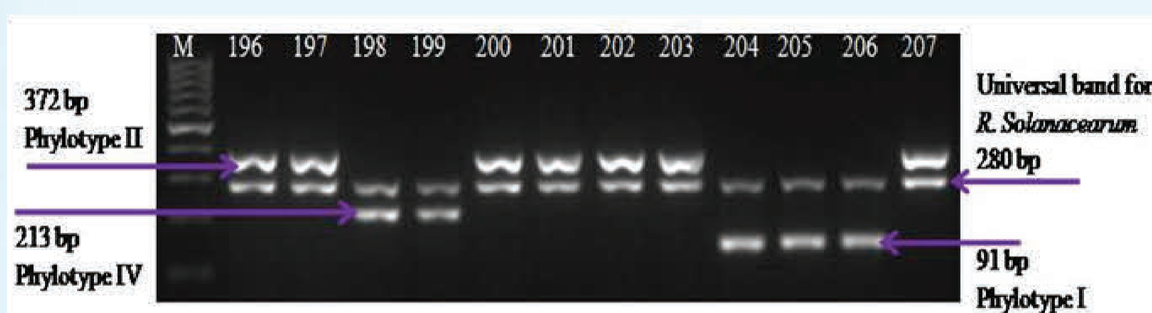

Fig. 71: PCR based identification and phylotype affiliation of representative *R. solanacearum* strains from Meghalaya

Phylogenetic analysis using *egl* gene

PCR amplifications of a 750- bp region of the *egl* gene were performed using the primer pair Endo-F and Endo-R (Fig. 71). Phylogenetic analysis of *egl* gene sequences of 114 *R. solnacearum* isolates from East Khasi hills and West Khasi hills districts of Meghalaya clustered all 41 phylotype II strains with reference strain IPO1609 (phylotype II-sequevar 1), three phylotype I strains with reference strain GMI8254 (phylotype I- sequevar 47), three phylotype I strains with reference strain MAFF211479 (phylotype I- sequevar 45) and 59 phylotype IV strains closely clustered with references strain MAFF301558 (phylotype IV- sequevar 8). However, eight phylotype IV strains did not cluster with any known sequevar reference strain (Fig. 72).

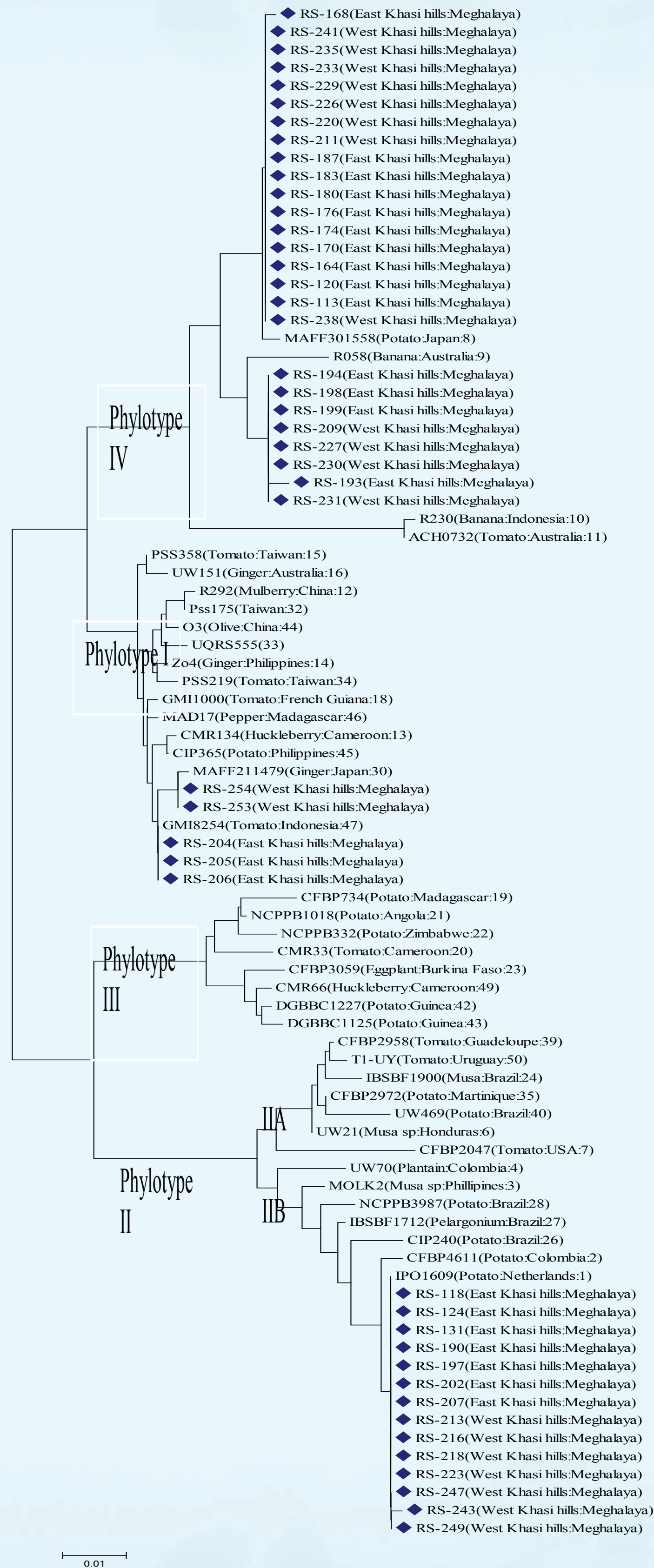

Fig. 72: Phylogenetic neighbour-joining tree based on the partial egl gene sequences of strains from Meghalaya (NEH region of India) (blue circles) and *Ralstonia solanacearum* species complex reference strains, generated using MEGA-5 software. The number at each node is the bootstrap value (1000 replications). Scale bar represents 1 nucleotide substitution per 100 nucleotides. The phylotype branch includes representative strains from each location out of 114 strains.

PhytoFuRa
AN ICAR NETWORK PROJECT ON Phytophthora, Fusarium & Ralstonia

INDIAN AGRICULTURAL RESEARCH INSTITUTE, NEW DELHI

Principal Investigator:
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Co-investigator:
Dr. K K Mondal

1. BIO DIVERSITY

Twelve new isolates of *R. solanacearum* were collected from bacterial wilt affected tomato and potato plants of Uttarakhand State in 2013. Total 171 isolates of *R. solancearum* were preserved from tomato, brinjal, chilli, capsicum.

2. HOST-RESISTANCE

a. Expression of defense related gene (s) in tomato cultivars against *Ralstonia solanacearum*

Expression levels of PIN2, LOXA genes (jasmonic acid pathway), PR-1b, Osmotin like (ethylene pathway) and GluA (salicylic pathway) were analyzed by using real time PCR in response to pathogen cell densities. Expression of genes using resistant (HWAII-7996) and susceptible (Pusa Ruby) cultivars of tomato against the *R. solanacearum* strain UTT-25 were assessed after treatments with chemical inducers of defense like jasmonic acid and salicylic acid with and without *Bacillus subtilis* prior to inoculation of *R. solanacearum* to determine the up regulation and down regulation process in respective genes. Tomato seedlings at 2-3 leaves stage were drenched with jasmonic acid 100 µM and salicylic acid 100 µM with and without *B. subtilis* (OD at 600 nm = 0.1) antagonist were poured at root zone of the plants prior to inoculation of *R. solanacearum* inoculation (10^9 cfu/mL) alone and in combinations. Un-inoculated plant samples were used as a control. Eight different treatment (T1- Un-inoculated plant sample, T2- *R. solanacearum*, T3- *B. subtilis*, T4- *R. solanacearum* + *B. subtilis*, T5- Salicylic acid + *R. solanacearum*, T6- Salicylic acid + *R. solanacearum* + *B. subtilis*, T7- Jasmonic acid + *R. solanacearum*, T8- Jasmonic acid + *R. solanacearum* + *B. subtilis*).

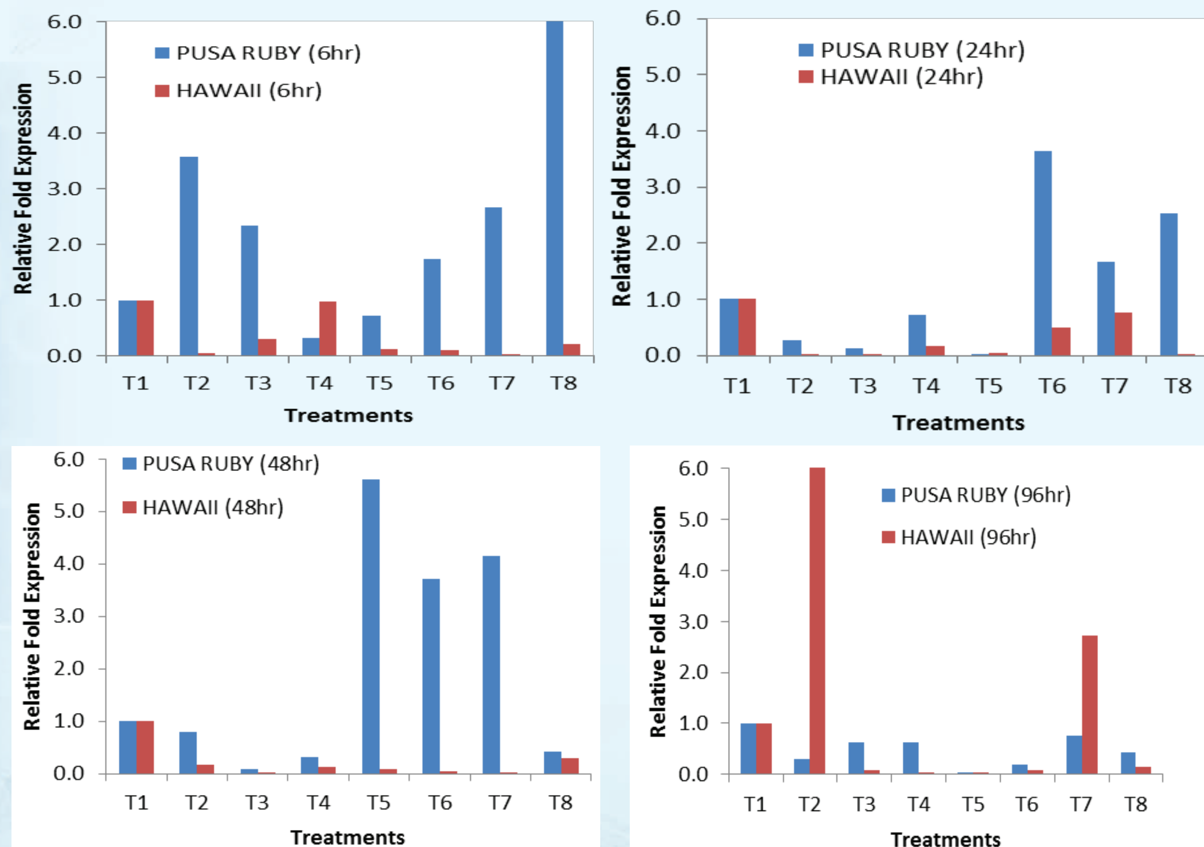


Fig. 74: Expression of LOX A gene in Pusa Ruby (Susceptible) and Hawaii 7996 (Resistant) cvs. of tomato at different intervals using RT- PCR.

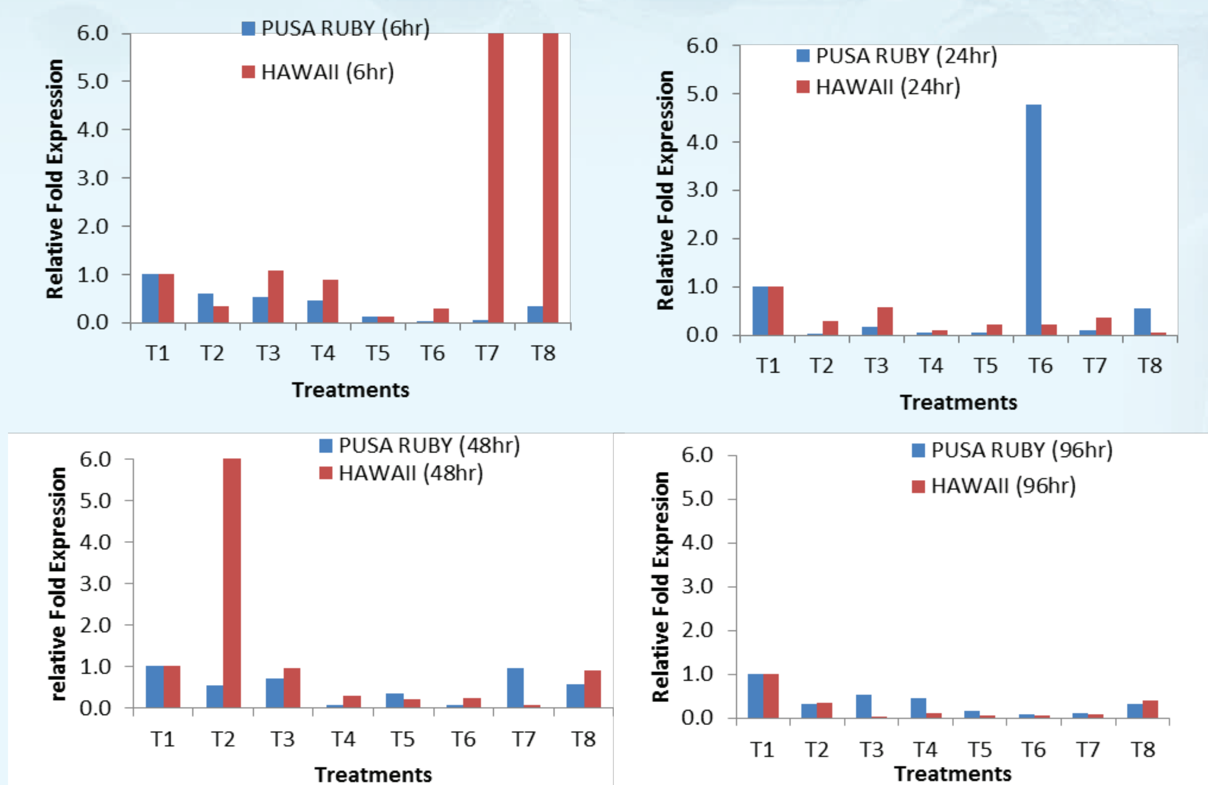


Fig. 74: Expression of LOX A gene in Pusa Ruby (Susceptible) and Hawaii 7996 (Resistant) cvs. of tomato at different intervals using RT- PCR.

PR1a gene was upregulated in susceptible cultivar in all eight treatments at different time intervals compared to resistant cultivar (Fig. 73). Expression levels of PR-1b gene in control plant showed more expression of PR-1b gene as compared to inoculated. The relative expression of PR-1b protein under different treatments showed difference in susceptible cultivar, whereas in resistant cultivar Hawaii 7996, it was down-regulated in all the eight treatments at different time intervals. The expression of Glucanase A gene had up-regulation in susceptible in all the treatments and down-regulation in T3, T5 & T7. The resistant cultivar Hawaii-7996 had high expression in T3 & T5. LOX A gene showed down regulation in all the treatments except T5, whereas the resistant cultivar (Hawaii 7996) recorded high expression in all except T5 (Fig. 74).

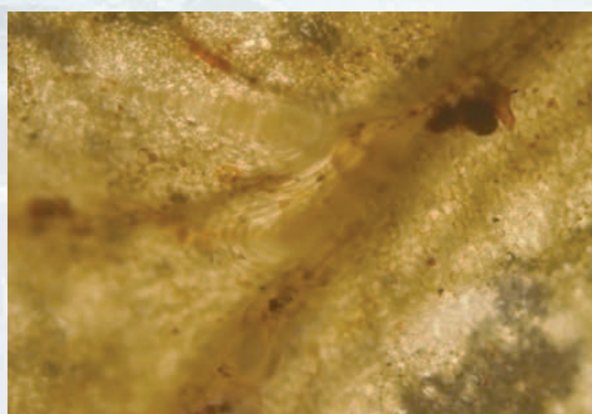
b. ROS detection in tomato leaves and stems tissue

Accumulation of reactive oxygen species (ROS) in tomato leaves and stems tissues was determined in susceptible (Pusa Ruby) and resistant (Hawaii 7996) tomato plants after infection with salicylic acid, jasmonic acid and *Bacillus subtilis* either alone or combinations prior to pathogen *R. solanacearum* inoculation (10^9 cfu/mL) along with water (control). Stem sections of 1 mm thick and 5 mm diameter and the whole leaf were taken, stained with 3,39- diaminobenzidine (DAB, Sigma, USA) and incubated overnight under dark. Oxidation of DAB by ROS created a visible brown precipitate in the host tissue. Resistant cultivar showed more appearance of ROS activity after 24 h of inoculation, while, less production of ROS was found in untreated with pathogen as control (Fig. 75 & 76; Table 24).

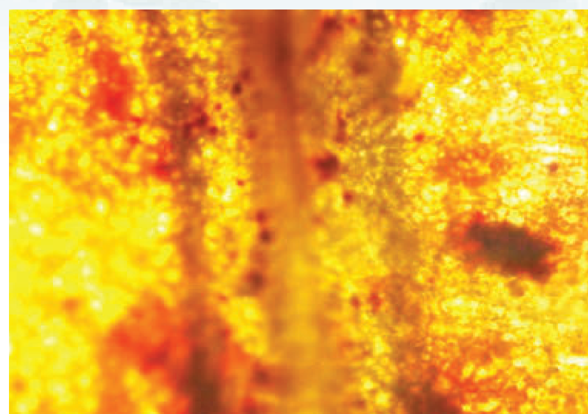
Pusa Ruby (Susceptible)

Hawaii 7996 (Resistant)

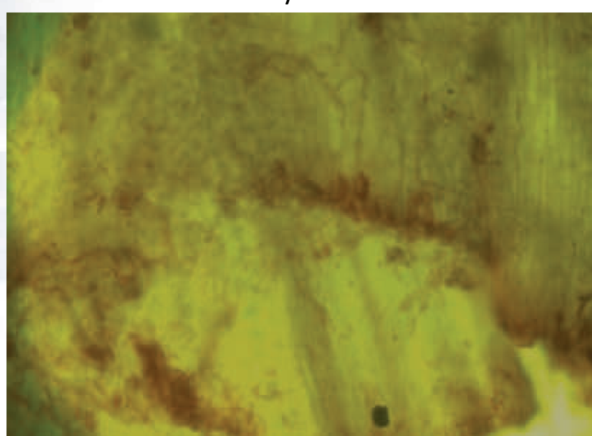
Fig 75: ROS accumulation in leaves of resistant and susceptible cultivars of tomato treated with chemical elicitors and *Bacillus subtilis* against *R. solanacearum*.



Pusa Ruby



Hawaii-7996



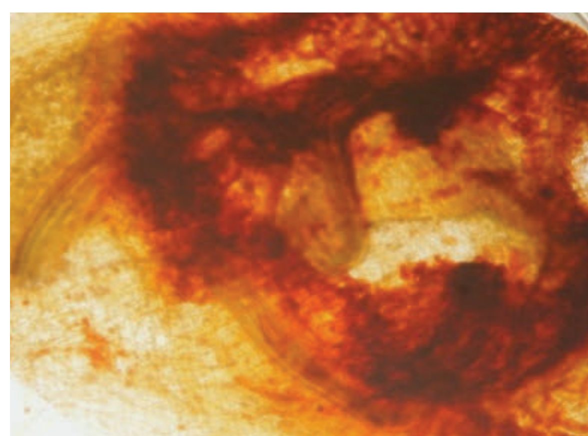


Fig. 76: ROS accumulation in leaf and stem tissues in resistant and susceptible cultivars of tomato treated with Jasmonic acid and *Bacillus subtilis* against *R. solanacearum* under microscope (10 X).

Table. 25. Microscopic observation of reactive oxygen species (ROS) in tomato cultivars treated with chemical elicitors and bioagents *Bacillus subtilis* against *R. solanacearum* after 24h of inoculation.

Treatment	No. of cell death/ microscopic area under 10X			
	Hawaii-7996		Pusa Ruby	
	Leaves	Stem	Leaves	Stem
Uninoculated (control)	9.00	12.33	11.00	10.00
R. solanacearum	30.33	18.00	12.33	9.00
Bacillus subtilis	22.66	13.00	22.00	10.33
R. solanacearum + *B. subtilis*	28.33	12.33	14.00	13.00
Salicylic acid + *R. solanacearum*	34.00	16.33	11.66	11.66
Salicylic acid + *R. solanacearum* + *B. subtilis*	35.00	22.33	16.00	13.00
Jasmonic acid + *R. solanacearum*	30.66	11.33	15.00	10.33
Jasmonic acid + *R. solanacearum* + *B. subtilis*	18.33	9.66	13.33	9.66

c. Effect of chemical inducers against *Ralstonia solanacearum*

Four chemical inducers *viz.* ,ascorbic acid (AsA), isonicotinic acid (INA), salicylic acid (SA) and beta-butyric acid (BABA) at different concentrations to inhibit the bacterial growth was analyzed at different time intervals. The growth of *R. solanacearum* was significantly inhibited at concentration of 150 µM of chemical inducers *i.e.* ascorbic acid, isonicotinic (INA) and beta-butyric acid (BABA), whereas salicylic acid (SA) inhibited the growth of bacteria at 100 µM *in vitro* when the bacterial population was measured at optical density (OD600) using a spectrophotometer (Fig. 77).

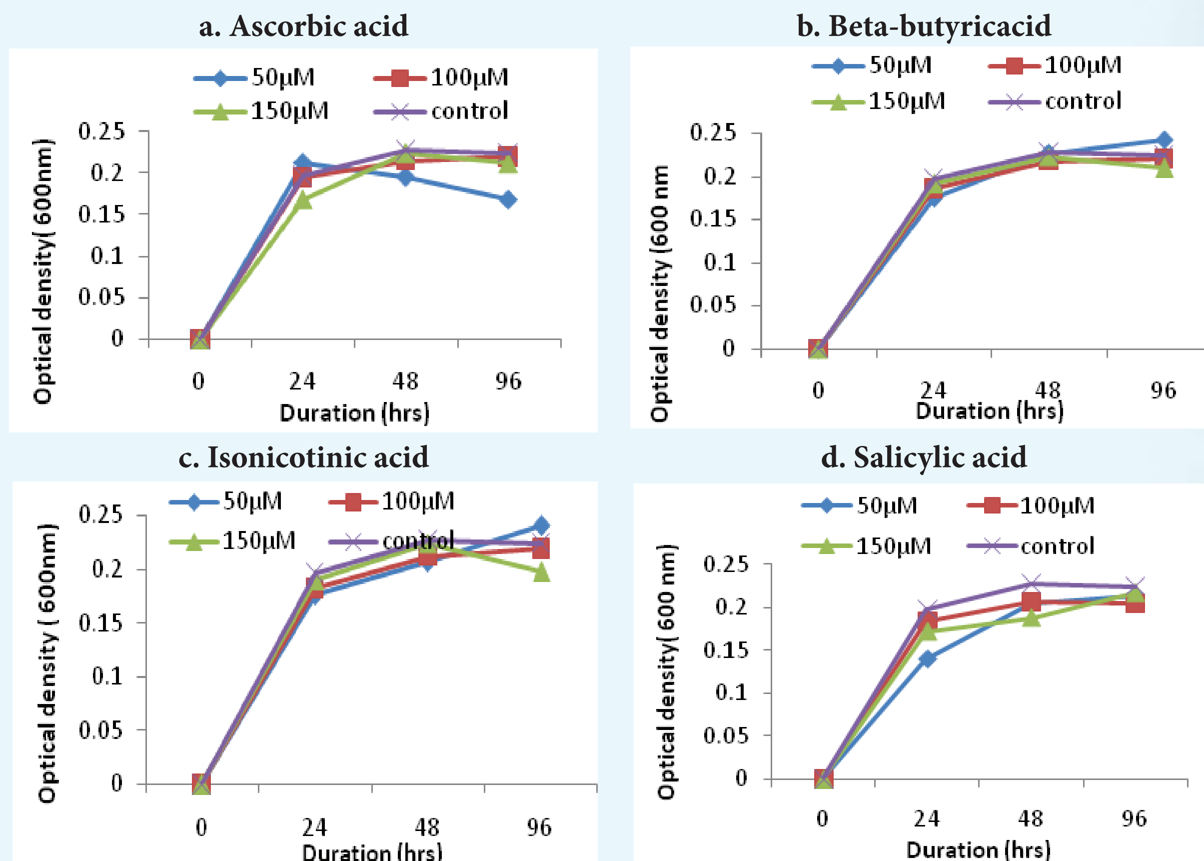


Fig. 77: *In vitro* effect of chemical inducers with different concentrations on growth of *Ralstonia solanacearum*.

3. DISEASE MANAGEMENT

a. Effect of antagonistic bacterium *Bacillus amyloliquefaciens* on induction of defense enzymes in tomato against *R. solanacearum*

An experiment was conducted at Phytotron, IARI, New Delhi. Seeds tomato cultivars *viz.* Arka Abha (Resistant) and Pusa Ruby (susceptible) to bacterial wilt treated with *B. amyloliquefaciens* (10^9cfu/ml) and sown in seed starter tray at 28 ± 2ºC. Sequential inoculation on 30 day old plants, using pathogen *R. solanacearum* (10^9cfu/ml) and bioagent *B. amyloliquefaciens* (10^9cfu/ml) at root zone of each plant and enzyme extraction at different intervals such as 0, 24, 48, 72 and 96 h after pathogen inoculation was done. Minimum wilt disease intensity was found 33.3 and 41.3% in Arka Abha and Pusa Ruby treated with *B. amyloliquefaciens* after 45 days of inoculation of the pathogen. Plant growth promoting activity in *B. amyloliquefaciens* was found to enhance plant height and plant dry weight of in both the cultivars which increased GPE 17.16 in Pusa Ruby and 15.90 % in Arka Abha cultivars of tomato.

The dynamics in the activity of PAL, PPO, POD, SOD and catalase enzymes by *B. amyloliquefaciens* and *R. solanacearum* was noticed in both the cultivars of tomato. The results suggests that *B. amyloliquefaciens* treatments significantly increased the activity in resistant cultivar, while the usceptible cultivar (Pusa Ruby) treated with *R. solanacearum* showed higher activity than treated with bioagent *B. amyloliquefaciens* after 96 hai.

b. Integrated disease management of bacterial wilt of tomato caused by *Ralstonia solanacearum*

The efficacy of formulation *viz. Trichoderma harzianum* Th3, *Pseudomonas fluorescens* PUSA PF 3, *Bacillus subtilis* PUSA BS5 were assessed either alone or in combination

at root zone @ 4 g/litre of each plant during transplantation using drenching method as integrated disease management of bacterial wilt at farmer's field at Chorgaliya, Nanital, Uttarkhand in August 2013. The tomato cultivar 3038 was used for this experiment. Minimum wilt disease incidence was recorded in *P. fluorescens* + *T. harzianum* (22.13) followed by *P. fluorescens* (28.73 %) and *P. fluorescens* (31.73 %).

c. Detection of antibiotics of *Bacillus amyloliquefaciens* by PCR

Bacillus amyloliquefaciens (DSBA-11 DSBA-12), *B. subtilis*, *B. licheniformis* and *B. cereus* were found to harbor the antibiotic genes BacillomycinD and Iturin A by PCR amplification of these genes (BacillomycinD -504 bp, Iturin A -617 bp) in 1.2% agrose gel (Fig. 78 & 79).

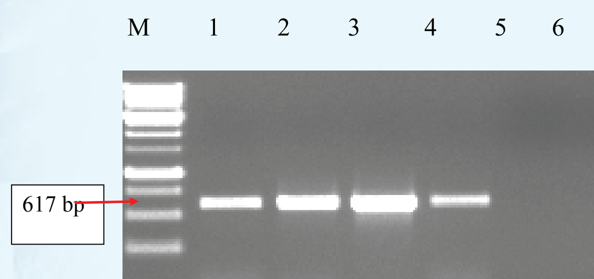


Fig. 78: The amplified DNA fragments at 617 bp produced strains of *B. amyloliquifaciens* by Iturin A primer. LaneM: 1kb DNA Ladder, lanes 1: *B. amyloliquifaciens* DSBA-11, 2: *B. amyloliquifaciens* DSBA-12, 3: *B. subtilis*, 4: *B. licheniformis* 5: *B. cereus,* 6:- ve control

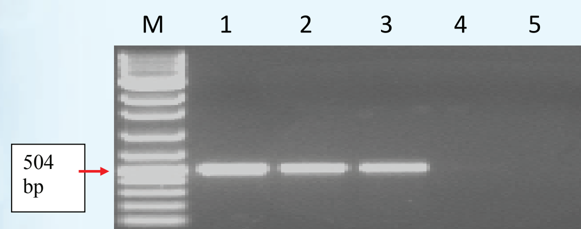


Fig. 79: The amplified DNA fragments at 504 bp produced strains of *B. amyloliquifaciens* by bacillomycinD printment Language : 100 bp DNA Ladder, lanes 1: DSBA-11, 2: DSBA-12, 3: *B. subtilis*, 4: *B. licheniformis*, 5: *B. cereus.*

d. Cloning of Zwittermycine A gene of *Bacillus cereus* (JHTBE-14)

The PCR product of gene was purified by using RBC mini purification kit and cloned using T&A cloning kit the cloned colonies were confirmed by colony PCR of each gene and also tested as a biological against *R. solanacearum* by dual culture technique with parent culture of *B. cereus*. Zwittermycine A antibiotic was produced by the strains of *B. cereus*, *B. thuringiensis, B. anthracis* and *B. toyonensis*. The strain *B. cereus* JHTBE 14 was distinguished from other strains reported from NCBI data base and formed separate cluster in the dendrogram (Fig. 80).

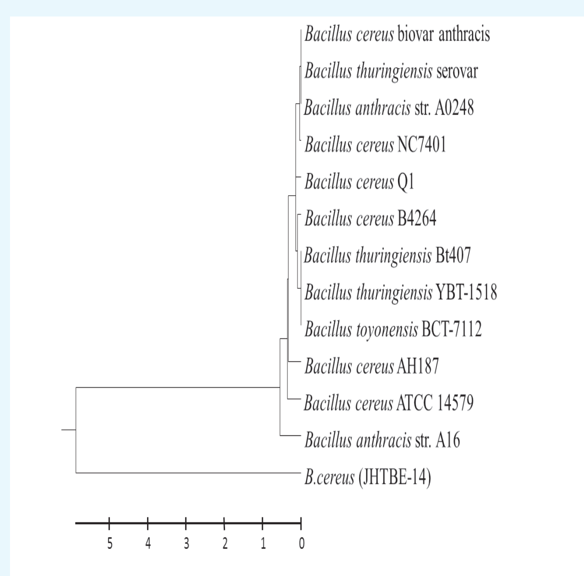


Fig. 80: Evolutionary relationship between *Bacillus cereus* (JHTBE-14) and other *Bacillus* isolates based on the similarity in zwittermycin A sequences.

ICAR RESEARCH COMPLEX, GOA

Principal Investigator:

Dr. R RAMESH

1. BIO DIVERSITY

a. Collection of *Ralstonia solanacearum* isolates

R. solanacearum strains (8) from eggplant (West Bengal), two strains from brinjal (Orissa) obtained from IARI, New Delhi, seven isolates (tomato-1, chilli-3, capsicum-1, and ginger-2) were added to the collection. The new isolates were confirmed based on phenotypic characterization, biovar characterization and PCR assay. All the new isolates are biovar 3 and phylotype I. A total of 246 isolates were deposited in the repository of IISR, Calicut.

b. Analysis of genetic diversity of *R. solanacearum*

Nearly full length of *egl* gene, *pga* gene and *hrpB* genes from 95 representative isolates were PCR amplified and sequenced. Out of 95 isolates, 50 isolates were selected for further analysis. Phylogenetic and taxometric trees were generated using the Neighbour. Joining (NJ) and UPGMA algorithm, respectively. Despite different mathematical algorithms, both analysis produced dendrograms that separated the previously defined phylotypes into distinct clades.

Based on the *egl* sequences, 22 isolates from the selected 95 are designated sequevar numbers. All other isolates constitute unknown sequevars. All isolates are phylotpe I and are most distinct from the other phylotypes. Within phylotype I, isolates are clustered into two distinct clades *viz*. Sub group 1 and Sub group 2. Sub group 1 consists majority of the isolates collected in this study and only two known sequevars *viz.* sequevar 44 (Rs-09-151) and sequevar 14 (Rs-10-250) are present. In Sub group 2, all the isolates were assigned sequevar numbers *viz.*, sequevar 17, 47 and 48. The predominant known sequevar from this data set is sequevar 47 (11/20 isolates), which comprises isolates from all the geographical areas studied. From the dataset, it was observed that only 6.9% isolates from Goa, 33.3% isolates from Karnataka and 23.1% isolates from Kerala are assigned with sequevars. However, 100% isolates from Maharashtra and A&N Islands are assigned sequevar numbers and 76.8% isolates from our dataset are unknown sequevars (Fig. 81).

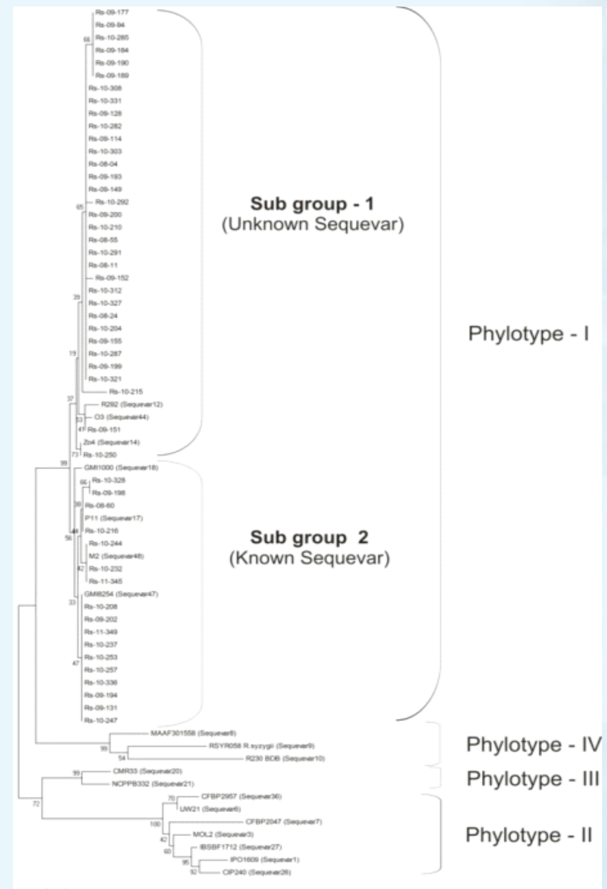


Fig. 81: Phylogenetic neighbor-joining tree based on the partial endoglucanase (*egl*) gene sequences of isolates of *R. solanacearum* infecting solanaceous vegetables from India and *R. solanacearum* species complex reference strains of sequevars.

Based on polygalacturonase precursor gene all isolates clustered together in a single group along with the other *R. solanacearum* isolates described. Isolates in the cluster could not be delineated according to host, origin or biovar characterization and the grouping was not in consistent with phylotyping classification as observed in *egl* sequences. Within the major cluster, our isolates are grouped into two distinct clades *viz.* Sub group 1 and Sub group 2. Sub group 1 consists the same isolates of Subgroup 1 of *egl* tree. In Sub group 2, all the isolates were the same as in Sub group 2 of the *egl* tree.

hrpB transcriptional regulator protein gene based grouping showed within phylotype I, isolates clustered into three distinct clades *viz.*, Sub group 1, Sub group 2

and Sub group 3. within phylotype Sub group 1 and Sub group 3 comprises the isolates with known sequevar numbers *viz.*, sequevar 17, 44, 47 and 48 based on *egl* sequences. Sub group 2 consists of the isolates with unknown sequevars collected in this study. This sub group consists only two known sequevars *viz.*, sequevar 17 (Rs-08-60) and sequevar 47 (Rs-09-202). Isolates within the major cluster (Phylotype I) did not group based on the host or geographical location. Isolates from Andaman & Nicobar islands were clustered into two different sub clusters, Sub group 1 and 3.

2. HOST RESISTANCE

a. Development of suitable mapping population (F1, F2)

F3 population was developed using 25 lines from F2 population (Surya X Agassaim) which were resistant to bacterial wilt during the last season. Two replications maintained for each line data were being collected for segregation of various traits with respect to fruit colour, fruit shape, color pattern and bearing habit (Fig. 82). F4 is in progress with selected 50 lines.

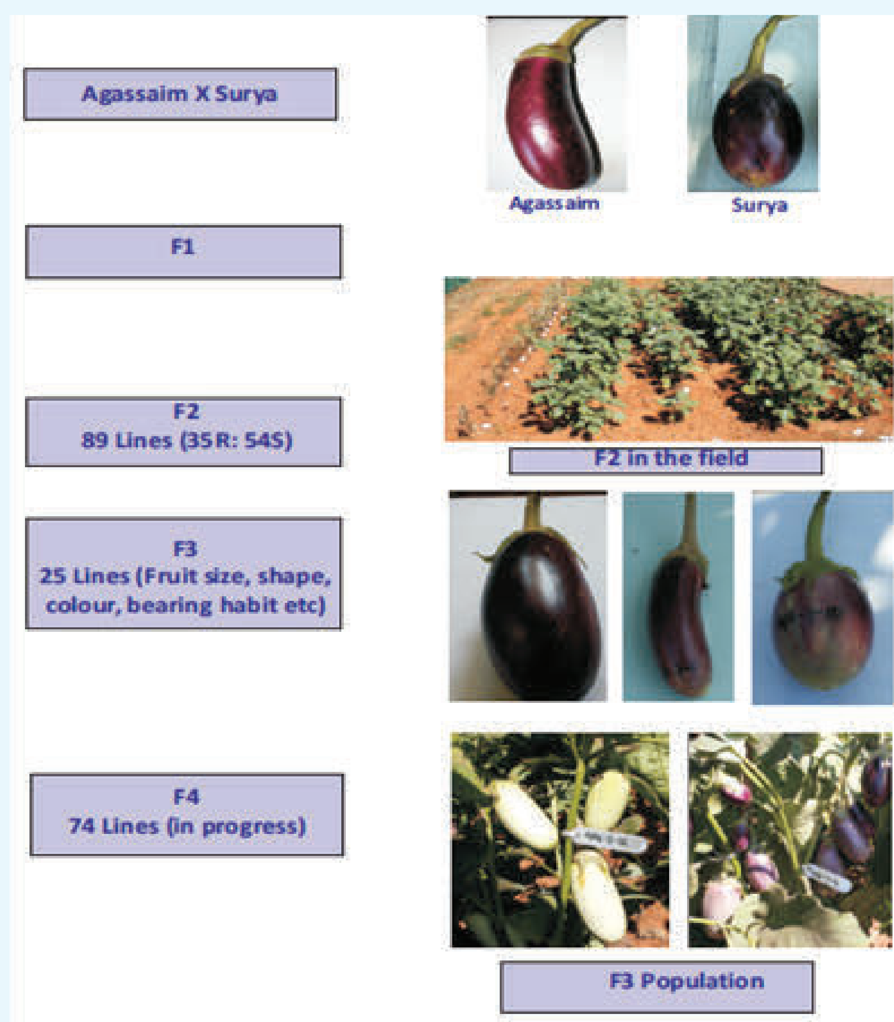


Fig. 82: Development of mapping population in brinjal

b. Studying the mechanism of host resistance

Bacterial wilt resistant cv. Surya and susceptible cv. Agassaim were inoculated with 10 ml of 8 Log 10 CFU ml^{-1} of Rs-09-161. Leaf samples were analyzed for the expression of defense enzymes *viz.,* peroxidase, polyphenol oxidase and changes in the concentration of phenolic compounds before inoculation and after 6 h, 12 h, 24 h, 48 h and 72 h of inoculation. Increase in the defense enzyme activity was observed in resistant variety in response to *R. solanacearum* inoculation whereas no significant change in the parameters in case of susceptible variety.

3. GENOMI CS

Based on the diversity analysis, pathogenicity on three hosts and geographical origin, two *R. solanacearum* isolates were selected for genome sequencing and to un-

derstand the role of various virulence factors in infection and colonization.

The nulcleotide sequences of genomes were obtained from a paired end library with an average insert size of 300-500 bp using Illumina HiSeq 2000. The trimmed reads were assembled into contigs by using SOAP denovo (1.05) and Velvet (1.2.07). Meta assembly was done using progressive Mauve (2.3.1) with *R. solanacearum* strain GMI1000 as the reference genome. Structural annotation was carried out using EuGene-P with *R. solanacearum* strain GMI1000 as the reference proteome. Compared with the published *R. solanacearum* genomes our genomes are most closely related to GMI 1000 and FQY_4. T3E genes present in these genomes are identified according to the latest nomenclature.

a. Study on T3 effectors in virulence of *R. solanacearum*

A total of 71 and 76 effector molecules are identified in strain Rs-09-161 and Rs-10-244, respectively (Table. 25). The identification is carried out based on various criteria's including homology to known T3E in other bacterial species, presence of a *hrpII* box in the promoter region, existence of specific amino acid distribution biases in the 50 N-terminal domain and presence of structural motifs predicting functions in eukaryotic cells.

Table 25. Uncommon type III effectors among phylotype I strains (Indian strains) based on sequence information

Effector name	GMI1000	Isolate 1	Isolate 2
RipC2	Pseudogene	Absent	Present
RipE2	Absent	Absent	Present
RipP1	Present	Absent	Present
RipP3	Pseudogene	Absent	Present
RipS1	Present	Present	Absent
RipS8*	Pseudogene	Present	Present
RipT	Present	Absent	Present
RipAH	Present	Absent	Present
RipAK	Present	Absent	Present
RipAL	Absent	Present	Present
RipAP	Pseudogene	Present	Present
RipAT	Pseudogene	Present	Present
RipAX1	Present	Pseudogene	Present
RipBA*	Pseudogene	Multiple copies	Multiple copies
RipTPS	Present	Multiple copies	Multiple copies
Rs_T3E_hyp-6*	Absent	Present	Absent
Rs_T3E_hyp-15*	Pseudogene	Present	Pseudogene
Rs_T3E_hyp-16	Present	Absent	Absent

4. HOST-PATHOGEN INTERACTION

a. Study on the role of T3E on the virulence of *R. solanacearum*

Attempt was made towards the detection of Type III effectors (T3E) with the help of a reporter based delivery system. The pRCG-GWY based delivery system has been obtained for proving the translocation of the cloned T3E. An insertion and deletion mutant of *hrpB* gene of *R. solanacearum* was created using TA cloning kit by amplifying the internal regions of the *hrpB* gene and the mutants were confirmed.

b. Colonization of xylem residing bacteria (XRB) in eggplant

Twenty eight antagonistic XRB were evaluated to determine their re-colonization ability. Ten ml suspension of XRB containing 8 Log10 CFU. ml^{-1} added and colonization was examined after 15 and 30 days post inoculation. XB1, XB99, XB123 were found to colonize rhizosphere and endophytic tissues where as XB7 and XB122 only colonized rhizosphere at 15 and 30 DAI. Endophytic population was found to be 2 Log10 CFU g^{-1} of tissue while the rhizosphere population ranged between 3 Log10 and 4 Log10 CFU ml^{-1}.

c. Esterase production by XRB

Xylem residing bacteria were analyzed for ability to produce an esterase that could hydrolyse 3-hydroxy palmitic acid methyl ester (3OHPAME, a quorum sensing molecule of *R. solanacearum*) by plate based bioassay using a quorum sensing indicator strain *R. solanacearum* AW1-3. Strains XB7 (*Pseudomonas aeruginosa*), XB19 (unidentified), XB102 (*Pseudomonas hibiscicola*), XB109 (*Rhodococcus corynebacteroidetes*), XB115 (*Rhodococcus corynebacteroidetes*), XB122 (*Pseudomonas aeruginosa*), XB174 (*Acinetobacter* sp.) and XB179 (unidentified) were found to degrade 10 nM 3OHPAME. These strains were identified by 16s rRNA gene sequencing.

5. DISEASE MANAGEMENT

a. Evaluation of different strategies for the management of *R. solanacearum*

Grafting of cultivated brinjal on wild brinjal: Susceptible cultivated type (agassaim) plants were grafted on the seedlings of the wildtypes (resistant). The hardened grafts when inoculated with the pathogen were resistant to wilting and the endophytic colonization and rhizosphere population of *R. solanacearum* indicated the failure of the pathogen to multiply in the root tissues sufficiently to cause wilt. Successful grafts were planted

in the field which produced fruits of the Agassaim type. Consumer preference for the fruits from grafts is similar to the fruits from the seedling type.

b. Application of lime to the soil and its effect on bacterial wilt incidence in brinjal

Under greenhouse conditions, the bacterial wilt susceptible Agassaim plants were grown in the experimental soil applied with four levels of lime (@ 2, 4, 10, 20 t/ha) and were inoculated with different levels of *R. solanacearum* inocula. Incidence of bacterial wilt was recorded regularly. Results indicated that bacterial wilt incidence reduced at 10 and 20 t/ha applied treatments in all the inoculum levels. Analysis of soil pH during the experimental time indicated that the soil pH of above 7.0 was maintained where the lime application was 10 and 20 t/ha. It indicates soil pH may play a role in the incidence and its manipulation would help in containing the bacterial wilt incidence.

c. Actinomycetes for biocontrol of *R. solanacearum*

Fourteen actinomycetes strains were isolated from rhizosphere of eggplant, cowpea and paddy based on typical colony characters. Ten strains were tested against *R. solanacearum* for *in vitro* inhibition by standard method. Six strains were antagonistic based on zones of inhibition ranging from 2-4 mm in radius. Evaluation on growth promotion and wilt prevention under greenhouse conditions is underway.

d. Evaluation of microbial candidates for use against *R. solanacearum* under field condition

Field experiments were laid out to evaluate the efficiency of the eight biocontrol agents in reducing bacterial wilt in eggplant. Four strains of endophytic bacteria (EB69, EC13, Rs-08-72 and K1), three strains of xylem residing bacteria (XB86, XB177, XB202) and one rhizobacterium (RP 7) are used in field trial. Nursery was treated with talc formulation of the biocontrol agents @ 50 gm^{-2} area. One treatment of consortium (EB69+ Rs-08-72) was also included based on the compatibility study. Seedlings raised in the treated nursery and control nursery (not treated with biocontrol agents) were planted in the field in January 2014. The plants were drenched with the biocontrol suspension (50 g lit^{-1}) @ 50 ml/plant. Appropriate controls were maintained. Incidence of wilt and other yield parameters are being recorded.

INDIAN INSTITUTE OF HORTICULTURAL RESEARCH, BENGALURU

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Co-investigators:

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Dr. S Sriram

1. BIO DIVERSITY

a. Molecular characterization of strains of *R. solanacearum* isolates by DNA fingerprinting -BOX-PCR

Ralstonia isolates which were confirmed by RS specific primers were further characterized by BOX PCR. Substantial variation was observed among the different isolates (Fig. 83).

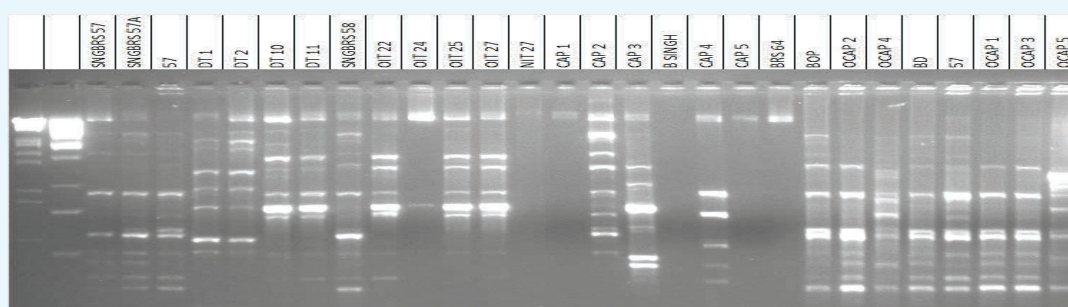

Fig. 83: Genomic fingerprinting of *Ralstonia* isolates by BOX PCR

b. Effect of storage of *Ralstonia solanacearum* at various media and temperatures on its virulence

The effect of storage of *R. solanacearum* culture in terms of virulence at various media and temperature was assessed. The culture stored at various media at different temperature was periodically retrieved and sub-cultured on TTC media and further multiplied on 523 media for testing its virulence. The virulence was tested on 18 day old tomato (Arka Vikas) seedlings (100 seedlings/ sampling) in pro-trays by drenching the roots with inoculum concentration of 1.0 x 10^8 cfu/ml. Observations were recorded on incubation period and per cent wilting. Results showed that *R. solanaceraum* culture could be stored at room temperature (28±2°C) in sterile distilled water for a period of 152 days with virulence intact (100% wilting) and a maximum period of 293 days with more than 50.0% wilting to tomato seedlings. Culture slants with 523 media was better than NA and TTC by recording a maximum of 129 days period of storage with 78.0% wilting when tested against tomato seedlings. Storage of *Ralstonia* in 30% glycerol under deep freezer showed that at -20 and -80°C the culture can be stored for 150 days with cent per cent wilting to tomato seedlings (Table 26).

Table 26. Effect of storage of *Ralstonia solanacearum* at various media and temperature on its virulence

Media	Storage temperature °C	Storage duration with high virulence		Storage duration with reduced virulence	
		Days	Pathogenicity (% wilt)	Days	Pathogenicity (% wilt)
Sterile distilled water	0	—	—	8	29.0
	4	40	100.0	46	71.0
	28±2*	152	100.0	329	29.0
Glycerol 30%	0	58	100.0	—	—
	4	120	100.0	—	—
	-20	150	100.0	—	—
	-80	150	100.0	181	66.0
NA slant	0	8	—	8	66.0
	4	40	100.0	—	—
	28±2	21	100.0	—	
TTC Slant	0	8	—	8	
	4	46	100.0	—	—
	28±2	21	100.0	—	
523 slant	0	21	100.0	31	60.0
	4	46	100.0	129	78.0
	28±2	21	100.0	31	65.0

*182 days (75.0%); 293 days (58.0%)

2. DIAGNOSTICS

a. Development of a quick diagnostic kit for detection of *Ralstonia solanacearum* in soil as well as in plants by DAC-ELISA

Production of polyclonal antiserum using the whole bacterial cells of *Ralstonia* fixed with glutaraldehyde and the purified extracellular polysaccharides as antigens for immunisation to produce a high titre polyclonal antiserum was done, since the other serological method such as DAC-ELISA was developed for detection has sensitivity detecting as few as 101 cells/ml of *R. solanacearum* with a titre of 1:4000. Both the antiserum produced successfully detected original bacterial culture of different concentrations as well as natural infection of *Ralstonia* in infected tomato, brinjal and chilli crops and infected soil at 1:4000 dilutions. The OD values in soil samples were less when compared to plant samples (Table. 27)

Table 27. Specificities of two PAbs of *R. solanacearum* for detection and identification of different antigens

	Pre bleed	Reaction	Ab1	Reaction	Ab2	Reaction
	OD405		OD405		OD405	
Buffer	0.14	-	0.18	-	0.17	-
Tomato healthy	0.11	-	0.18	-	0.17	-
Brinjal healthy	0.10	-	0.18	-	0.16	-
Hot pepper healthy	0.10	-	0.22	-	0.16	-
Soil (autoclaved)	0.11	-	0.18	-	0.15	-
10^{12}	0.14	-	1.27	++	1.17	++
10^{10}	0.13	-	1.37	++	1.00	++
10^{8}	0.13	-	1.47	++	0.99	++
10^{6}	0.12	-	1.58	++	0.83	+
10^{4}	0.11	-	1.53	++	0.70	+
10^{2}	0.11	-	1.12	++	0.64	+
10^{1}	0.11	-	0.96	++	0.54	+
Tomato soil	0.13	-	0.58	+	0.52	+
Brinjal soil	0.10	-	0.62	+	0.56	+
Hot pepper soil	0.10	-	0.97	++	0.89	+
Tomato root	0.10	-	0.83	+	0.69	+
Brinjal root	0.11	-	0.61	+	0.58	+
Hot pepper root	0.12	-	1.14	++	1.02	++
Tomato ooze	0.11	-	0.78	+	0.80	+
Brinjal ooze	0.12	-	0.88	+	0.75	+
Hot pepper ooze	0.10	-	1.02	++	0.9	++

Value of OD (405 nm) = average of two replications. Absorbance value measured using Versa-max ELISA reader from Molecular Devices

3. GENOMICS

a. Whole genome sequencing of *Ralstonia solanacearum*

Among the confirmed isolates, the whole genome of the highly virulent brinjal isolate was sequenced using the Ion Torrent™ Technology. The total number of contigs were 1031. The shortest contig was 350 bases and the longest 3080 bases. *De novo* assembly was done using 1031 contigs using CLC Assembly Cell. Using Ugene, the fasta files were converted to genbank format and opened with MAUVE and comparative analysis was done with the following three reference sequences (Table 28).

[1] *Ralstonia solanacearum* PSI07 which has one chromosome sequence and one plasmid sequence. Chromosome sequence - NC_014311.1, Plasmid sequence - NC_014310.1

[2] *Ralstonia solanacearum* CFBP2957 which has only one chromosome sequence. Chromosome sequence - NC_014307.1

[3] *Ralstonia solanacearum* GMI1000 which has one chromosome sequence and one plasmid sequence. Chromosome sequence - NC_003295.1, Plasmid sequence - NC_003296.1

Using the RAST- NMPDR (http://rast.nmpdr.org/), SEED-based, prokaryotic genome annotation service, the genome was annotated (Fig. 84).

Table 29. Comparative analysis of *Ralstonia* genomes using MAUVE

Reference genome	Total no. of reads	Total no. of reads mapped	No. of reads mapped to chr	No. of reads mapped to plasmid	Average base coverage depth	Genome covered		
						with 1x	with 10x	with 20x
R. solancearum CFBP295 7 chromosomes	1985937	1701824 (85.69%)	1701824	NA	37.41	96.07%	82.87%	76.59%
R. solancearum GMI1000 chromo-some plus plasmid	1985937	1859903 (93.65 %)	1246336	613567	41.15	93.27%	88.90%	83.81%
R. solancearum PSI07 chromosome plus plasmid	1985937	1709999 (86.11%)	1181250	528749	31.49	87.73%	74.81%	68.07%

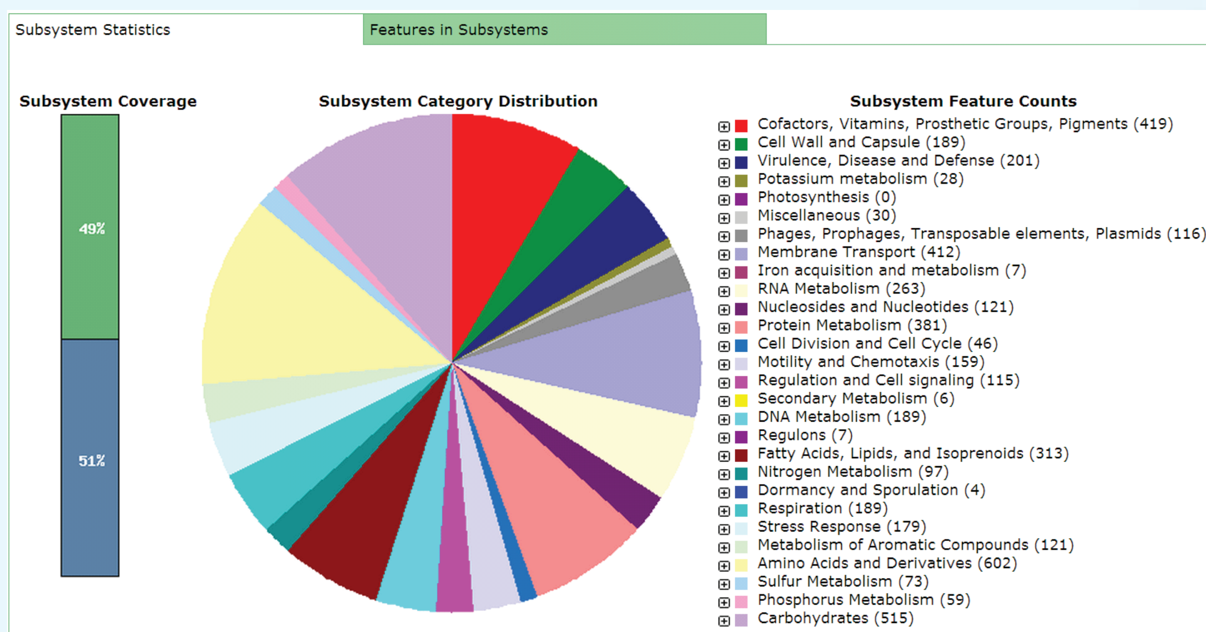

Fig. 84: Functional genome annotation of *Ralstonia* using the RAST NMPDR

4. DISEASE MANAGEMENT

a. Effect of bactericides and botanicals against *R. solanacearum* under *in vitro*

The antibacterial activity of two bactericides and 23 botanicals were investigated against the bacterial wilt pathogen, *R. solanacearum* under *in vitro* by well diffusion method (Table 29). Fresh extracts from the leaf, bulb (garlic and onion) rhizome (ginger) and fine powder (turmeric and asafetida) were used. The commercial essential oils, *viz., Azadirachta indica* A. Juss., *Pongamia glabra* L. and *Syzygium aromaticum* (L.) Merril & Perry were diluted using the solvent Di-Ethyl ether. The bactericide Streptocycline and Bronopol were used as standards and sterile distilled water as control. Results showed that of the 23 botanicals tested, only four, *viz., Allium sativum, Lawsonia inermis*, *Piper betle* and *Syzygium aromaticum* exhibited antibacterial activity against *R. solanacearum*. *S. aromaticum* showed maximum inhibition zone of 52.0 mm diameter followed by *A. sativum*, *L. inermis* and *P. betle*, exhibiting inhibition zone of 49.0, 16.0 and 15.0 mm diameter, respectively. The antibacterial activity of *S. aromaticum* and *A. sativum* was significantly high as compared to the commercial Streptocyline, whereas the bacterial activity of *L. inermis* and *P. betle* as compared to Bronopol and the commercial bactericide was superior and on par respectively (Table 30).

Table 30. Antibacterial effect of bactericides and botanicals against *R. solanacearum*

Botanical name	Common name	Name of family	*Mean zone of inhibition (mm, diameter)
Allium cepa L.	Onion	Amaryllidaceae	0.00 (0.00) f
Allium sativum L.	Garlic	Amaryllidaceae	49.00 (3.91)b
Azadirachta indica A. Juss. (leaf)	Neem	Meliaceae	0.00 (0.00) f
A. indica A. Juss. (oil)	Neem	Meliaceae	0.00 (0.00) f
Calotrophis gigantea R.Br..	Milk weed	Apocynaceae	0.00 (0.00) f
Catharanthus roseus (L). G. Don	Periwinkle	Apocynaceae	0.00 (0.00) f
Coleus forskohlii Brig.	Coleus	Lamiaceae	0.00 (0.00) f
Curcuma longa L.	Turmeric	Zingiberaceae	0.00 (0.00) f
Datura stramonium L.	Datura	Solanaceae	0.00 (0.00) f
Ferula assa-foetida L.	Asafetida	Apiaceae	0.00 (0.00) f
Lawsonia inermis L.	Henna	Lythraceae	16.00 (2.83)d
Leucas aspera Willd. (L.)	Thumbai	Lamiaceae	0.00 (0.00) f
Mentha longifolia (L.) Huds.	Mentha	Lamiaceae	0.00 (0.00) f
Nerium oleander L.	Oleander	Apocynaceae	0.00 (0.00) f
Ocimum sanctum L.	Tulsi	Lamiaceae	0.00 (0.00) f
Piper betle L.	Betel	Piperaceae	16.00 (2.83)d
Plectranthus amboinicus (Lour.) Spreng	Indian mint	Lamiaceae	0.00 (0.00) f
Pongamia glabra L.	Pongamia	Fabaceae	0.00 (0.00) f
Syzygium aromaticum (L.) Merril & Perry	Clove	Myrtaceae	52.00 (3.97)a
Tinospora cordifolia (Thunb.) Miers	Guduchi	Menispermaceae	0.00 (0.00) f
Vitex negundo L.	Chaste tree	Verbenaceae	0.00 (0.00) f
Withania somnifera (L.) Dunal	Ashwagandha	Solanaceae	0.00 (0.00) f
Zingiber officinale Roscoe	Ginger	Zingiberaceae	0.00 (0.00) f
Streptocycline (200 ppm)	---	---	20.00 (3.04)c
Bronopol (200 ppm)	---	---	15.00 (2.77)e
Control (water)	---	---	0.00 (0.00)f

*Mean of three replications
Figures in parentheses are x + 0.5 log transformations; Values followed by same letter in a column are not significantly different (P=0.05)

b. Formulation of potential bioagents against *Ralstonia solanacearum*

Talc based formulation of potential strains of *Pseudomonas fluorescens* (IHRPf24) and *Bacillus subtilis* (IHRBs39) were developed. *P. fluorescens* and *B. subtilis* cultures were grown separately in nutrient broth for 48 h as shake culture in shaking incubator at temperature 28 ±2°C at 150 rpm. Ten grams of carboxymethyl cellulose was added to 1 kg of sterile talc as carrier material and mixed well. About 400 ml of bacterial suspension containing 2.5 x 10^8 cfu/ml was added and mixed well under sterile condition. Further studies on rhizosphere colonization of the bioagents through seed priming, seedling root dip and efficacy against *R. solanacearum* under glass house condition are in progress.

c. Development of integrated disease management strategy for bacterial wilt in tomato

A tield trial was carried out for the management of bacterial wilt in tomato variety Shivam (Bacterial wilt susceptible). Results showed that the wilt incidence was significantly low (8.50 and 10.0%) in treatments T8 (FYM+GM+Pf+COC) and T9 (FYM+GM+Bs+COC). This was followed by treatments T4 (Pf - seed treatment and soil drenching) and T5 (Bs - seed treatment and soil drenching), which recorded 18.20 and 19.65 per cent wilt incidence, respectively. However, the untreated control plot recorded significantly highest wilt incidence of 68.0 per cent (Table 31). Similarly, the yield was significantly high (40.8 t/ha) in treatment T8 (FYM + GM + Pf

+ COC), followed by treatments T9 (FYM+GM+Bs+COC) and T10 (FYM+ GM+Pf+Bs), which recorded 38.5 and 36.8 t/ha, respectively. However, the untreated control plot recorded lowest yield of 7.22 t/ha.

Table 30. Effect of various treatments (bioagents, botanicals and bactericide) on bacterial wilt incidence and harvest yield of potato

Treatment	Mean % wilt*	Yield (t/ha)*
FYM @ 20 t/ha	32.60 (34.77)fg	12.25d
FYM + Green manure (GM) (sunnhemp) @ 25kg/ha	22.50 (28.26)d	16.50d
GM @ 25kg/ha	28.82 (32.45)e	14.28d
Pseudomonas fluorescens (Pf) @ 10^8cfu/ml (seed treatment & Soil drenching)	18.20 (25.14)c	29.50c
Bacillus subtilis (Bs) @ 10^8cfu/ml (seed treatment & Soil drenching)	19.65 (26.21)c	28.00c
Neem cake @ 150 kg/ha	32.00 (34.41)efg	14.00d
Pongamia cake @ 150 kg/ha	35.00 (36.22)g	12.80d
FYM + GM + Pf seed treatment and soil drenching + COC	8.50 (16.88)a	40.80a
FYM + GM + Bs seed treatment and soil drenching + COC	10.00 (18.19)a	38.50b
FYM + GM + Pf + Bs	14.00 (21.91)b	36.80b
Streptocycline 250 ppm + COC 0.2 %	29.79 (33.05)ef	14.90d
COC 0.2%	32.84 (34.93)fg	13.85d
Un treated Control	68.0 (55.54)h	7.22e
CD (P=0.05)	3.39	4.38
S.Em±	1.18	1.52
CV%	7.72	14.21

*Mean of four replications

Figures in parenthesis are arc sine transformed values.

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1. BIO DIVERSITY

A new *R. solanacearum* was isolated from wilted capsicum plant and confirmed by PCR based detection using species-specific universal primer 759/760 and the isolates were found to be biovar 3.

a. Study on genetic divergence among *R. solanacearum* isolates from North-east India using Multi Locus Sequence Typing (MLST)

MLST studies for *R. solanacearum* isolates from tomato (RsRC-T1), potato (RsRC-P1), brinjal (RsRC-B1), ginger (RsRC-G1) and capsicum (RsRC-C1) with five chromosomal housekeeping genes *viz., ppsA, gyrB, adk, gdhA, gapA*, and three megaplasmid virulence-related genes *hrpB, fliC* and *egl* was done. The clustering pattern of *hrpB* gene indicated close association of *R. solanacearum* isolates from North-East India with previously reported isolates from different parts of India (Fig. 85). The relationship and diversity of RCR-226 strain of *R. solanacearum* from Meghalaya with previously reported Indian strain of *R. solanacearum* was established by comparing the sequence with earlier reported 42 partial *fliC* gene sequences of *R. solanacearum* from India and with a single strain of *R. syzygii* available in NCBI database. In Neighbor-Joining phylogeny of partial *fliC* gene, the RCR-226 strain grouped with Indian strain of *R. solanacearum* (Fig. 86). The phylogenetic tree was constructed on the matrices of aligned sequences with 1000 bootstrap replicates following neighbour-joining phylogeny of MEGA 5.

2. DIAGNOSTICS

a. PCR based rapid detection of *Ralstonia solanacearum* directly from infected plants

The infected plant samples collected randomly from different fields were examined by ooze-test, the positive samples were cut into small pieces aseptically and were placed in 1-ml sterile nuclease and protease free water and incubated at room temperature for 5 min or until the water became turbid followed by a final incubation at 96ºC for 6 min in thermal cycler. The suspension was directly used as template for PCR amplification using Rsol_fliC primers (Forward: 5'GAA CGC CAA CGG TGC GAA CT 3'; Reverse: 5' GGC GGC CTT CAG GGA GGT C 3'). PCR amplification yielded an expected product of 400 bp in size from infected samples (Fig. 87). Cloning and sequencing of the amplified product showed 99% nucleotide identity with the previously reported GM strain.

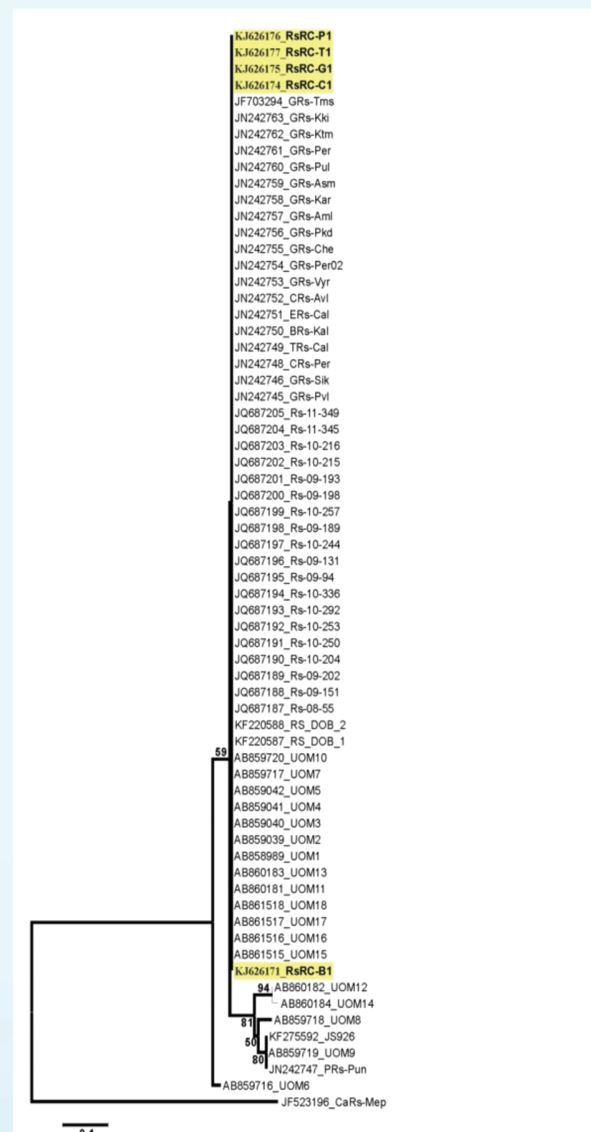

Fig. 85: Phylogenetic relationship based on nucleotide sequences of *hrpB* gene of *R. solanacearum* isolates from NE India.

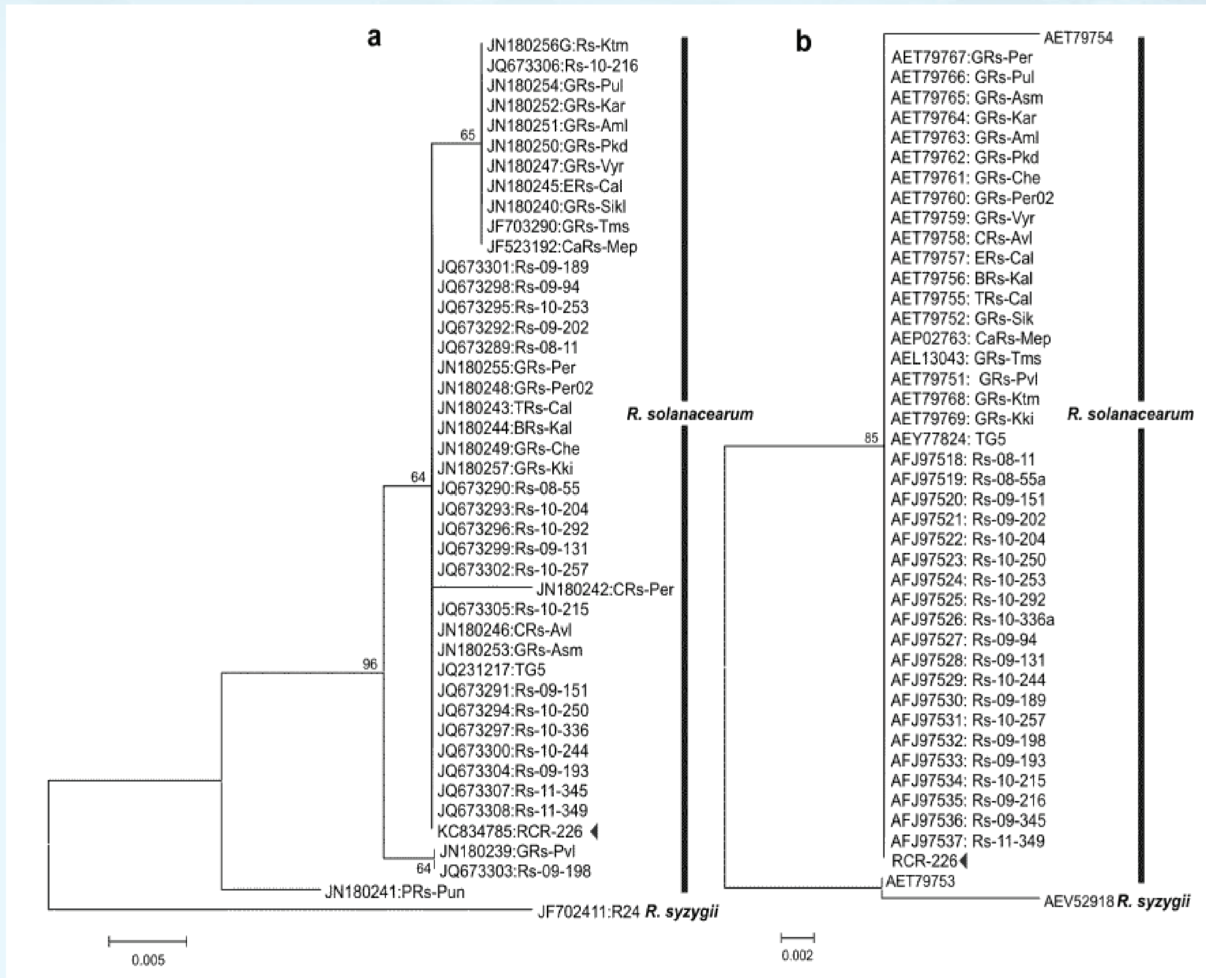

Fig. 86: Phylogenetic relationships based on nucleotide and amino acid sequences of partial *fliC* gene (a, b) of the *R. solanacearum* strain (RCR-226) along with previously reported Indian strains. A single strain of a related species, *R. syzygii* was also considered for comparison as an out group member. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (shown only when >50%).

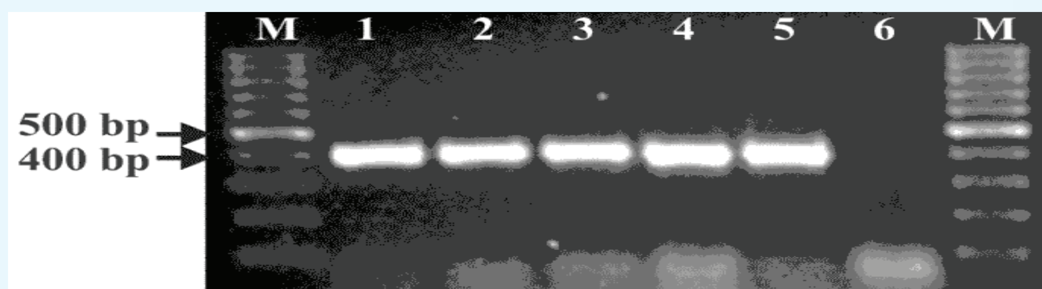

Fig. 87: PCR-based detection of *R. solancearum* using bacterial ooze extracted from infected plant materials as template M: 100 bp DNA ladder; lanes 1-4: test samples from field of tomato; lane 5: positive control; lane 6: negative control.

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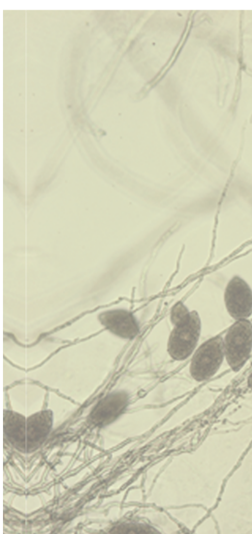
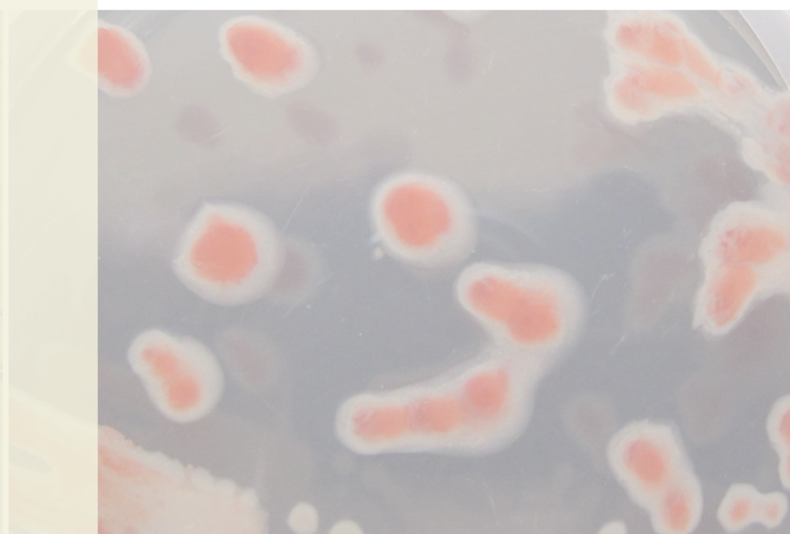
BUDGET UTLIZATION DURING 2013 – 14 (Rupees in lakhs)

Name of Institute		Unspent balance carried forward from previous year	Allocation during 2013-14	Total	Utilization during 2013-14	Balance
IISR, Kozhikode		0.74	60.91	61.65	56.87	4.78
ICAR RC NEH, Umiam		1.96	12.18	14.14	13.76	0.38
IARI , New Delhi	*Fusarium*	1.35	10.12	11.47	8.52	2.95
	Ralstonia	-1.39	10.12	8.73	9.22	-0.49
CPRI, Shimla		32.80	39.43	72.23	60.06	12.17
IIHR, Bengaluru		7.12	16.81	23.93	13.33	10.6
CPCRI, Kasaragod		11.34	9.75	21.09	6.92	14.17
CTCRI,Thiruvanathapuram		5.51	10.18	15.69	9.73	5.96
NRC Citrus, Nagpur		8.14	10.18	18.32	10.65	7.67
YSPUHF, RC Kullu		1.84	9.44	11.28	6.97	4.31
IIPR, Kanpur		0.23	9.68	9.91	8.44	1.47
DOR, Hyderabad		0.00	10.12	10.12	9.46	0.66
CISH, Lucknow		2.01	5.50	7.51	1.13	6.38
IIVR, Varanasi		2.62	9.18	11.80	6.97	4.83
NRC Banana, Tiruchirapalli		0.99	8.61	9.60	6.75	2.85
NBAIM, Mau		1.87	5.41	7.28	3.26	4.02
ICAR-RC, Goa		3.84	9.93	13.77	9.31	4.46
Total		**80.97**	**247.55**	**328.52**	**241.35**	**87.17**





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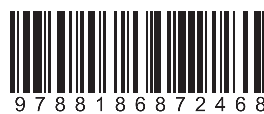

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