PhytoFuRa
AN ICAR NETWORK PROJECT ON
Phytophthora, Fusarium & Ralstonia

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

वार्षिक रिपोर्ट 2014/15
Annual Report 2014/15

भा. कृ. अनु. प. – भारतीय मसाला फसल अनुसंधान संस्थान   ICAR-Indian Institute of Spices Research

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ICAR - INDIAN INSTITUTE OF SPICES RESEARCH
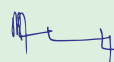P.B. No. 1701, Kozhikode - 673 012, Kerala, India.

Contents

PREFACE

We started the year in an environment of change where XII Plan has been launched and three new research partners have joined the network. The problems undertaken are *Phytophthora* in rubber, *Fusarium* in sugarcane and seed spices. As we look at the past, we have made impressive strides over the past twelve months, thanks to the untiring efforts of all the research partners.

During 2014-15, 192 and 60 more isolates were added in the *Phytophthora* and *Ralstonia* repositories, respectively. In the diagnostics front, SCAR markers for *P. nicotianae*, qPCR protocols for detection of *P. meadii* and *P. colocasiae*, LAMP-PCR protocols for detection of *R.solanacearum* from soil and seed rhizomes of ginger were developed. Cutting edge technologies like SSH, targeted expression sequencing, RNAi mediated gene silencing and transcriptomics are now in vogue for deciphering host-pathogen interactions. Our endeavor for locating resistant lines continues relentlessly. This year resistant accessions were identified in taro, citrus, castor against *Phytophthora*; in chickpea and pigeon pea against *Fusarium*, and in brinjal against *Ralstonia*. Efficacy of fungicides like dazomet and cymoxanil + mancozeb in suppressing *Phytophthora, Pythium* etc. is noteworthy. More strains of *R. solanacearum* were sequenced and efforts are on to make use of the genomic information in a constructive manner.

Out of the 36 research papers published from this project, more than 60% have a NAAS rating of >6 and over 30% are above 7 rating, which is a testament to the passion and dedication of all those involved. Several new technologies started emanating from the project and let us be cautiously optimistic about the year ahead by ensuring optimal performance and create value for the farmers and prospective entrepreneurs.We also need to work for the furtherance of technologies that are relevant to the farming community before the close of the project. Therefore, the coming couple of years are very critical and we have no option but to improve on our performance and to grow continuously at unprecedented rates, not just nationally but also globally. Let us sustain the high level of productivity and efforts to strengthen our commitment to farmers and end users.

Although the primary purpose of this report is to provide the peers with an accurate description of the progress made during 2014-15, we recognize that the report is also a valuable source of information for the research community. My special thanks to all my fellow investigators, who have made a substantial contribution to the success of this Project. We look forward to continuing our cooperative partnership in the future. I profusely thank Dr. S. Ayyappan, Secretary, DARE and Director General, ICAR for his support to continue this project as envisaged. The support and guidance received from Dr. N.K. Krishna Kumar, Deputy Director General (Horticultural Sciences) and Dr T. Janakiram, Assistant Director General (HS) are gratefully acknowledged. I would like to thank all the project staff once again and express my sincere appreciation to all of them on behalf of the Council.

M. Anandaraj
National Coordinator &
Director, ICAR - IISR, Kozhikode

Kozhikode
20 May 2015

EXECUTIVE SUMMARY

PHYTOPHTHORA

DIVERSITY

A total of 192 of *Phytophthora* spp. from Maharashtra, Karnataka, Punjab, Mizoram, Himachal Pradesh, Meghalaya, Uttarakhand and Uttar Pradesh were collected and maintained. In India, *P. infestans* population are polyploid consisting of diploids, triploids and tetraploids. The frequency of diploids was highest (85%) followed by triploids (14%) and tetraploids (1%) in Indian hills and sub-tropical plains while in plateau and in Madhya Pradesh only diploids were found. Multi Locus Sequence Typing (MLST) analysis of *Phytophthora* infecting black pepper showed a separate group for isolates from black pepper.

DIAGNOSTICS

SCAR markers and specific primers from extracellular cystatin-like protease inhibitor (epiC1) gene were developed for the detection and identification of *P. nicotianae*. Real-time PCR detection protocols were standardized for *P. meadii*. Duplex PCR protocol along with SCAR marker for the simultaneous detection of *P. infestans* and *Alternaria solani* in a single reaction using both genomic DNA and infected tissues of potato were revalidated at CPRI Shimla. A rapid and reliable method to quantify the pathogen load in the infected plant tissue towards resistance screening in taro cultivars has been established using qPCR assay.

HOST-PATHOGEN INTERACTION

RNAi construct was developed at CPRI, Shimla for RNAi-mediated silencing of *P. infestans Pol* gene for imparting late blight resistance. Complete mitochondrial genome of *P. infestans* A2 mating type was sequenced and mt DNA of A2 mating was found to have 60.49 per cent homology with *P. andina*. Specific genes that have a putative role in pathogenicity of *P. colocasiae* were identified by SSH approach. Targeted expression analysis of defense related genes in black pepper showed constitutive expression of β 1, 3-glucanase in IISR Shakthi during initial hours where as in susceptible variety, Subhakara, down regulation of PnBGlu was observed. Quantitative RT-PCR was used to assess the level of expression of pathogenicity genes of *P. capsici-P. colubrinum* interaction .

HOST PLANT RESISTANCE

Citrus rootstocks Khasi Papeda and NRCC-2 showed moderately tolerant reaction against *Phytophthora* spp. The 14 potato R genes conferring resistance to late blight were used for allele mining in 11 highly resistant wild species. Three taro accessions U64, TCR 125, IC204065, two castor cultivars GCH 4 and GCH 7 showed resistant reaction against *Phytophthora* leaf blight under field conditions. Phenotyping of progenies of the cross Panniyur 1 and Subhakara and open pollinated progenies of IISR Shakthi for *Phytophthora* resistance showed five progenies of the cross and four progenies of IISR Sakthi tolerate stem infection. Transcriptome sequencing was done for RRIM 600, a susceptible cultivated rubber clone and FX 516, an interspecific hybrid clone tolerant to *Phytophthora* pathogen. Evaluation of seedlings of pollinizer cultivars of apple indicated that *Malus floribunda* showed tolerance to *Phytophthora* with minimum mortality.

EPIDEMIOLOGY

Epidemiological studies on apple collar rot disease indicated that the disease is positively correlated with rainfall, soil moisture and minimum soil temperature and was negatively correlated with soil temperature at 5 cm.

DISEASE MANAGEMENT

The fungicide dazomet granules were found to be effective in reducing soil-borne inocula of *Phytophthora* and *Pythium* sp. in citrus nursery soils. Field trial on management of fruit rot disease of arecanut was conducted in CPCRI, RC, Kidu, Vittal showed fruit rot incidence was low when Bordeaux mixture (1%) spray followed by Mandipropamid 250 SC. Mass production of black pepper plants (Sreekara) through somatic embryogenesis was standardized and tested for the presence of mycoendophytes. Soil drenching with cymoxanil 8% + mancozeb 64% at 0.3% concentration was highly effective in managing apple collar rot under nursery and orchard conditions.

BIOINFORMATICS

Secretome analyses of different *Phytophthora* species namely *P. capsici* (05-06 and 98-93), *P. sojae, P. infestans, P. ramorum* were completed at ICAR-IISR, Kozhikode and proteins with signal peptides were identified for further comparative genomics studies.

FUSARIUM

DIVERSITY

SRAP primers and 28s nuclear large rDNA region were used for the genetic diversity analysis of *Fusarium oxysporum* f. sp. *ciceris* (Foc) isolates. Morphological and molecular characterisation of *F. oxysporum* f. sp. *gladioli* and *F. oxysporum* f. sp. *dianthi* isolates from wilt affected gladiolus and carnation plants was studied. The isolates of *F. oxysporum* f. sp. *lycopersici* infecting chilli were characterised based on multi-gene analysis , RAPD-PCR anal-

ysis, BOX-PCR analysis and mating type sequences.

DIAGNOSTICS

A simple and rapid procedure was developed for molecular detection of *F. udum* and *F. oxysporum* f. sp. *ciceri* from soil using PCR detection. Seven avirulence genes from *F. oxysporum* f. sp. *ciceri* races were isolated and characterization.

HOST PATHOGEN INTERACTION

Differential expression pattern of mitogen-activated protein (MAP) kinase and MAP kinase kinase kinase (MAP-KKK) genes was studied using conventional and real time PCR assays in *Fusarium* infected resistant and susceptible chickpea plants.

HOST PLANT RESISTANCE

A grafting technique was standardized to establish seedlings with brinjal as root stock and tomato (cv. Kashiamman) as scion. Pigeon pea genotypes ICP 8862, ICP 89049, ICP 8858, BDN 1 showed resistant reaction to all the six variants of *F. udum*. Chickpea lines were screened for resistance, 19 desi chickpea lines and five Kabuli chickpea genotypes were found resistant to race 2 of *F. oxysporum* f.sp. *ciceri*.

DISEASE MANAGEMENT

Cymoxanil 8% + Mancozeb 64% (Curzate-M) @0.2% and *T. harzianum* Th4d SC @ 2 ml/kg were found to be effective in managing safflower wilt. In tomato, among the *Trichoderma* different isolates, Phyto 13 followed by Phyto 1, Phyto 3, Phyto 7, Phyto 4 showed significant reduction in wilt severity and higher yield. Soil application of wild endophytic *Trichoderma asperellum* recorded significant decrease in nematodes in *Fusarium* - nematode complex system of banana.

GENOMICS

Markers were identified and validated for molecular breeding and marker assisted selection in safflower for *Fusarium* wilt resistance. Differentially expressed genes were identified during the interaction between *Fusarium* and effective biocontrol agent in banana by suppressive subtractive hybridization.

RALSTONIA

DIVERSITY

Sixty new *Ralstonia solanacearum* isolates were collected from tomato, potato, brinjal, capsicum and ginger from different locations. Characterization of 30 *R. solanacear-* um isolates from Meghalaya, West Bengal and Chhatisgarh into biovars based on the ability to utilize disaccharides and oxidize hexose alcohols showed that isolates from East Khasi hills belonged to phylotype II and phylotype I, whereas isolates from West Bengal and Chhattisgarh belonged to phylotype II of the pathogen.

DIAGNOSTICS

Detection of *R. solanacearum* from soil using LAMP PCR was standardized and the sensitivity of detection was comparable with other techniques like PCR. The real time LAMP technique was standardized for the detection of *R. solanacearum* in ginger seed material at IISR Kozhikode.

HOST-PATHOGEN INTERACTION

Production of extracellular polysaccharide expression with *epsB* gene and reactive oxygen species (ROS) accumulation after infection *R. solanacearum* in susceptible and resistant tomato cultivars was studied. Pathway analysis of transcriptomes of ginger and mango ginger and tissue specific expression analysis of shortlisted genes/ ESTs in ginger using qPCR was completed. Inoculated and un inoculated sections of roots of *C. amada* and ginger were observed under light and fluorescent microscope.

HOST PLANT RESISTANCE

The local brinjal variety RL22 showed resistance against bacterial wilt similar to resistant variety Bholanath.

DISEASE MANAGEMENT

A grafting technique using cultivated brinjal on wild type was standardized of management of bacterial wilt. The mechanism of suppression of *R. solanacearum* with cloned product of Iturine gene was tested under *in vitro* conditions. Treatment with *P. fluorescens* DTPF-3 + *T. harzianum* Th3 in tomato crop resulted in reduction of bacterial wilt disease incidence. A formulation with *P. fluorescens* (IHRPf24) and *B. subtilis* (IHRBs39) was developed with talc, farmyard manure and coco peat at IIHR, Bengaluru. Apoplastic bacteria isolated from ginger apoplastic fluid were evaluated against bacterial wilt disease.

GENOMICS and BIOINFORMATICS

Genomes of four strains of *R. solanacearum* that cause potato bacterial wilt and *R. solanacearum* strain UTT-25 isolated from wilted tomato were sequenced. Whole genome sequencing of two strains of *R. solanacearum* from ginger was completed and the raw data has been assembled. Proteomes of both strains were compared against 10 other available strains of *R. solanacearum*.

Phytophthora

ICAR – CENTRAL CITRUS RESEARCH INSTITUTE, NAGPUR

Principal Investigator:
Dr. AK Das

Co-investigator:
Dr. IP Singh

A. DIVERSITY

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

A total of 23 *Phytophthora* spp. (11 isolates of *Phytophthora nicotianae*, one isolate each of *P. palmivora* and *P. insolita*, four isolates of *P. citrophthora*, two isolates of *P. tropicalis* and four *Phytophthora* spp. isolates) were isolated and purified from soil, root, leaf, fruit and water samples collected from citrus orchards situated in Vidarbha region of Maharashtra, Kodagu District of Karnataka, parts of Punjab and Mizoram states. These isolates (NRCPh-178- 200) are being maintained in sterile distilled water at 25ºC in *Phytophthora* Repository of NRCC. The same sets of cultures were also maintained in CMA plates by periodical subculturing in a BOD incubator at 25ºC.

Diversity analysis

Colony characteristics: Colonies of *P. nicotianae* isolates showed dense cottony mycelium to cottony aerial mycelium with no specific pattern of growth. *P. palmivora* isolate showed stellate pattern with uniform margin on V8 agar whereas less defined petalloid pattern with irregular margin was observed on PDA. *P. citrophthora* and *P. tropicalis* showed chrysanthemum pattern colony morphology on V8 agar (Fig. 1). The growth rate varied from 6.25 – 17.2 mm/ day in V8 agar and 4.5 – 11.6 mm/day in PDA for the above isolates.

Sporangial morphology of 23 isolates were 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 showed simple sympodial branching. All the isolates were found A1 mating types.

The colony, sporangial and oogonial morphology of *P. tropicalis* are shown in Fig 2. Sporangia have conspicuous papillae, usually single. Sporangial shapes were uniform, oboviod, pyriform or ellipsoidal. The culture was heterothallic, oogonia are spherical, with amphigynous antheridia. The two *P. tropicalis* isolates (NRCPh-179 and -182) did not grow at 35ºC. Both the isolates were found to be A1 mating type.
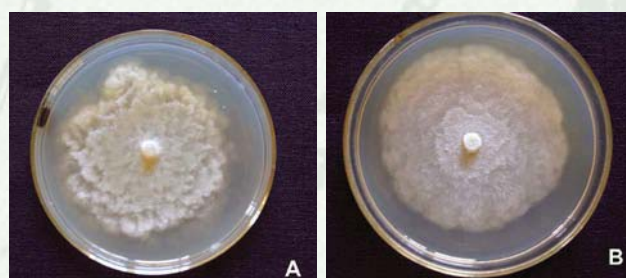

Fig. 1: Colony morphology of A. *Phytophthora citrophthora* (NRCPH-194) and B. *P. insolita* (NRCPH – 186) on V8 juice agar.
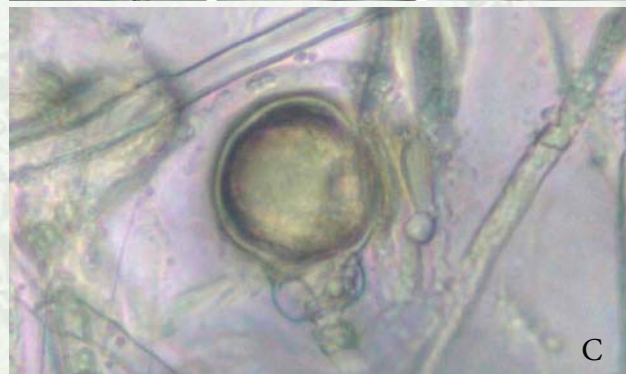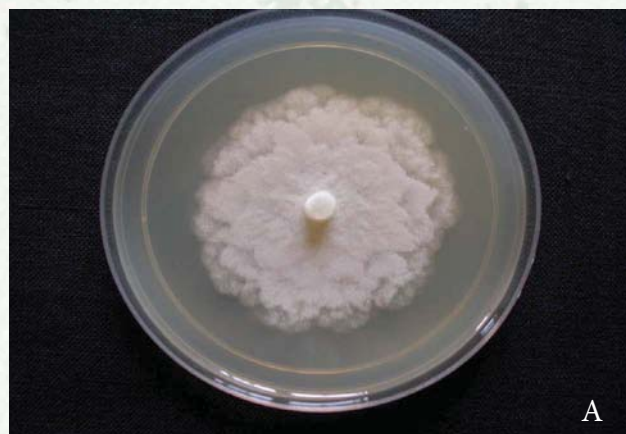

Fig. 2: Morphology of *Phytophthora tropicalis*. A. Chrysanthemum colony pattern on V8 juice agar. B. Sporangium C. Oogonium with amphigynous antheridium

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 29 (NRCPh 155 – NRCPh 183) isolates were found sensitive.

Molecular diversity analysis

Twenty seven isolates (Acc no. NRCPh 169-195) were analysed by PCR amplification of the ITS region using the primers ITS4 and ITS6. The PCR amplicons (~900 bp) obtained from all the isolates were subjected to ITS-RFLP using the restriction enzymes MspI and AluI. The results of ITS-RFLP analysis of all the isolates revealed difference in their restriction pattern. The RFLP banding patterns (that are characteristic of a species) identified four different *Phytophthora* spp. among the fifteen isolates (Fig. 3). A total of nine ITS sequences were submitted for *Phytophthora* spp. isolates at the GenBank database.
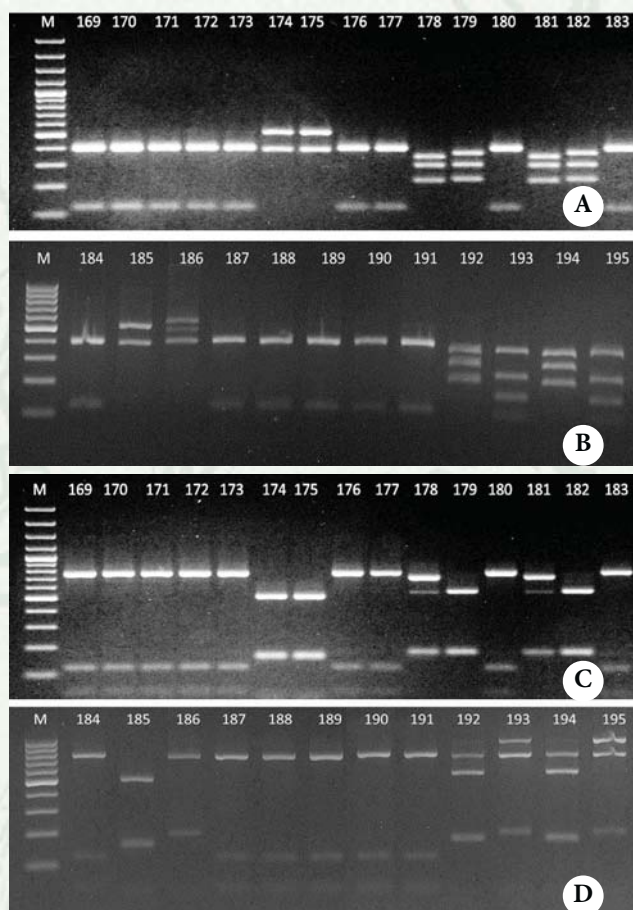

Fig. 3: RFLP profiles of *Phytophthora* isolates obtained after digestion of ITS4/ITS6 amplification products with AluI. Lane 169-195 – corresponding isolates; M – 100 bp marker.

B. DIAGNOSTICS

Development of SCAR marker for detection and identification of *P. nicotianae*

Towards developing a SCAR marker for *P. nicotianae* 20 RAPD primers (OPA1 to OPA 20) were checked on DNA isolated from pure cultures of eight *P. nicotianae* isolates (NRCPh-56, NRCPh-66, NRCPh-108, NRCPh-59, NRCPh-72, NRCPh-90, NRCPh-155 and NRCPh-109), *P. lacustris* (NRCPh-112), *P. boehmeriae* (NRCPh-114), *P. insolita* (NRCPh-119), *P. citrophthora* (NRCPh-147) and *P. palmivora* (NRCPh-148) isolate. All selected *Phytophthora* spp. isolates were from citrus but have different geographic origin. Out of 20 RAPD primers OPA 4 and OPA 10 showed amplification of distinct monomorphic band of ~380 bp in all the *P. nicotianae* isolates along with other polymorphic bands. In case of OPA 4 the ~380 bp band was more distinct than OPA10 hence RAPD primer OPA 4 was found to be more promising than primer OPA 10 for the development of species specific SCAR marker. Monomorphic band of ~380 bp from all eight *P. nicotianae* isolates used in this study was sequenced. Multiple sequence alignment of 341 bp core sequence from all nine *P. nicotianae* isolates was performed using MEGA 5 and a consensus sequence was obtained for primer designing. Manual designing of primer was done so as to get a full length amplification of desired product. These manual primers were analysed for GC content and Tm using Oligoanalyzer.

Out of three primers designed, SCAR4F -5' TACTCCTC-CATTCCACACG 3' and SCAR4R1 -5'AATCGGGCTGTGGGA-TAGCC 3' gave the desired result in all the *P. nicotianae* isolates with amplicon size of 330 bp (Fig. 4). The primer pair was further tested for the sensitivity and cross reactivity; in sensitivity it has given the amplification up to 10^{-2} dilutions. In cross reactivity check it has given amplification only in case of *P. nicotianae* isolates.
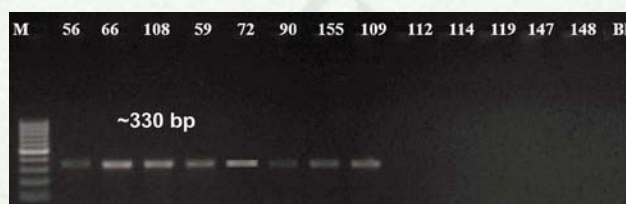
Fig. 4: Detection of *Phytophthora nicotianae* with SCAR primers SCAR4F-SCAR4R1. First eight lanes – Isolates of *P. nicotiana*; Next five lanes – *P. lacustris* (NRCPh-114), *P. insolita* (NRCPh-119), *P. citrophthora* (NRCPh-147) and *P. palmivora* (NRCPh-148); Bl – Blank; M – 100 bp marker.

Development of *P. nicotianae*-specific primers from extracellular cystatin-like protease inhibitor (epiC1) gene

Developing novel gene region for diagnosis of *P. nicotianae* Mining of the genomic data of *P. nicotianae* available at Broad institute, Cambridge, MA, USA website (https://

olive.broadinstitute.org/projects/phytophthora_parasitica) was carried out. EPIC region unique to *P. nicotianae* was selected from the genomic data, specific primer pairs (FEPIC1F/FEPIC1R) for this region were designed and validated for its use as diagnostic tool for species specific PCR based detection system. Cross reactivity of primers was tested on six species of *Phytophthora i.e. P. nicotianae, P. palmivora, P. citrophthora, P. boehmeriae, P. lacustris* and *P. insolita* (Fig. 5). Detection by these primers was done effectively upto 100 pg/μl of DNA isolated from pure cultures of *P. nicotianae*.
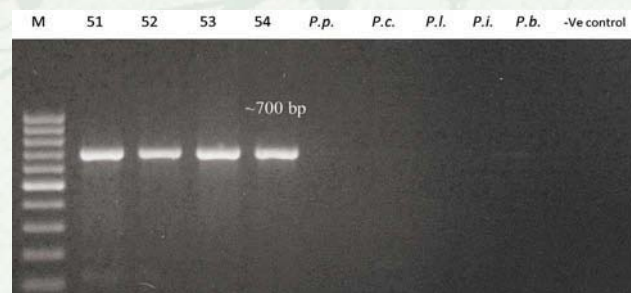
Fig. 5: Species specific detection of *Phytophthora nicotianae* using specific FEPIC1F/FEPIC1R primer. M – 100 bp ladder; First 4 lanes – *P. nicotianae* isolates followed by other *Phytophthora* spp.

C. HOST-PATHOGEN INTERACTION

Search for plant natural compounds against *Phytophthora*

In silico screening: The cell wall of *P. nicotianae* is mainly composed of two components *viz.,* ß- 1, 3 glucans and cellulose. All four cellulose synthase genes (CesA1 to CesA4) are coordinately upregulated during pre- and early infection stages of potato including NLP and EPIC proteins from different *Phytophthora* spp. were selected to serve as substrate in the docking study. Plant natural compounds from *Citrus* spp. that act as fungicides were selected from Dukes database (http://www.ars-grin.gov/duke/) and shortlisted as ligands (plant natural compounds) to be used for molecular docking in order to find the suitability/efficacy of plant natural compound as inhibitor for *P. nicotianae*. EPIC2B, cellulose synthase gene 1, necrosis inducing NLP proteins, cellulose synthase gene 2, cellulose synthase gene 3 and cellulose synthase gene 4 with the selected ligands *viz.,* 1, 8-cineole, alpha-phellandrene, citronellal, ferulic acid, geraniol, limonene, linalool, nobiletin, p-cymene, perillaldehyde, selenium, seselin, sinensetin, tangeretin, terpenen-4-ol and terpinolene.

D. HOST RESISTANCE

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

Nine rootstock seedlings (including six hybrid rootstocks) were raised in polybags for screening against *Phytoph-*

thora *viz.,* NRCC-1 (Rough lemon x Troyer citrange) 113/L10P1, NRCC-2 (Rough lemon x Troyer citrange) 113/L2P2, NRCC-3 (Rough lemon x Troyer citrange) 113/ L1P1, NRCC-4 (Rough lemon x Trifoliate) 113/ L3P1 , NRCC-5 (Rough lemon x Troyer citrange) 113/ L11P3, NRCC-6 (Rough lemon x Troyer citrange) 113/ L11P2, Galgal (*C. pseudolimon*), Volkamer lemon (*C. volkameriana*) 113/L7P1, and Khasi papeda (*C. latipes*). These rootstock seedlings were inoculated with *P. nicotianae* (chlamydospores and freshly released zoospores). Results indicated that all the rootstocks were susceptible to highly susceptible (Fig. 6) except Khasi Papeda and NRCC-2 which showed moderately tolerant reaction.

Fig. 6: Reaction of citrus rootstocks to *Phytophthora* inoculation under glasshouse conditions

A new set of eight rootstock seedlings were being raised in polybags in glasshouse conditions for screening against *Phytophthora* root rot *viz.,* Smooth Flat Seville (SFS), Rough lemon Rahuri, Sour orange Tirupati, SFS x Argentina trifoliate orange, Florida Rough lemon, Sun Chu Sha (mandarin), X-639 and Kusai Rangpur.

E. DISEASE MANAGEMENT

Efficacy of dazomet in reducing soil-borne pathogen population

Nursery soil naturally infested with *Phytophthora* spp. was fumigated with dazomet granules (Basamid, ob-

tained from M/S Margo Pvt. Ltd., Pune) @ 40 g / m^3 of soil and covered with polythene sheet for 10 days. After 10 days, the soil was made loose for evaporation of left-over residues of the fumigant. The soil samples were tested for *Phytophthora* and *Pythium* spp. on selective media. No propagule count was recorded, indicating the efficacy of dazomet fumigation in eliminating *Phytophthora* from nursery soil.

In vitro efficacy of bacterial bioagents

The rhizospheric soil was collected from the citrus orchards in NRCC farm site, Nagpur and six isolates of *P. fluorescens* were obtained. Among all the *P. fluorescens* isolates, Ps5 was found to be promising by completely arresting the growth of *P. nicotianae*.

One-week-old mycelial mat showing green sporulation grown in potato dextrose broth was aseptically separat-ed from the broth and mixed with 1 kg talcum powder and 0.5 g of carboxy methyl cellulose sodium salt (CMC). The formulation was then packed in plastic bags under laminar air flow. Periodic counting of *Trichoderma* as cfu/g in the formulation kept at room temperature was carried out for consecutive nine months. Results revealed that even after eight months of storage, formulation had viable propagule count of 25 x 10^6/g.

Integrated management of *Phytophthora* root rot and gummosis in Nagpur mandarin

A field trial was laid out at a farmer's orchard in Nagpur mandarin on rough lemon rootstock with Bordeaux paste, two strains of *T. harzianum* and Fosetyl Al alone and in combination. Observations with respect to disease and growth parameters are being recorded.

ICAR-CENTRAL PLANTATION CROPS RESEARCH INSTITUTE, KASARAGOD

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Co-investigators:
Dr. Prathibha VH and Ms. Chaitra M

A. DIVERSITY

Isolation and characterization of *Phytophthora*

Survey for arecanut fruit rot disease incidence in endemic areas of Karnataka State and collection of disease infected samples for *Phytophthora* isolation during 2014-15 monsoon season yielded 13 isolates, 12 from fruit rot infected samples and one from a single isolate crown rot of an arecanut palm. Among the disease endemic areas, the highest incidence of fruit rot was observed in Karkala of Udupi District, closely followed by Sirsi of Uttara Kannada District of Karnataka. Including the new isolates, a total of 51 isolates of *P. meadii* causing fruit rot of arecanut in major areca growing areas were characterized and maintained.

Cultural and morphological characters of *Phytophthora* isolates were studied to identify the species and the extent of variability. The colony diameter of *P. meadii* isolates of arecanut varied from 42.33 mm (CPCRI-PT-048) to 67.00 mm (CPCRI-PT-053). Variations were also observed in shape and size of the sporangia and pedicel length. Majority of the isolates exhibited stellate/striate/rosaceous pattern of growth with ovoid to ellipsoidal in shape with a round base and conspicuous papilla type sporangia. Sporangial stalk was thin, slender and short. Thus all the arecanut isolates collected from disease infected arecanut gardens were identified as *P. meadii*.

Genetic diversity in *Phytophthora meadii*

RAPD analysis: Cultural, morphological and pathogenic characterization of *Phytophthora* isolates of arecanut collected from different locations of South India revealed distinct variations not only between species but also among the isolates within a species, especially *P. meadii*. Based on initial screening of 24 RAPD primers, 12 primers were selected for analysis of genetic variation on 28 isolates of *P. meadii* and two isolates of *P. heveae* of arecanut fruit rot. Both of the *P. heveae* could easily be distinguished with the 12 selected primers (Fig. 1). Thirty *Phytophthora* isolates analysed were forming into two distinct main clusters. There were two sub clusters under the first main cluster (Fig. 2). *P. meadii* isolates formed one group whereas the two *P. heveae* formed a separate cluster. The patterns within *P. meadii* were quite similar with

some intra-specific variations. The *Phytophthora* isolates did not cluster according to geographical origin, virulence or morphological variations.

Fig. 1: Genetic diversity in *P. meadii* isolates as evidenced by PCR fingerprint patterns using RAPD primers.
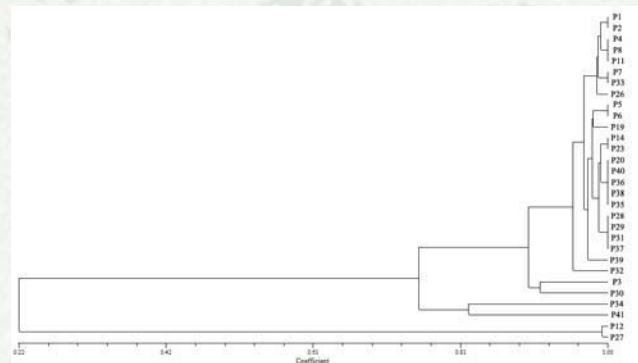
Fig. 2: Dendrogram from UPGMA analysis using simple matching coefficient based on RAPD bands of *Phytophthora* isolates from infected arecanut

B. DIAGNOSTICS

Real- time PCR detection of *P. meadii*

A total of five pairs of primers from ITS region were designed using Primer 3 software. Under optimal conditions, the primer PM1F and PM1R specifically amplified at 180 bp sequence of DNA. Real time PCR cyber green reaction was carried out in Biorad Mini Opticon system. The primers PM1F and PM1R specifically amplified *P. meadii*. Amplification also happened with closely related species in the Clade II *viz.* *Phytophthora citrophthora* and *P. colocasiae*, but not with any other species of *Phytophthora*.

C. DISEASE MANAGEMENT

Cultural and morphological characterization of selected *Trichoderma* isolates

Cultural and morphological characters of 18 *Trichoderma*

isolates were studied. The colony morphology was depressed, cottony to fluffy with aerial mycelium and the colony color varied from green, yellowish green to white mycelial growth.

Antagonistic activities of 18 isolates of *Trichoderma* spp. against common pathogens of arecanut, cocoa and coconut namely *P. meadii, P. palmivora, Ganoderma lucidum, Theilaviopsis paradoxa* and *Lasiodiplodia theobromae* were analyzed. *Trichoderma harzianum* isolate CPCRI-TD-28 was found to be the most promising isolate. There was a wide variability among the *Trichoderma* isolates in their antagonistic potential against the pathogens tested. *L. theobromae* was effectively inhibited by isolate CPCRI-TD-03 (76.23%), whereas *T. paradoxa* by isolate CPCRI-TD-04 (72.76%), *P. meadii* by CPCRI-TD-07 (75.47%) and *G. lucidum* showed 95.60% inhibition by isolate CPCRI-TD-28.

Screening of *Trichoderma* isolates for cellulase enzyme activity and their effect on plant growth promotion

Growth promoting activities of 18 *Trichoderma* isolates were studied on green gram seeds by evaluating shoot length, root length, fresh weight, dry weight, germination percentage and vigor index. Statistical analysis showed significant positive effects on germination in treatments with *Trichoderma* isolates *viz*, CPCRI-TD-28, TD-13, TD-11, TD-16, TD-03, TD-02 and TD-06. There was wide variability among the *Trichoderma* isolates in promoting seed germination, ranged from 66.66 to 100.0%. But all the *Trichoderma* isolates showed significant effect on shoot length, root length, fresh weight, dry weight and vigor index. *Trichoderma* isolate CPCRI-TD-16 was found to be superior in plant growth promoting activity compared to other *Trichoderma* isolates (Fig. 3).

Cellulase enzyme activity of all the *Trichoderma* isolates was quantitatively measured by DNS spectrophotometric assay. Reaction mixture consisted of 0.5 ml of 1% CMC in 0.1 M sodium citrate buffer (pH, 4.8) and 0.5 ml of supernatant added, then incubated at 50°C for 30 min. The reaction was stopped by adding 3 ml DNS and incubated in a boiling water bath for 5 min and cooled. The absorbance was measured at 540 nm. Our research reveals that there was slight variation in cellulase activity by *Trichoderma* spp. during mycoparasitism. Highest cellulase activity was recorded by CPCRI-TD-16 (28.04 mg glucose liberated/mg protein/30 min) whereas for CPCRI-TD-28 and TD-3 cellulase activity was 26.88, 24.96 mg glucose liberated/mg protein/30 min, respectively. Lower activities were detected with TD-13 and TD-15 (16.77, 17.98 mg glucose liberated/mg protein/30 min).
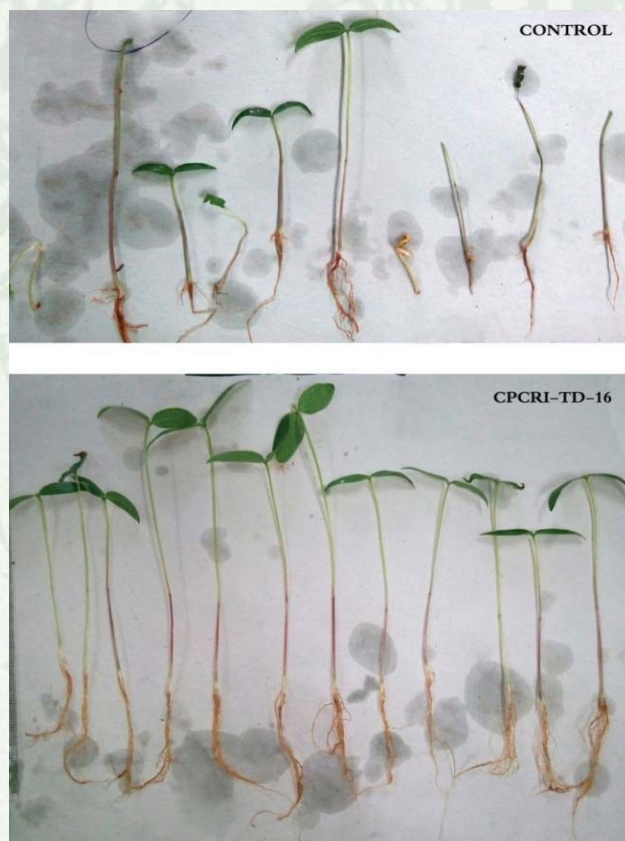
Fig. 3: Effect of *Trichoderma* on growth of green gram plant in treated seeds and untreated seeds

Effect of environmental factors on *Phytophthora* disease

Data regarding climatic factors such as temperature, relative humidity, rainfall on arecanut fruit rot incidence were recorded daily from three locations *viz.*, Kidu, Vittal and Dharmasthala. The disease incidence was noticed from 1st week of August in Kidu, Vittal and 3rd week of August in Dharmasthala. The disease reached to maximum during 4th week of August in Kidu, Vittal and reached maximum during 2nd week of September in Dharmasthala. The compiled climatic data were correlated with the disease incidence. Development of a disease prediction model for arecanut fruit rot is in progress.

Field trials on management of fruit rot disease of arecanut

Field trial on management of fruit rot disease of arecanut was conducted in CPCRI, RC, Kidu, Vittal and farmer's field at Dharmasthala with nine treatments. The observation on fruit rot disease incidence was recorded on alternate days. Lower fruit rot disease incidence was observed in 1% Bordeaux mixture sprayed plots followed by Mandipropamid 250 SC in all three locations.

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A. DIVERSITY

Collection, maintenance and DNA isolation of *P. infestans*

Over 130 *P. infestans* isolates were purified on rye agar medium from infected potato samples collected from different geographic locations *viz.,* Himachal Pradesh, Meghalaya, Karnataka, Uttarakhand and Uttar Pradesh and maintained. DNA from 210 isolates was extracted and stored.

Determination of ploidy status of *P. infestans* population

Ploidy status of 100 isolates of *P. infestans* collected from Himachal Pradesh, Meghalaya, Tamil Nadu, Punjab, Uttar Pradesh, Madhya Pradesh, Bihar, West Bengal and Karnataka was determined using Feulgen cytophotometry. Results revealed that *P. infestans* population in the country are polyploid consisting of diploids, triploids and tetraploids. However, frequency of different polyploids varied from region to region. The frequency of diploids, triploids and tetraploids was 86, 10 and 4%, respectively. In Indian hills (North-western, north-eastern and southern hills), frequency of diploids was highest (85%) followed by triploids (14%) and tetraploids (1%). The same trend was observed in sub-tropical plains while in plateau and in Madhya Pradesh only diploids were found.

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

Out of total 20 published SSR markers only five markers *viz*, PinfSSR1, Pi04, PinfSSR2, 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 133 isolates of *P. infestans* (Fig. 1). Three alleles (164, 172 and 186 bp) were observed with primer Pi04 (Fig. 2) in 21 isolates of *P. infestans*. Among three alleles, 172 bp size loci were observed in most of the isolates. Mostly two alleles (170 and 184 bp) were observed with primer Pinf SSR2 (Fig. 3).
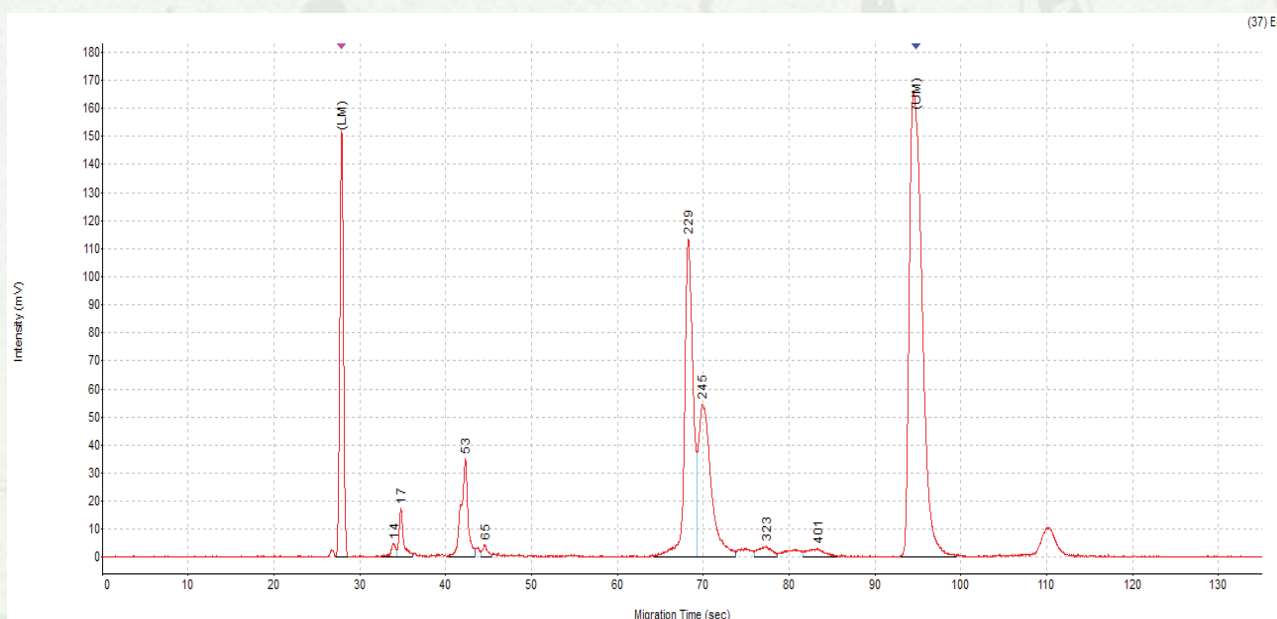
Fig.1: Electropherogram of *P. infestans* isolate with Pinf SSR1 primer
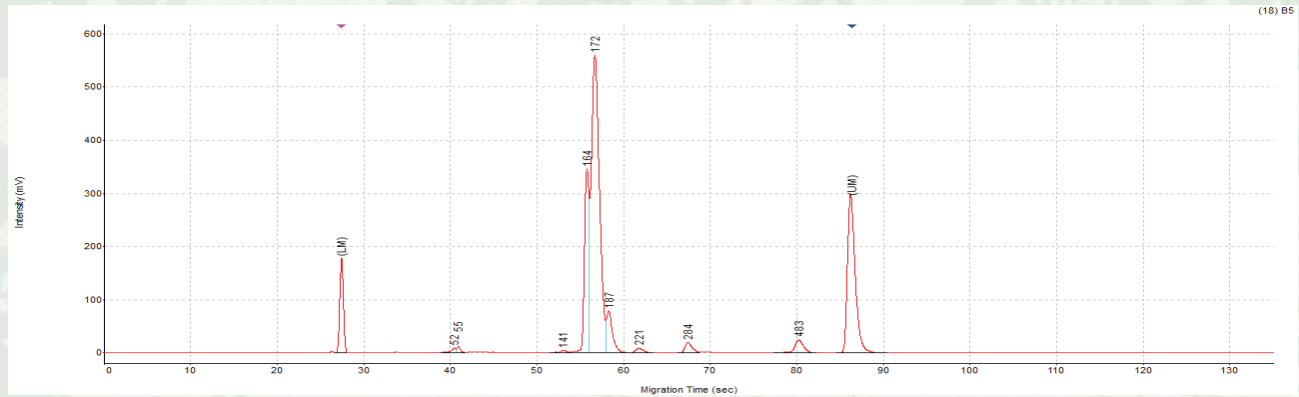
Fig. 2: Electropherogram of *P. infestans* isolate with Pi04 primer
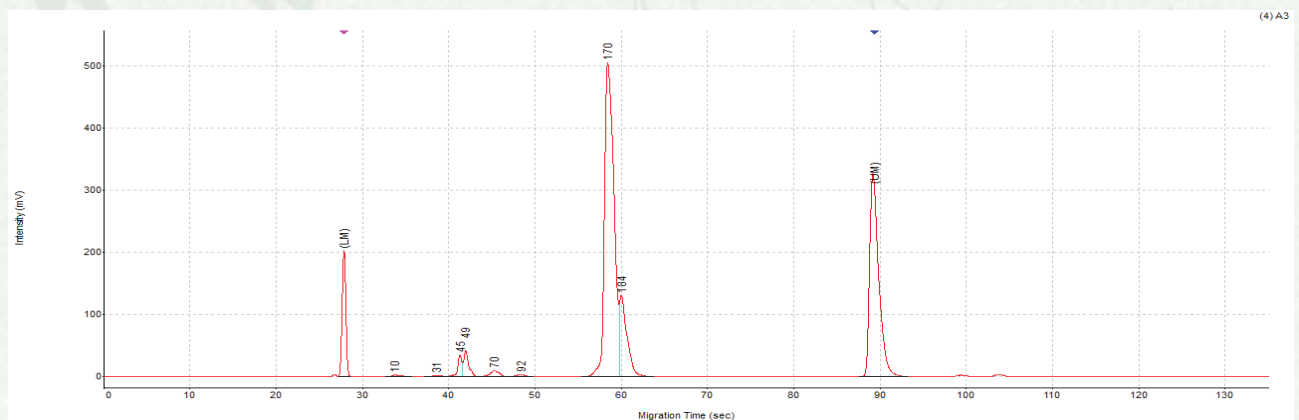
Fig. 3: Electropherogram of *P. infestans* isolate with SSR2 primer showing two alleles

Mt haplotype analysis for lineage determination

Mitochondrial haplotyping was done with the sub set of population comprising 72 isolates collected from Himachal Pradesh, Meghalaya and Karnataka with primer F2-R2 (digested with *MspI*) and F4-R4 (digested with *EcoR1*) and found that all the isolates belonged to the Ia haplotype (Fig. 4). 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.
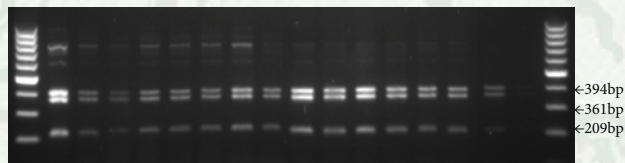
Fig. 4: Mt haplotyping with primer F4F4 & F4R4 digested with *EcoR1* Lane: 1-15 *P. infestans* isolates M: 100 bp N: Negative control

B. DIAGNOSTICS

Detection and quantification of *P. infestans* in host tissues

Duplex PCR protocol was revalidated along with SCAR marker for the simultaneous detection of *P. infestans* and *Alternaria solani* in a single reaction using both genomic DNA and infected tissues of potato as such (Figs. 5 & 6). Further, new primers specific to *P. infestans* were designed using bioinformatics tools and synthesized.
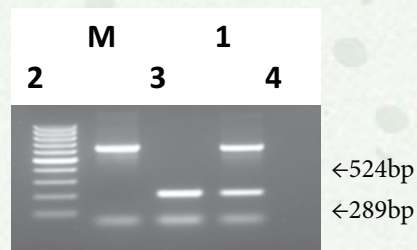
Fig. 5: PCR detection of genomic DNA of *P. infestans* and *A. solani* in duplex PCR Lane: 1= *P. infestans*, 2= *A. solani*, 3= combined *P. infestans* and *A. solani,* N= negative control, M=100 bp DNA ladder

Fig. 6: PCR detection of infected tissues with *P. infestans* and *A. solani* in duplex PCR, Lane 1: *P. infestans* infected leaves with SCAR maker, 2: *A. solani* infected leaves, with NBAIMSbt primer, 3: Duplex PCR products of *P. infestans* and *A. solani*, M: 100 bp DNA ladder (Fermentas)

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

Polyclonal antibody of cell wall bound cellulose binding protein (CBD) of *P. infestans* was raised in rabbits, purified, analyzed by SDS PAGE (90%) and validated through DAC-ELISA. The expression of CBD1 was detected two fold over buffer control in mycelium, sporangium and zoospores as well as in infected leaf samples using DAC-ELISA. Data indicated that the produced recombinant antiserum was efficient and accurate in determining negative and positive results in ELISA tests. Though it is efficient in detecting the *P. infestans* it also showed cross reactivity with other *Phytophthora* spp. like *P. capsici*.

C. HOST-PATHOGEN INTERACTION

Development of *P. infestans Pol* gene RNAi construct

RNAi-mediated silencing of *P. infestans Pol* gene for imparting late blight resistance: Towards developing the RNAi construct to silence the *Pol* gene expression in transgenic potato, a 307 bp cDNA sequence of *Pol* was included for construction of sense and antisense fragment (Fig. 7) of iIR-GypsyPi-1 (*Phytophthora* Gypsy like element GypsyPi-1a transposon gene). Invertase GBSS intron fragment of 105 bp in length corresponds to 3821-3925 of potato GBSS genomic DNA sequence out of which 3835-3919 sequence is intron (Ref. Gene Bank accession no. X58453). The iIR-GypsyPi-1a gene construct under the control of constitutive promoter CaMV 35S was placed on MCS of binary plant transformation vector pRI101.pRI201::iIR-GypsyPi-1a and was introduced into *Agrobacterium tumefaciens* strain, EHA105, by freeze-thaw method for genetic transformation of popular Indian cultivars.

Fig. 7: (A) PCR amplification of *Phytophthora infestans* gypsy-like retrotransposon GypsyPi-1a gene sequence cDNA for use in sense (S) or antisense (A) orientation for RNAi gene construct. (B) Restriction digestion of pUC19 cloning vector confirming the sense (Xba1 & BamH1), antisense and intron orientation of *P. infestans* gypsy-like retrotransposon GypsyPi-1a gene sequence.

Whole genome sequencing of *Phytophthora infestans* A2 mating type

Complete mitochondrial genome of *P. infestans* A2 mating type was sequenced. The genome size of 37,845 bp, possesses a GC content of 22.38% which is slightly higher than all A1 types. A total of 61 protein coding genes are predicted on strands which included twenty six transfer RNA (one tRNA coding for an unknown amino acid), two ribosomal RNA, and 18 respiratory protein coding genes. The gene order of A2 mating type was consistent with that established in all A1 types, despite of high level of polymorphism in both coding and non-coding regions. The highest variation was observed in non-coding region. A large spacer flanked by the gene coding for tRNA-Tyr (*trnY*) was found that varied in size with all A1 types (Fig. 8). The mt DNA of A2 mating was found to have 60.49% homology with *P. andina*. Homology study of complete *cox1* gene sequence revealed the relationship among 50 different *Phytophthora* species.

Fig. 8: Mitochondrial genome map of *P. infestans* A2 mating type (Ia)

D. HOST PLANT RESISTANCE

Sequence analyses of RB-homologous gene fragments isolated from wild *Solanum* species in potato

The seventeen non-redundant sequences of RB-homologous gene fragments identified from the 15 wild potato species with un-interrupted open reading frames (ORFs) and high amino acid sequence homologies to various nucleotide binding site leucine-rich repeat (NBS-LRR) proteins from GenBank database. Alignment of the DNA (protein) sequence analysis of the NBS domain revealed 17 RGAs belong to the disease resistance protein. The RGAs consisted of three major conserved domains such

as i) Leucine-rich repeat containing protein, ii) disease resistance protein (TIN-NBS-LRR class), and iii) L-domain like structures .

Screening of wild *Solanum* species for late blight resistance

In vitro grown plants from wild potato accessions (Table 1) were screened for late blight resistance by challenge inoculation of *P. infestans*. Except three accessions of *S. polytrichon* (CGN17750, CGN17751 and CGN22361) which were susceptible, other accessions showed varying degree of resistance. Three accessions of *S. jamesii* and two accessions of *S. trifidum* (CGN18335 and PI255538) could not be tested due to poor survival/growth in the pot under glasshouse conditions.

Table 1: Wild accessions selected for re-testing for late blight resistance after challenge inoculation of *P. infestans*.

Species	Genbank accessions
S. berthaultii (BER)	PI265858, PI310925, PI498096
S. cardiophyllum (CPH)	CGN18325, CGN18326, PI283063, PI595465
S. iopetalum (IOP)	PI239402, PI275180
S. jamesii (JAM)	CGN18349, CIP762777, PI558089
S. lesteri (LES)	CGN18337, CGN23988
S. microdontum (MCD)	PI473171, PI195185
S. pinnatisectum (PNT)	CGN17740, CGN17741, CGN17744, CGN18331, CGN23011, CGN23012
S. polyadenium (PLD)	CGN17746, CGN17748, CGN17749, CGN23013, CGN23014, CIP761014, CIP760724, PI275237, PI275238, PI558443, PI558445, PI310963, PI320342
S. polytrichon (PLT)	CGN17750, CGN17751, CGN18318, CGN22361
S. trifidum (TRF)	CGN18335, PI255537, PI255538, PI255539, PI255541, PI255542, PI283064, PI283065, PI283104
S. verrucosum (VER)	PI275256, PI275257, PI275258, PI275259, PI275260

Allele mining for late blight resistance in *Solanum* species

Out of total 39 wild species (99 accessions) tested for late blight resistance by challenge inoculation of *P. infestans* over the last three years, 11 highly resistant wild species were selected for allele mining of known potato R genes. The selected species were; *S. berthaultii* (BER), *S. cardiophyllum* (CPH), *S. iopetalum* (IOP), *S. jamesii* (JAM), *S. lesteri* (LES), *S. microdontum* (MCD), *S. pinnatisectum* (PNT), *S. polyadenium* (PLD), *S. polytrichon* (PLT), *S. trifidum* (TRF) and *S. verrucosum* (VER). The known potato R genes conferring resistance to late blight namely Rpi-pta1, Rpi-edn1.1, EDNR2GH7, Rpi-hjt1.1, SNKR2GH5, Rpi-snk1.1, Rpi-blb1/RB, Rpi-vnt1.1, Rpi-bt1, Rpi-sto1, Rpi-blb2, RGA1, RGA3 and RGA4 were used for allele mining in these species. Several full gene-specific primers were designed and amplified in the species (Fig. 9). These were cloned in TA vector and sequenced for analysis of allelic variants.
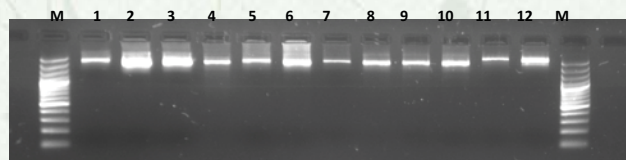

Fig. 9: PCR amplification of R gene from the wild species. Lane 1 – 3PTA/CPH62 (Rpi-pta1); Lane 2 – 3PTA/MCD24 (Rpi-pta1); Lane 3 – 3PTA/CPH87 (Rpi-pta1); Lane 4 – 3PTA/LES34 (Rpi-pta1); Lane 5 – EDN1.1/BER457 (rpi-edn1.1); Lane 6 – SNKR2GH5/LES34 (SNKR2GH5); Lane 7 – SNK1.1/JAM07 (Rpi-snk1.1); Lane 8 – BLB1/PLT62 (Rpi-blb1); Lane 9 – BLB2/STO61 (Rpi-blb1); Lane 10 – BLB1/STO40 (Rpi-blb1); Lane 11 – BLB2/LES29 (Rpi-blb2); Lane 12 – BLB2/PLD80 (Rpi-blb1) and M - marker

Cloning of late blight resistance genes from wild potato species

Allelic variant of the late blight resistance gene Rpi-pta1 (2.5 kb) isolated from the late blight resistant wild potato species *S. cardiophyllum* was cloned in a binary vector pRI101AN at the restriction sites of Kpn I-EcoRI. Gene insert was confirmed by colony PCR and restriction digestion and sequenced for analysis of allelic variants in progress.

Identification of genotypes with multiple resistance genes

The 16 clones identified last year possessing R1 and R3a genes in stacked conditions were further evaluated for late blight resistance and for desirable agronomic traits and were advanced to F1C2 generation. Another 560 clones possessing multiple disease resistance genes were advanced to F1C1 generation. Hybridization was attempted to combine multiple resistance genes to late blight (R1 and R3a), PVY (Ryadg, Rysto) and potato cyst nematodes (H1, HC_QRL and Gro1-4) using molecular markers. Seeds were extracted from eight successful crosses which were raised under controlled glass house conditions. Validation of genotypes possessing

known novel late blight resistance genes (blb1, blb2) and PVY(Rychc) was done through PCR based markers. Further, core collection of exotic and indigenous tuberosum and andigena parental lines is being screened through these PCR markers. Two genotypes viz., Sarpo Mira and Toluca possessing novel late blight resistance genes were imported from The Netherlands and conserved in vitro for further multiplication and validation of genes.

E. DISEASE MANAGEMENT

Testing of rhamnolipid based formulation against P. infestans under field condition

Different concentrations (2.5%, 3%, 4%, 5%, 7%, 9%, and 11%) of botanical formulation were tested with rhamnolipid bioformulation (0.25%) for its phytotoxicity on potato leaves using detached leaf method and whole plant method. No phytotoxicity was observed at 4% concentration of botanical and 0.25% rhamnolipid.
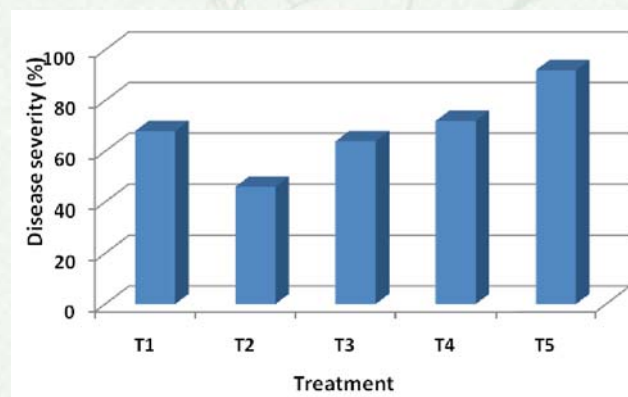

Fig. 10: Efficacy of botanical +rhamnolipid formulation against late blight in field conditions. (T1: Mancozeb, T2: Chlorothalonil, T3: Propineb, T4: Botanical+rhamnolipid, T5: Control)

Therefore, this combination was tested under field conditions in Shimla hills against standard contact fungicides. Results revealed that minimum terminal disease severity (46%) was observed in chlorothalonil treated plots followed by propineb (64%), mancozeb (68%) and botanical+rhamnolipid (72%) as against of 92% in untreated plots (Fig. 10).

Thermal adaptation of P. infestans isolates

A subset of 47 isolates of P. infestans was analyzed for their thermal adaptation at 25°C and 28°C. Results revealed that 53.33% isolates from Uttar Pradesh and hundred per cent from Shimla and Shillong were fast growing and 46.67% were slow growing from UP at 25°C (Fig. 11). Whereas 14.29% isolates were fast growing and 85.71% were slow growing from Uttrakahand at 25°C. On the other hand, all isolates were slow growing at 28°C. In another study, a subset of 29 isolates which grew at 28°C were further studied at 30°C for their thermal adaptation. Results revealed that the isolates from all regions (western hills, Tarai region, north central plain and Bengal) could grow (diameter from 0.91 to1.26 cm) at 30°C.
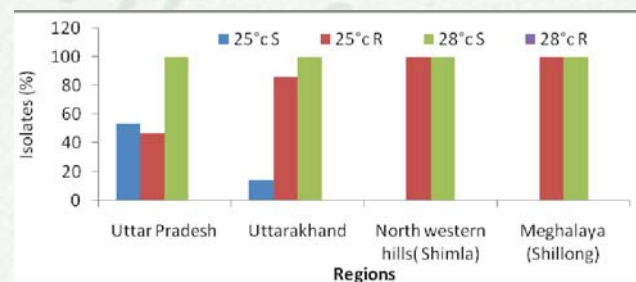

Fig.11: Thermal adaptation of P. infestans isolates at 25°C and 28°C

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A. DIVERSITY

P. colocasiae isolation and PCR confirmation

Twenty *P. colocasiae* isolates were added to the existing culture collection. Yearly variation in *P. colocasiae* isolates from the same field was studied for all the isolates. Species level identification was done using RL-F/RL-R primer pairs.

Genetic diversity analysis using RAMS markers

The genetic diversity of the *P. colocasiae* isolates were performed using Random Amplified Microsatellite (RAMS) markers. A total of 40 isolates were amplified using the primers described by Hantula *et al.* (1997). The results showed that a considerable amount of genetic diversity exists among *P. colocasiae* collected from various geographical boundaries of India. The isolates from similar origins were grouped into sub-populations and their degree of diversity was calculated. Genetic diversity, varied among populations with the PPB values ranging from 45.45 % (Kerala) to 72.73 % (Andhra Pradesh), with an average of 61.365%. The average Nei's gene diversity (H) was estimated to be 0.10 within populations and 0.11 for the pooled populations. The dendrogram generated from the RAMS data grouped *P. colocasiae* isolates irrespective of their geographical origin or phenotypic characters. This reinforces the fact that *P. colocasiae* frequently move across the country creating new strains in the process.

Multigene molecular phylogeny of P. colocasiae isolates

Multigene molecular phylogeny was carried out using selected target genes/loci. A total of 50 *P. colocasiae* isolates representing the major taro growing regions of India were used for the study. Amplification of targets ITS rDNA, Beta Tubulin, Larger Subunit (LSU) has been completed and currently sequencing and phylogenetic analysis is under progress.

B. DIAGNOSTICS

qPCR based resistance screening of taro

A rapid and reliable method to quantify the pathogen load in the infected plant tissue towards resistance screening in taro cultivars has been established using qPCR assay.
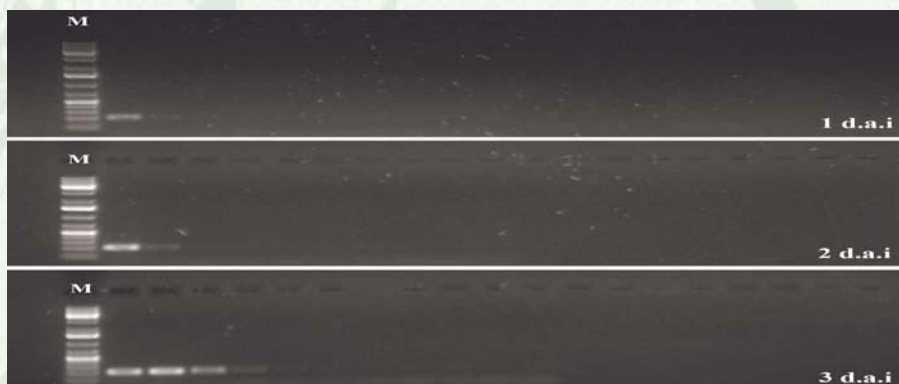

Fig. 1: Agarose gel image showing the extent of *P. colocasiae* infection in cultivar Sree Kiran at various days after inoculation (d.a.i.). A 1:10 dilution series of the total genomic DNA from infected sample was used as a template for the amplification.

C. HOST-PATHOGEN INTERACTION

Identification of P. colocasiae genes differentially expressed during infection on taro

Specific suite of genes that may have a putative role in pathogenicity of *P. colocasiae* were identified by SSH approach. The expressed genes validation by reverse northern and qRT-PCR analysis is in progress. The study revealed that a greater portion of the genes identified belong to the biological process category followed by molecular and cellular component category.
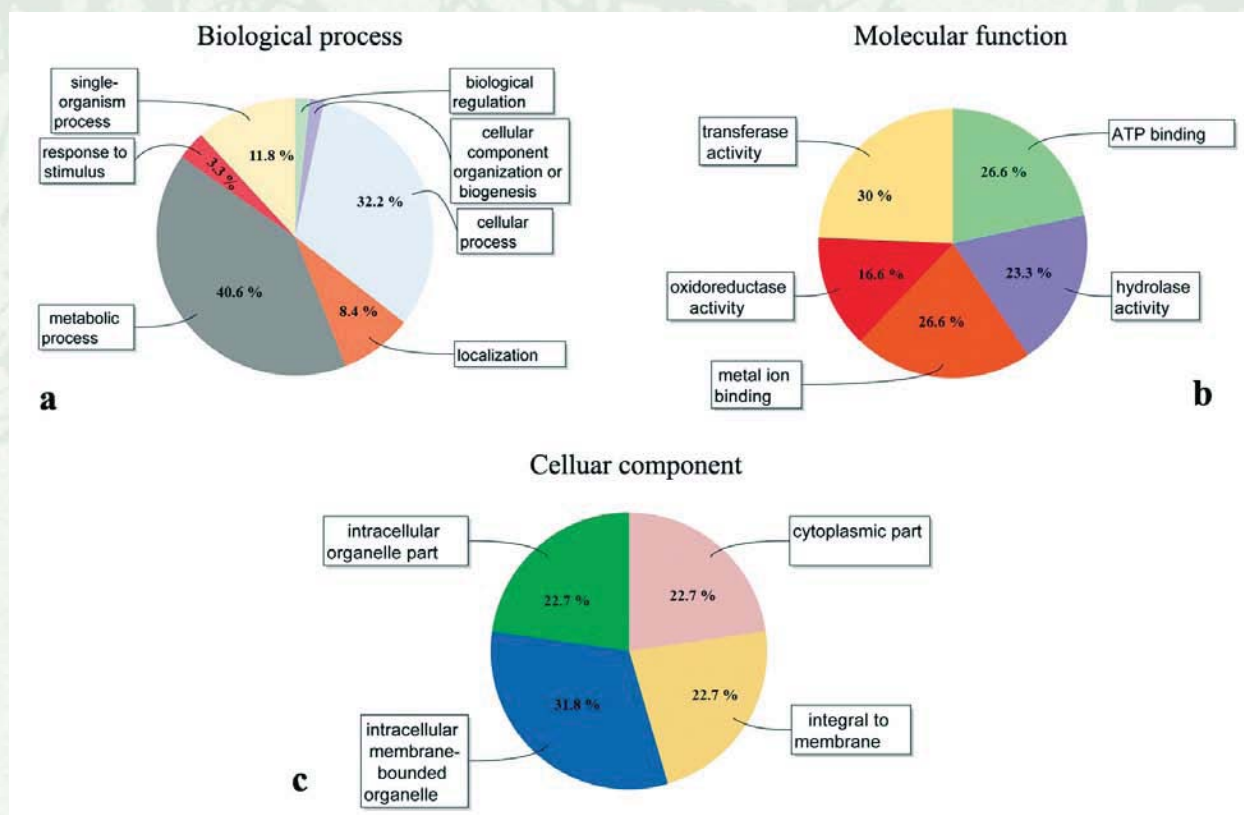
Fig. 2: Gene ontology annotations of *P. colocasiae* ESTs obtained through the SSH technique (a) biological process (b) molecular function (c) cellular function

D. HOST RESISTANCE

Grouping of accessions based on pathogenicity assay

A total of 234 accessions were evaluated in the field condition using the disease incidence scale. Three accessions (U64, TCR 125 and IC204065) were found to be resistant (R) to leaf blight, 39 were found to be moderately resistant (MR) and the remaining were susceptible to leaf blight.

Identification of resistant gene analogues (RGAs) in taro

Reported degenerate primers targeting various motifs were used to isolate the putative RGAs (NBS-LRR domains) from representative taro cultivars previously classified as resistant (R), moderately resistant (MR), susceptible (S) and highly susceptible (HS). PCR cycling conditions have been standardized and sequencing of the amplified fragments (approx. 500 bp) is in progress (Fig. 3).
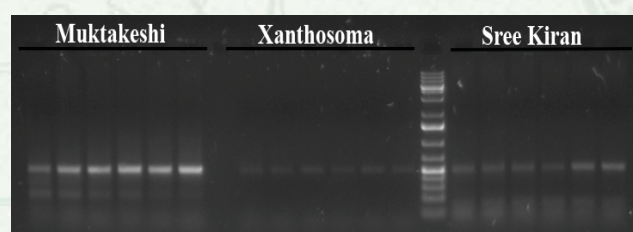

Fig. 3: Amplification of RGAs using degenerate primers

E. DISEASE MANAGEMENT

Field evaluation of the effective biocontrol agent

The effective biocontrol agent identified *in vitro* was evaluated for its efficacy in controlling leaf blight in field conditions.

T1- Tuber treatment with *Trichoderma harzianum* (CTCRI-T7)

T2- Tuber treatment with bacteria (CTCRI- 2B17)

T3- Soil + tuber treatment with *Trichoderma*

T4- Soil + tuber treatment with bacteria

T5- Soil+ tuber + spraying with *Trichoderma*

T6- Soil + tuber + spraying with bacteria

T7- Soil + tuber treatment with *Trichoderma* + spraying fungicide (0.5 % of TATA Samarth: Hexaconazole, 2% SC)

T8- Tuber treatment with fungicide

T9- Cowdung slurry alone

T10- Soil + tuber treatment with bacteria + spraying fungicide

T11- Tuber treatment + spraying fungicide

T12- Control

There was no significant difference in disease severity between treatments, the plant height was significantly higher when tubers were treated with *Trichoderma* (T1, T3, T5 and T7). Application of *Trichoderma* in soil along with tuber treatment and spraying 0.5% of TATA Samarth (Hexaconazole, 2% SC) recorded the highest yield.

Table 1: Biometric parameters of plants observed in various treatments along with their disease incidence and tuber yield

Treatment	No. of leaves*	Plant height (cm) at 6 months*	PDI	Yield (tha-1)*
T1	6.00A	91.50A	57.50	13.1BC
T2	4.00I	46.00C	50	6.9FG
T3	5.00C	92.50A	55	12.2CD
T4	4.00H	38.50C	57.5	13.6BC
T5	4.00G	83.00AB	50.75	6.2G
T6	5.00D	38.00C	55.66	9.2EF
T7	5.00B	94.00A	50.27	17.7A
T8	3.00L	38.50C	52.50	13.1BC
T9	4.00F	74.00B	56.25	12.2CD
T10	4.00E	72.50B	50.50	15.1B
T11	3.00K	47.00C	51.25	7.2FG
T12	4.00J	75.00B	53.75	10.5DE

*Values with same letter do not differ significantly according to Tukey's test

Pot trial of PhytoFuRa cultures

A pot trial was performed with PhytoFuRa *Trichoderma* cultures for evaluating their efficacy in controlling leaf blight disease. Significant difference in the biometric parameters of the plant in different treatments (Table 2) was noticed with no correlation between plant height and the yield. The isolate PF 12 followed by 8, 9 and 10 showed maximum height whereas PF 11 followed by 14 showed highest yield.

Table 2: Biometric parameters of the pot trial along with yield

Treatment	Plant height at 6 months*	Yield (kg per plant)*
PF1	38.66a	0.77EF
PF2	42.00b	0.83DE
PF3	53.33d	0.70F
PF4	52.33d	0.60G
PF5	48.66c	0.70F
PF6	48.66c	0.75EF
PF7	57.33f	0.60G
PF8	60.00g	0.60G
PF9	59.33g	0.75EF
PF10	61.00g	0.75EF
PF11	55.66ef	1.20A
PF12	67.33h	0.75EF
PF13	55.66ef	0.90D
PF14	55.66ef	1.10B
PF15	54.00de	0.80E
PF16	38.66a	1.00C

*Values with same letter do not differ significantly according to Tukey's test

ICAR-INDIAN INSTITUTE OF OILSEEDS RESEARCH, HYDERABAD

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A. DIVERSITY

Pathogenic variability in *Phytophthora nicotianae*

A total of nine *P. nicotianae* isolates were tested for their pathogenic variability on five different castor (DCS-107, 48-1, GCH-4, Kranti & Harita) and safflower (Bhima, PBNS-12, SSF-708, SSF-658 & Phule Kusuma) cultivars. Two isolates of *P. parasitica* var. *nicotianae viz.*, Phy-3 collected from Andhra Pradesh and Phy-9 collected from Kerala were found to be highly virulent. Castor cultivars DCS-107 and Harita and safflower cultivars PBNS 12, SSF 658 & Phule Kusuma found to be highly susceptible to *P. nicotianae* leaf blight.
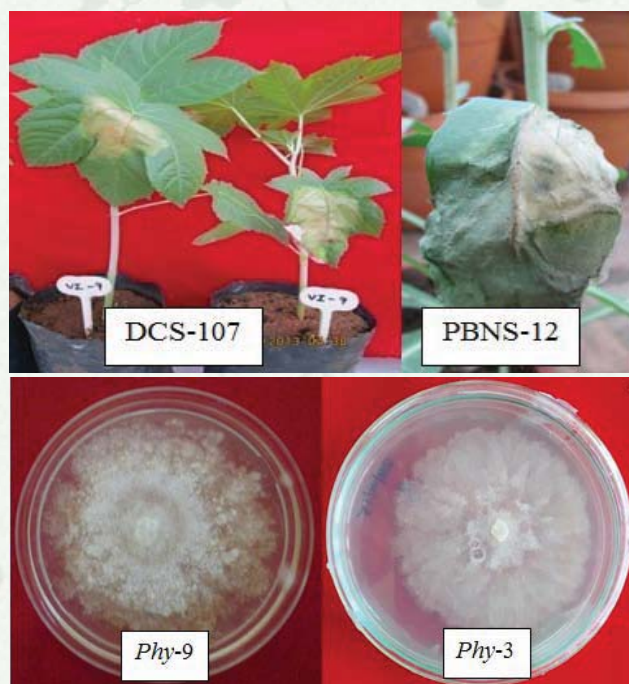

Fig. 1: Susceptible castor & safflower cultivars and highly virulent *Phytophthora nicotianae* isolates

B. HOST RESISTANCE

Screening and selection of castor and safflower lines resistant to *Phytophthora* leaf blight

A total of 20 castor germplasm lines and 40 safflower germplasm lines, popular varieties and parental lines were screened against *Phytophthora* seedling blight by agar bit inoculation method using 45 days old seedlings.

Reaction of castor cultivars to *Phytophthora nicotianae*

Two castor cultivars GCH 4 & GCH 7 were resistant showing less than 10% disease severity over two years screening (Fig. 2) and eight cultivars *viz.*, GCH 2, GCH 6, 3216 PCH 222, PCH 111, RG 3344, RG 907 and 1139 were moderately resistant with 10-25% disease severity. All the other cultivars were found to be susceptible.
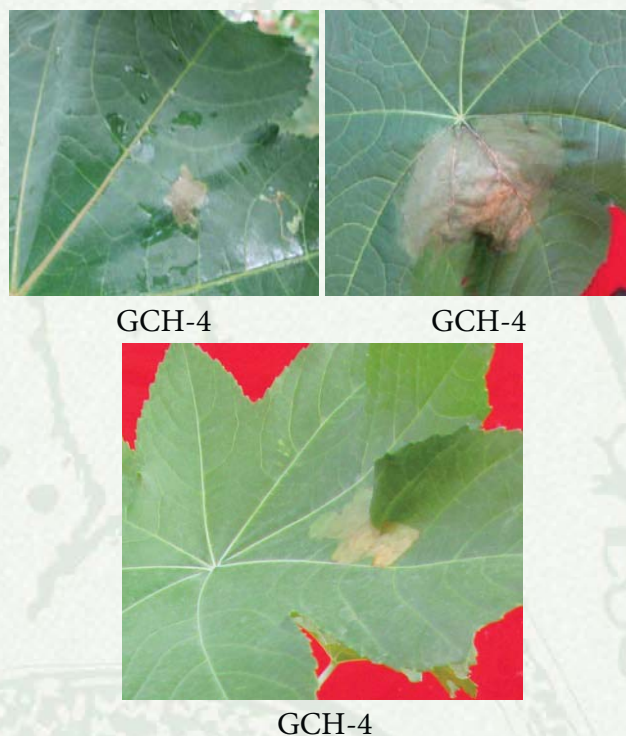

GCH-4   GCH-4

GCH-4

Fig. 2: Reaction of castor cultivars to *Phytophthora nicotianae*

Reaction of safflower cultivars to *Phytophthora nicotianae*

Eleven safflower cultivars *viz.*, SAF-1203-2(R), SSF-GMU-6878, PBNS-116, PBNS-123, PBNS-114, SAF-1205-(W), DSI-101, DSF-2014, DSI-118, SSF-GMU-4912 & Manjeera were found promising (Fig. 3) with less than 25% disease severity and other cultivars were susceptible to *Phytophthora*.

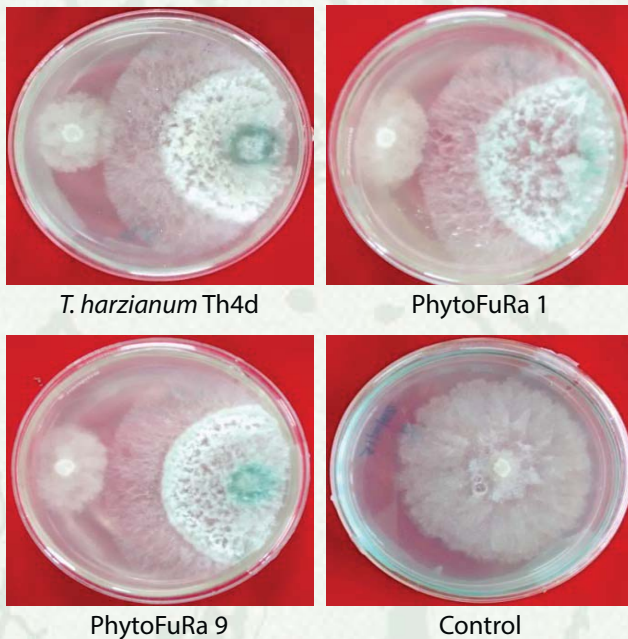| SSF-GMU-6878 | SAF-1203-2 (R) | PBNS-116 | DSI-101 | PBNS-12 |

Fig. 3: Reaction of safflower cultivars to *Phytophthora nicotianae*

C. DISEASE MANAGEMENT

Screening of biocontrol agents against *Phytophthora nicotianae*

Thirteen PhytoFuRa *Trichoderma* isolates and *T. harzianum* Th4d were screened against *P. nicotianae in vitro*.

| *T. harzianum* Th4d | PhytoFuRa 1 |
| PhytoFuRa 9 | Control |

Fig. 4: Screening of biocontrol agents against *Phytophthora nicotianae*

Among the all isolates *T. harzianum* showed the highest inhibition (54.1%) followed by PhytoFuRa 1, PhytoFuRa 9 & PhytoFuRa 13 (51.7%) (Fig. 4).

Screening of biocontrol agents against *Phytophthora* seedling blight in castor

Six *Trichoderma* isolates and *Pseudomonas fluorescens* Pf2 were screened against *Phytophthora* seedling blight of castor by zoospore spraying method on potted plants (Fig. 5). Among the six *Trichoderma* isolates, high disease reduction (35.0%) was obtained with *T. harzianum* Th4d and *P. fluorescens* Pf2 treatments followed by *T. asperellum* T673 and *T. asperellum* N13 with 28.3% reduced disease incidence.
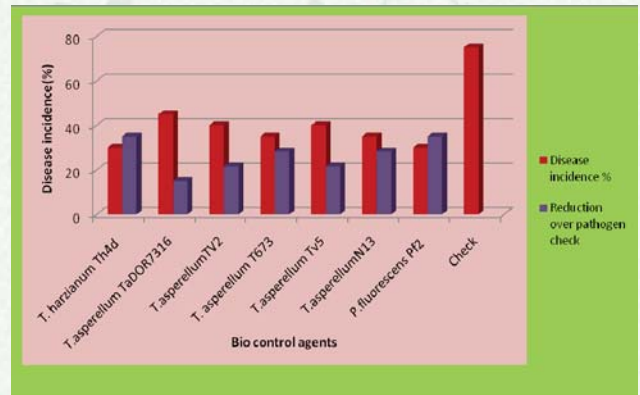

Fig 5: Screening of biocontrol agents against *Phytophthora* seedling blight in castor

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A. DIVERSITY

Collection and maintenance of *Phytophthora* isolates

During 2014-15 period, six new *Phytophthora* isolates from black pepper, colocasia and arecanut were added to the National Repository for *Phytophthora*. A total of 433 isolates of *Phytophthora* from different hosts are being maintained in the repository.

Characterization of black pepper *Phytophthora* spp. using MLST

To characterise and study the diversity of *Phytophthora* infecting black pepper, Multi Locus Sequence Typing (MLST) was done using eight nuclear genes *viz*. 28S ribosomal DNA, 60S ribosomal protein L10, beta-tubulin, elongation factor 1 α, enolase, heat shock protein 90, *TigA* gene fusion protein and mitochondrial genome region between gene *Cox2* and *Cox1* and Ras-related protein (*Ypt1*) gene. Phylogenetic analysis using Bayesian method showed a separate grouping for *Phytophthora* isolates from black pepper (Fig. 1).
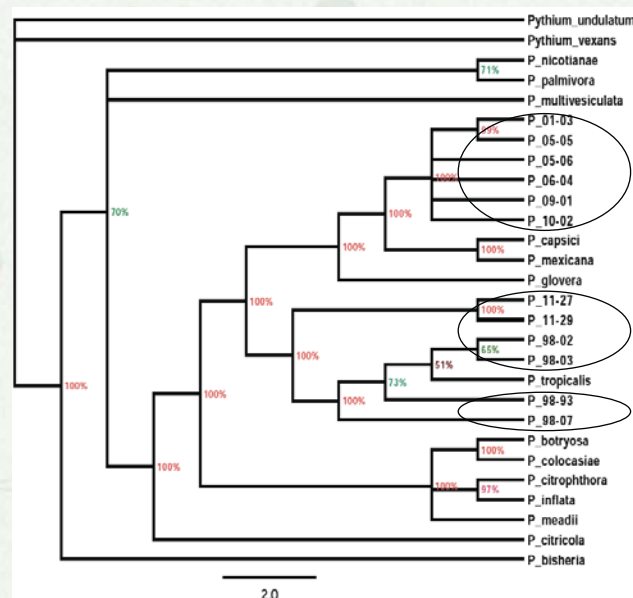

Fig. 1: Phylogenetic tree based on Multi Locus Sequence Typing of *Phytophthora* isolates from black pepper (within circles) using Bayesian method

Characterization of *Phytophthora* spp. causing outbreaks of leaf and nut fall of nutmeg

During the monsoon period of 2011, occurrence of a serious leaf fall and nut fall was observed in major nutmeg growing areas of Thrissur, Ernakulam, Idukki, Kottayam and Kozhikode districts of Kerala (Fig. 2). The disease is characterized by severe defoliation and nut fall. Symptoms appeared as dark brown water-soaked lesions on the midrib of the leaves which enlarged and spread along the lateral veins to leaf lamina resulting in blighting of the leaves which enlarged and spread along the lateral veins to leaf lamina resulting in blighting. Petioles of the infected leaves showed black discoloration. Black lesions were also observed on young shoots which enlarged in size resulting in rotting and drying up of shoots from the tip downwards. Leaf and stem infections resulted in extensive defoliation. A detailed study on morphology, temperature requirement and molecular characterization using ITS marker was undertaken. The sporangial morphology of *Phytophthora* isolates of nutmeg (13-01 to 13-06) is characterized by papillate/semi papillate sporangia, ovoid to obovoid, with intermediate pedicel lengths. The oogonia is amphigynous and formed on pairing with *P. meadii* from cocoa which is of A2 mating type except for 13-06 which formed oogonia in presence of 05-06 (A1) and designated as A2. All the isolates grow between 15-30°C with an optimum temperature of 25°C and no growth was observed at 35°C. In ITS sequencing the nutmeg *Phytophthora* isolates form a separate clade with *P. colocassiae* (Clade 2) and showed close similarity to *P. meadii.* In ITS sequence, 100% similarity was shown to *P. meadii* by isolates 13-02, 13-04 and 13-06 in Q-bank fungal identification database.
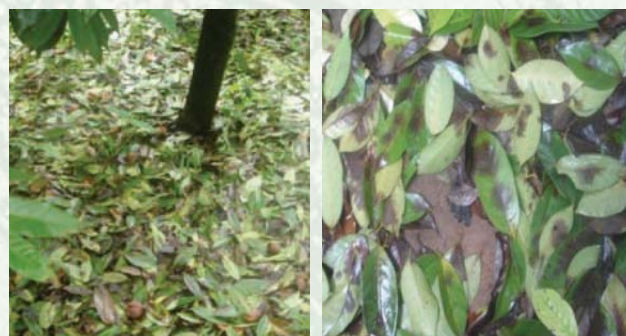

Fig. 2: Leaf fall in nutmeg due to *Phytophthora*

New target genes in *Radopholus similis*

Potential target genes of *R. similis* involved in parasitism such as FMRFamide-like peptides (nematode FLPs), β-1, 4, endoglucanase, transthyretin-like protein 3 precursor, serine-threonine phosphatases and survival such as glutathione-S-transferase(s), acetylcholinesterase, tetratricopeptide TPR-1, superoxide-dismutase and actin were amplified and sequenced. These were submitted to NCBI database (KP027004, KP027005, KM670015 to KM670018).

B. DIAGNOSTICS

In order to develop a real-time PCR based protocol for detection of *R. similis*, a standard graph was prepared by using four different dilutions of DNA isolated from *R. similis* pure culture. DNA sequences from the internal transcribed spacer (ITS) region of this nematode were used to design primers for real-time PCR. SYBR green reliably quantified as little as 100 fg of *Radopholus* nematode DNA, and could be used to quantify as few as five *Radopholus* nematodes. The *Radopholus* specific primer pair RAD F (GTCCTTTGGTGGGCAGTG) and RAD R (GGTCTGCGCTCATCAAGTC) did not detect other nematodes like *Meloidogyne incognita*.

C. HOST PLANT RESISTANCE

Isolation of resistance gene candidates in black pepper using NBS specific primers designed from *Phytophthora* Rgenes

Sequences of putative blight resistance protein from *Capsicum annum* that provides resistance to *Phytophthora capsici* namely CaRGA3 (GU295217.1), CaRGA1 (GQ386945.1), CaRGA4 (JQ219039.1), CaRGA5 (JQ219040.1), CaRGA2 (GU116570.1) were retrieved from NCBI. R gene specific primers from *C. annuum* (GU295217.1) were designed with parameters: GC content- 45-60%, primer Tm 60-65^{0}C and product size of 500-750 bp using Primerquest (IDT) software (Table 1).

Table 1: Details of R gene specific primers designed from *Capsicum annuum*

Primer ID	Primer Sequence (5'-3')	Product size	Tm (^{0}C)	GC%
RT CaRGA3F	TAGGCGGGAAA CAGGTTATG	650 bp	64.9	50
RT CaRGA3R	CGTCTGGAATCA ACTGGAAGA		63.2	50

A 72 hour old culture of virulent isolate of *P. capsici* (05-06), cultured on carrot agar media, was used as inoculum to challenge inoculate moderately resistant variety, IISR

Shakthi and susceptible variety of black pepper, Subhakara. Total RNA was isolated from both IISR Shakthi and Subhakara at 0.5, 1, 2, 4, 8, 16, 24, 48 and 72 hour after inoculation (hai) with *P. capsici* (05-06) and just before inoculation (0 hai) using TRIZOL (Invitrogen) and RT-PCR was done. A 650 bp fragment was amplified from IISR Shakthi and were cloned into pGEMT vectors and cloned into *E.coli* DH5α. Positive recombinant white clones were identified by colony PCR using M13 forward and reverse primers and sequenced. Sequenced amplicon has 31% identity with putative disease resistance RPP13-like protein 1-like [*Vitis vinifera*]. The sequence has been deposited in the NCBI with genbank number: KM058062. Analysis of amplified fragment from susceptible variety Subhakara is under progress.
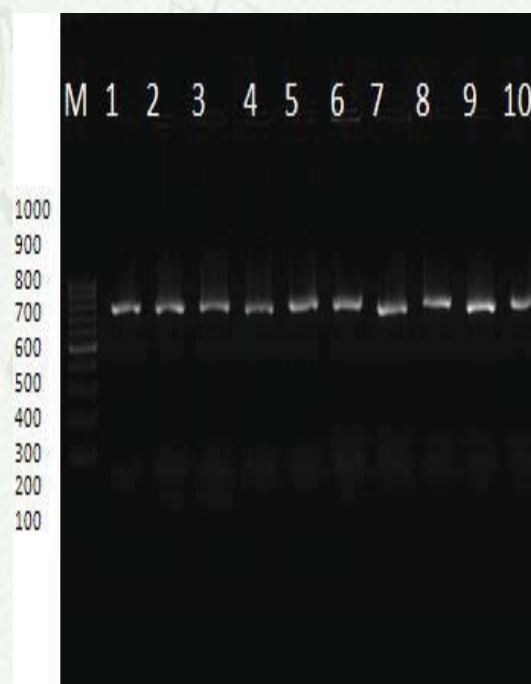

Fig. 3: Representative cDNA clones of IISR Shakthi containing inserts of size 650 bp, M- 100 bp ladder

Screening for *Phytophthora* resistance

Phenotyping of progenies of Panniyur 1 and Subhakara for *Phytophthora* resistance: One hundred and forty progenies of Panniyur 1 x Subhakara were screened for *Phytophthora* resistance by stem and leaf inoculation methods with the virulent isolate 05-06. Among them five progenies of Panniyur 1 x Subhakara were found to tolerate stem infection.

Phenotyping open pollinated progenies of IISR Sakthi and P24-0-4 for *Phytophthora* resistance: One hundred open pollinated progenies of IISR Sakthi and 27 open pollinated progenies of P24-0-4 were screened for *Phytophthora* resistance. Four of the progenies of IISR Sakthi were found to tolerate stem infection (Fig. 4). Only one progeny of IISR Sakthi could moderately resist leaf infection. None of the progenies of P24-0-4 were found to tolerate leaf and stem infections.
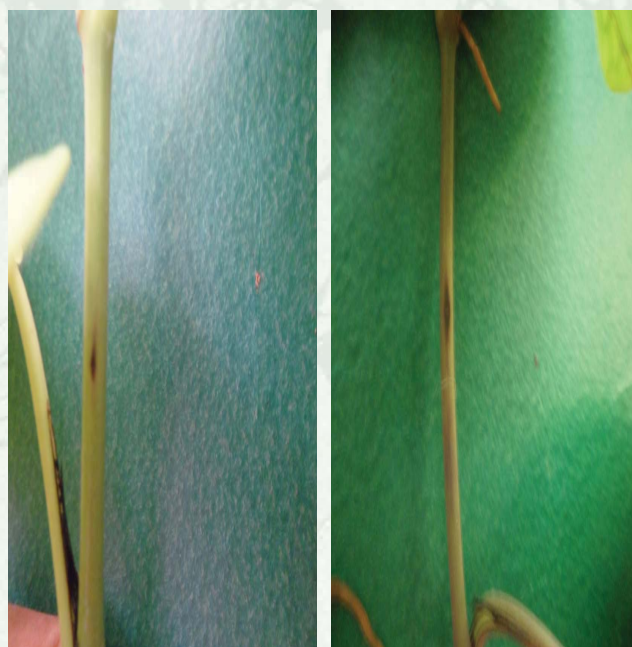
Fig. 4: Progenies of Panniyur 1 x Subhakara tolerant to stem infection by *Phytophthora* in black pepper

Molecular profiling to identify markers associated with *Phytophthora* **resistance in black pepper:** Nine SSR primers were tested on selected progenies of IISR Sakthi. Four genotypes were selected based on phenotyping data. Two primers namely PnD10 and PnE3 were found to be polymorphic among the progenies tested (Fig. 5).
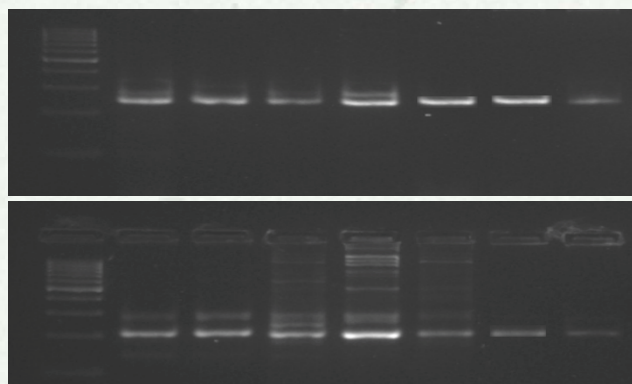

Fig. 5: Molecular profiling of progenies of IISR Shakthi with SSR primers PnE3 and PnD10. M -100 bp ladder, 1 - IISR Sakthi, 2 - P24-0-4, 3 - IISR Shakthi op- 46, 4 - 48, 5 - 188, 6 - 116 and 7 - Subhakara

D. HOST- PATHOGEN INTERACTION

Selection of reference genes for gene expression studies in *Piper nigrum- Phytophthora capsici* interaction

In order to find out proper reference gene for gene expression studies in *P. nigrum - P. capsici* interaction, reference genes namely actin, glyceraldehyde phosphate dehydrogenase, β-tubulin, ubiquitin conjugating enzyme, 18s rRNA and elongation factor-1-α were used. Moderately resistant variety of black pepper, IISR Shakthi, and

a susceptible variety, Subhakara, were inoculated at different time points (0.5, 1, 2, 4, 8, 16, 24, 48 and 72 hpi (hours post inoculation) and mock inoculated control was also kept. qRT-PCR primers were designed from *P. nigrum - P. capsici* transcriptome database with parameters 150-200 bp maximum length, optimal Tm at 60°C, GC% between 45-65% using primer 3plus software. The qRT-PCR expression analysis was performed with Rotor gene Q with a thermocycler profile of 94°C for 5 min, 35 cycles of 94°C for 30 seconds and 60°C for 30 seconds. A melt curve analysis was performed for the six reference gene primers which gave single peak showing specific amplification. The qRT-PCR products (118-232 bp) were sequenced and a local BLAST search with *Piper* transcriptome database proved their identity. The Ct values for each reference gene were analysed using different algorithms like geNorm, NormFinder and Bestkeeper were used for analysis (Fig. 6). PnGAPDH/ ubiquitin conjugating enzyme (PnUbCE) was optimised as set of genes to be used as internal control for expression in leaf tissue on *P. nigrum - P. capsici* interaction.
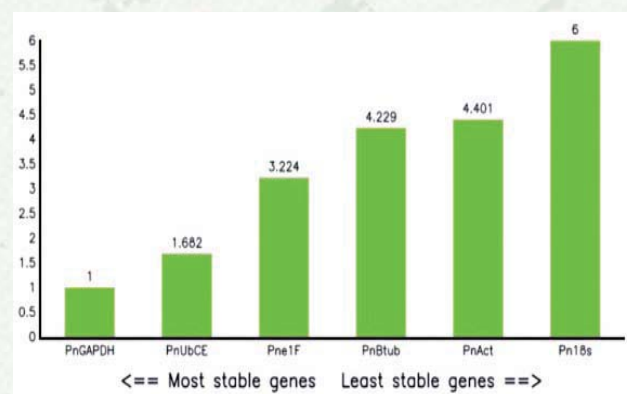

Fig. 6: Comprehensive gene stability of six reference genes in *Piper nigrum* analysed using Ref finder algorithm

Targeted expression analysis of defense related genes

Analysis of expression of locus_3839 annotated as β-1,3 glucanase was performed using leaf transcriptome of IISR Shakthi and Subhakara after challenge inoculating with *P. capsici* (05-06) different time courses ranging from 0.5 hai to 72 hai. A mock inoculated control of both plants were also kept. qRT-PCR primers were designed for locus_3839 (F: GGGCAACGAACAGATTCCTA and R: TGTGAACTGCAGTGGAGACC) with amplicon size-122 bp. The qRT-PCR expression analysis was performed with Rotor gene Q with thermocycler. A melt curve analysis was performed at $62-99^{o}$C which gave single peak showing specific amplification of locus_3839. The Ct values of Locus_3839 were compared with corresponding Ct values of normaliser gene and was found that maximum transcript abundance of PnBGlu in IISR Shakthi occurs at 72 hpi (8.5 fold) (Fig. 7). Constitutive expression of β 1, 3-glucanase was seen in IISR Shakthi starting from 0 hrs where as in susceptible variety, Subhakara, down regulation of PnBGlu was observed from 0.5 hai to 72 hai.
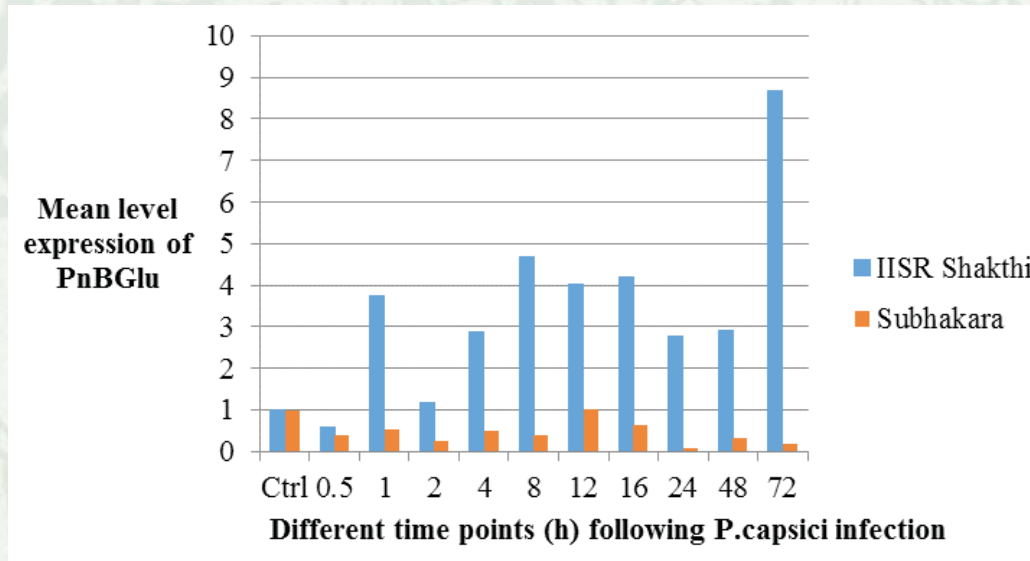
Fig. 7: Defense gene dynamics between resistant and susceptible black pepper consequent to inoculation with *Phytophthora capsici*

Targeted discovery of R genes

Molecular information of R gene and its expression will provide a critical foundation for screening germplasm and improving disease resistance breeding programs in black pepper. Nine resistance gene loci from transcriptome were taken for targeted gene expression analysis. Cloning of R gene c-DNA made from pooled RNA samples taken at 0.5 h, 2, 4, 6, 8, 12, 24, 48 and 72 hpi and further sequencing of clones, conserved domain search revealed that the R genes involved in black pepper- *P. capsici* interaction are of coiled coil type of R genes. The phylogentic analysis grouped the R genes into three different clades which is the characteristic of coiled coil groups of R gene. Black pepper R genes showed only 20% similarity to *Arabidopsis* R genes hence pointing out the structural difference of *P. nigrum* R genes.

Targeted expression analysis of resistance genes identified in black pepper- *P. capsici* interaction

Two step real time PCR was done to find out the expression pattern of the R gene locus 6113. Relative expression was calculated using REST 2009 software. Expression level of R gene locus_6113 was found to be induced in IISR Shakthi in the early hours of infection where as in Subhakara it was found to be down regulated after inoculation. The amplicons were sequenced and local BLAST search with *Piper* transcriptome database proved their identity. Expression level of R gene loci PN_6113 was found to be induced in IISR Shakthi in the early hours of infection (12 hai) where as in Subhakara it was found to be down regulated after inoculation. Basal level expression is present in both IISR Shakthi and Subhakara (Fig. 8).
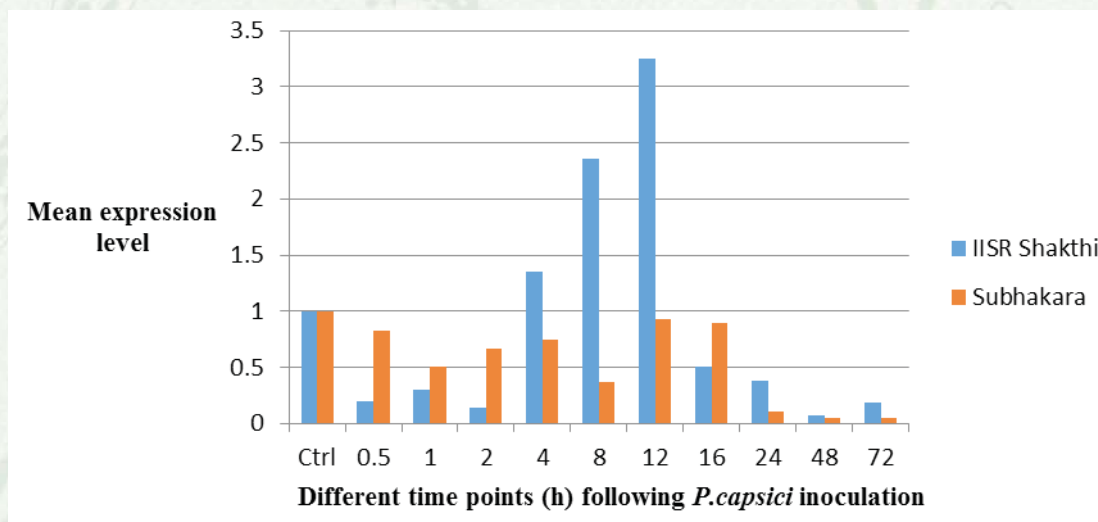

Fig. 8: Expression dynamics of R gene locus 6113 in *Piper nigrum – Phytophthora capsici* interaction

Isolation of full length sequence of R gene locus_6113 using Rapid Amplification of CONA End

Based on the motifs present in the conserved domain of locus_6113, 3'RACE and 5' RACE primers were designed with Tm greater than 70^0C. 3'RACE primers (GSP2) were designed from NB-ARC domain (AAA superfamily) and 5'RACE primers (GSP1) from PLN03210 containing LRR repeats. Using gsp1 and gsp2 internal fragments of 1400 bp were amplified from IISR Shakthi and Subhakara and were sequenced. 3' and 5' RACE cDNA was synthesized from challenge inoculated IISR Shakthi and Subhakara plants after 2 hai and amplified using gene specific primers as per manufacturer's instructions. In 5'RACE an amplicon of 2.5 Kb was obtained and in 3'RACE 4 Kb amplicon was obtained. Cloning and full length sequencing of locus_6113 is in progress.

Real time quantitative RT-PCR analysis of pathogenicity genes expressed during *Phytophthora capsici –Piper colubrinum* interaction

Quantitative RT-PCR was employed to assess the level of expression of some of the pathogenicity genes of *P. capsici* like glycoside hydrolase, NPP 1, RXLR and pectate lyase during *P. capsici-P. colubrinum* interaction (Fig. 9). Glycoside hydrolase and RXLR genes showed high levels of expression during early stages of infection (up to 16 hpi), whereas the NPP1 gene showed maximum expression at later stages of infection (at 72 hpi). Pectate lyase gene showed high level of expression at early stages of infection but was then down regulated during the later stages of infection. The expression of these genes during initial phase of infection gives an idea about the importance of these pathogenicity genes during host colonization. Phylogenetic analysis of these pathogenicity genes showed maximum similarity to *P. capsici* sequences in the database, except in the case of glycoside hydrolase which was grouped along with *P. sojae*.

In planta expression and docking studies of a glucanase inhibitor gene from *Phytophthora capsici* and beta 1, 3 glucanse gene from *Piper colubrinum*

The plant apoplast during plant–pathogen interactions is considered as a battleground for plant glucanases and other proteases, which are targeted by pathogen proteins such as glucanase inhibitor proteins (GIPs). Species of the oomycete genus *Phytophthora* employ a matching counter defense system by secreting glucanase inhibitor proteins (GIPs) that specifically bind and inhibit the activity of plant endo--1, 3-glucanases (EGases). The sequence characterization, *in planta* expression analysis and molecular docking studies of glucanase inhibitor
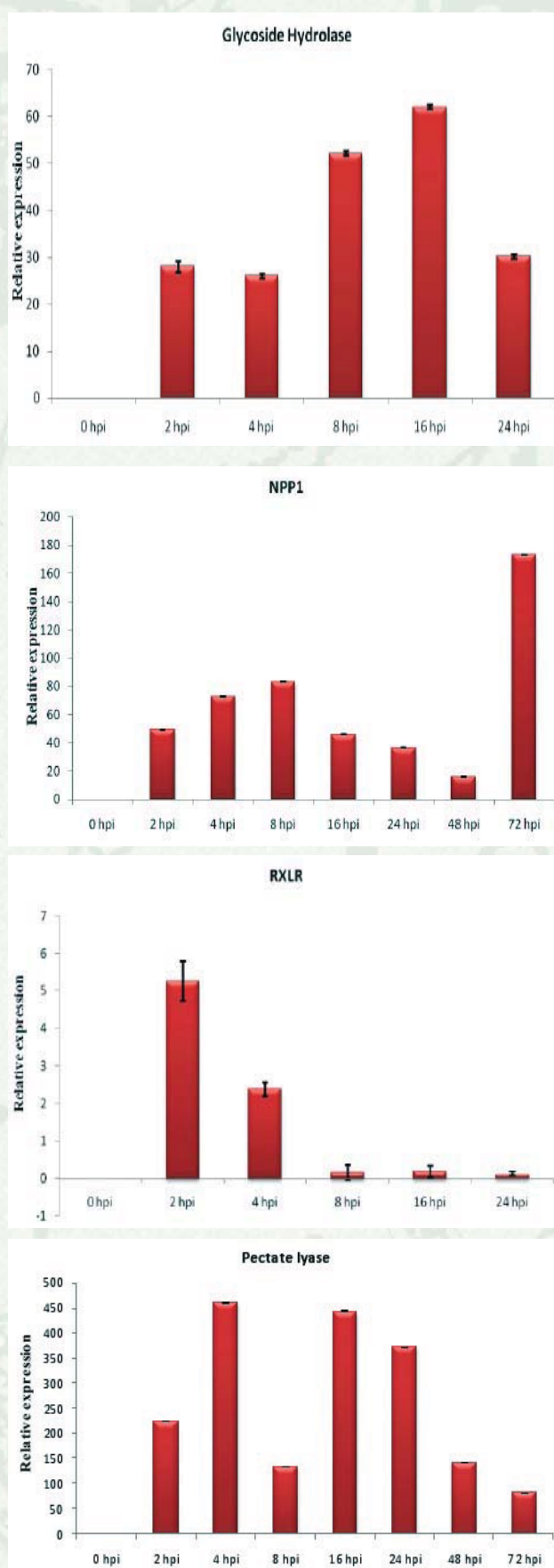

Fig. 9: Relative expression of pathogenicity genes expressed during *Phytophthora capsici – Piper colubrinum* interaction. (From Top) glycoside hydrolase; NPP1; pectate lyase and RXLR

protein (GIP) and *P. colubrinum* endo beta-1,3 glucanse genes (pc EGase) based on sequence information derived from the *P. capsici* whole genome sequence data and *P. colubrinum* transcriptome data, respectively, were done. The presence of domains in each functional coding sequences and proteins were confirmed with blastX, PSI-blast and conserved domain database. The GIP gene from *P. capsici* have a 1059 bp ORF, encoding a putative peptide of 353 amino acids and the partial sequence of (pcEGase) gene from *P. colubrinum* have a 936 bp ORF, encoding a putative peptide of 312 amino acids. The expression of these genes was studied *in planta* at different time points by qRT-PCR after challenge inoculation with the pathogen. The *in planta* expression of GIP gene from *P. capsici* was at its peak during initial hours of challenge inoculation and the expression of (pcEGase) gene was at its peak at 16 hpi (hours post inoculation). The peak expression of (pcEGase) gene from *P. colubrinum* at 16 hpi and sharp decrease in later periods indicate the successful neutralizing activity of the (pcEGase) gene against the GIP gene in this incompatible plant- pathogen interaction (Fig. 10).
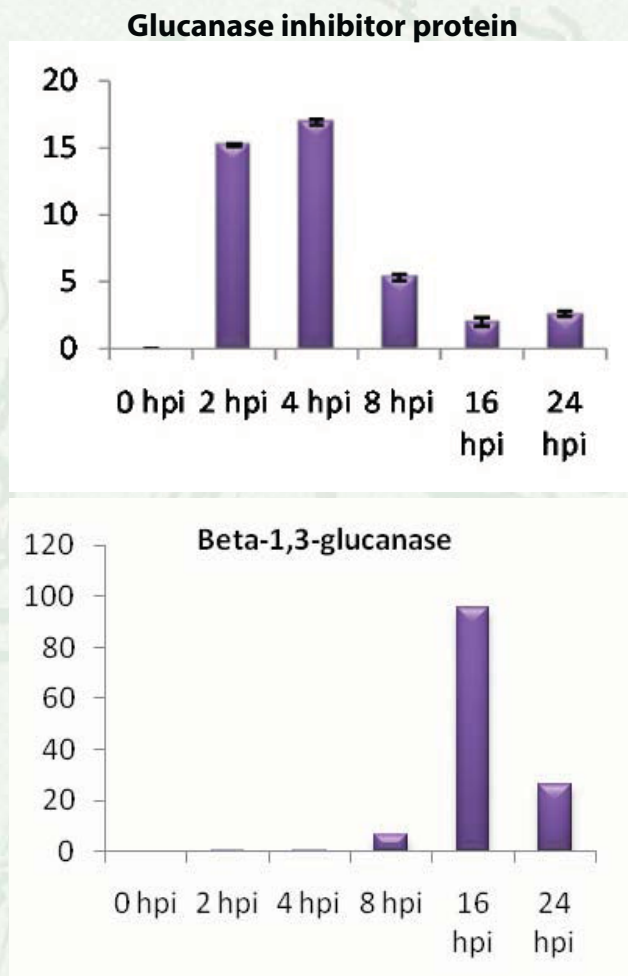
Three-dimensional model of GIP and (pcEGase) gene

were constructed and molecular docking studies predicted sites on the surfaces of (pcEGase) gene and GIP that may be involved in high affinity binding. Molecular docking studies between (pcEGase) gene and GIP revealed that substrate inhibition is obtained by recognizing arginine and isoleucine residues in the substrate molecule (Fig. 11). Docking studies of EGase with GIP indicates that GIP blocks the active site of EGase. Glucanase Inhibitor Protein was docked into the binding site of Endo- β -1, 3-Glucanases using Cluspro 2.0. The enzyme – substrate complex was analysed using SwissPDB viewer and PyMOL v1.7.2 in order to understand their mode of interactions and key residues involved in binding. The docked compelx of EGase in complex with GIP, revealed 21 polar hydrogen bond interactions.
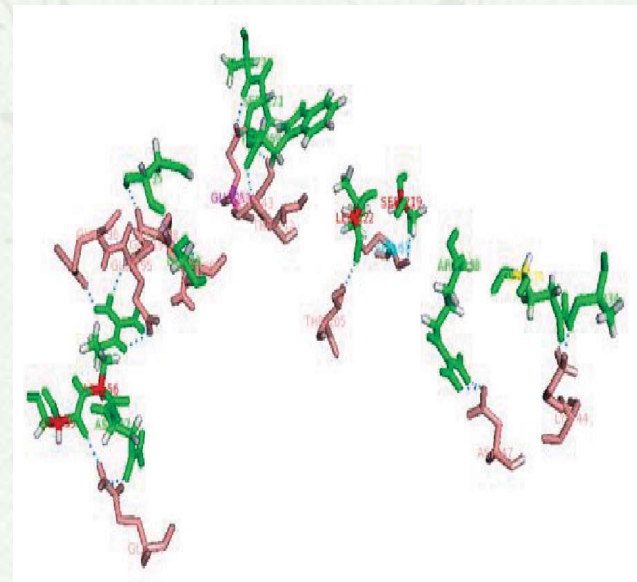
Fig. 11: Polar covalent interaction of GIP with pcEGase active site residues. The active site residues are shown as sticks Helix Sheet Loop and EGase shown in Helix Sheet Loop. The interactions are shown in blue dashed lines

Glucanase inhibitor protein

Beta-1,3-glucanase

Fig. 10: Real time PCR results of (Top) Glucanase inhibitor protein (GIP) and (Bottam) beta-1, 3-glucanase

Expression analysis of defense associated transcription factors

PAMP triggered immunity (PTI) and effector triggered immunity (ETI) leads to biotic stress signalling involving induction of defence- related transcription factors. Hence studies on expression analysis of these transcription factors is very important to understand their importance during host pathogen interaction. Quantitative RT-PCR analysis was done for three transcription factors viz, MYB, MYC and WRKY, to assess the transcriptional activity of these genes during *P. colubrinum - P. capsici* interaction (Fig. 12). Higher folds of expression of these genes were observed at initial stages of infection when compared to later stages. MYB gene showed up to 1.25 folds expression at 4 hpi, MYC gene showed 2.6 folds expression at 8 hpi and expression of WRKY gene was 1.65 folds at 4 hpi.

Fig.12. Relative expression of MYB, MYC and WRKY genes under *Piper colubrinum-Phytophthora capsici* interaction
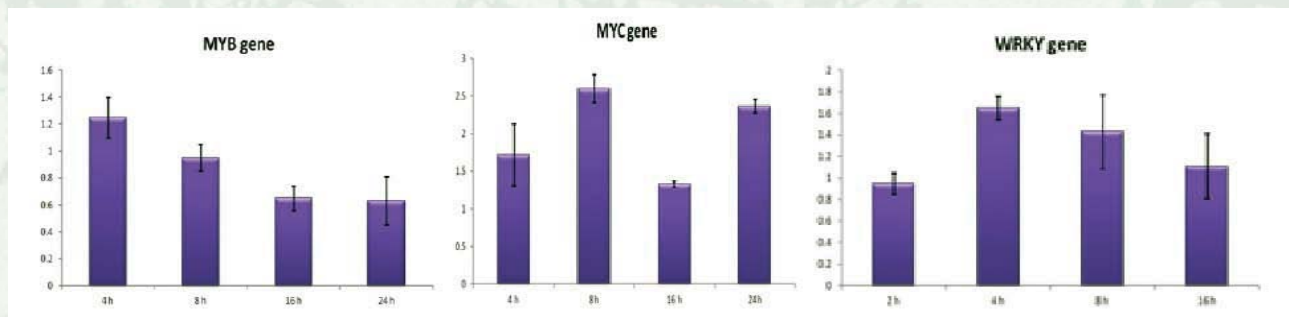
Identification and characterization of micro RNAs from *Piper colubrinum*

Dual RNA-seq data of disease-resistant *P. colubrinum* and its pathogen *P. capsici* (05-06) isolate from infected leaves in the early stages of infection (12 and 24 h post-inoculation) were analysed for microRNAs and their corresponding mRNA targets. Primary miRNA predicted from *Piper* transcripts of *P. colubrinum* against the precursor and mature sequences of known plant miRNAs deposited in miRBase version 16 and MIREAP programs. Sequences with an E-value of lower than 0.05 or a score > 32 were processed for further analysis, allowing for a maximum of 1 nt mismatches and 190 precursor miRNAs present in *P. colubrinum* transcriptome were identified.

To understand the corresponding mRNA targets from *P. colubrinum*, which are critical to understanding many pathways and biological systems in which miRNAs are involved, BLASTC searches performed against *P. colubrinum* assembled transcript database to identify putative targets for miRNAs. Similarities with an E-value of less than 0.05 were considered a hit. Around 4542 putative targets were identified from *P. colubrinum* coding transcripts for the predicted miRNAs. Out of these, 881 transcripts were predicted with putative functions and these predicted targets were involved inhibition and cleavage of various molecular functional genes, such as cytochrome c biogenesis protein, serine/threonine-protein phosphatase, NEDD8-like protein RUB2, translation, transcription etc.

Search for targets in *P. capsici* genomic exons/CDS for the 190 miRNAs from *P. colubrinum* were also done and three of the miRNAs had 13 mRNA targets corresponding targets in *P. capsici* transcripts. These studies will help in understanding miRNAs involvement in *P. colubrinum* that have interactions with *P. capsici*.
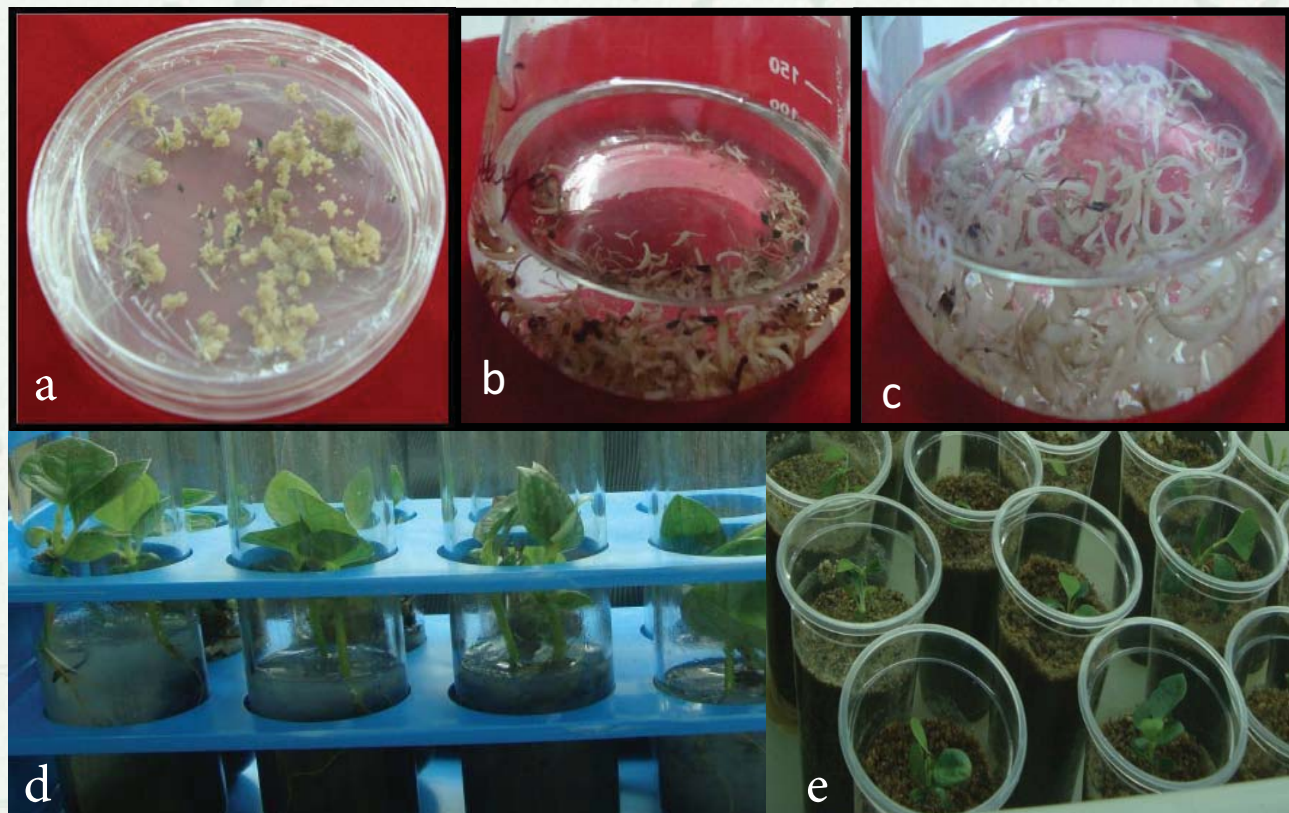

Fig. 13: Different stages of plant regeneration (a) embryogenic clumps in SH-15 medium; (b) growth after 10 days of incubation in SH-30 broth; (c) growth after 20 days; (d) maturation of plants in WPM; and (e) hardening of regenerated plants

E. DISEASE MANAGEMENT

Mass production of black pepper plants (Sreekara) through somatic embryogenesis and testing the presence of mycoendophytes in somatic plants

Secondary embryo proliferation and regeneration of plants: Embryogenic clumps (25 mg each) from secondary embryogenic cultures were inoculated into five 250 ml flasks containing 250 ml of liquid SH-15 medium and incubated under darkness in shakers for 20 days. After 20 d of culturing, embryos of similar developmental stages were allowed to develop further in SH-30 medium with 12 h light and dark cycles for 10 more days (Fig. 13). Twenty ml of medium was withdrawn at 5-days interval and replaced with the same quantity of fresh medium. After 30 days of growth in SH-30, cotyledon stage plantlets were developed and plants with one tap root were transferred to WPM for further growth and maturation. The developed plants were transferred to sterile sand for hardening. Tissues (leaf, stem, root) of somatic embryo derived plants were subjected to endophytic fungal isolation. No fungal growth was observed after 30 days of incubation.

F. BIOINFORMATICS

Comparative genomics of *Phytophthora* species

Secretome analyses of *Phytophthora* species were done using different softwares like SignalP, TMHMM and TargetP. Gene encoding proteins from five different *Phytopthora* species namely *P. capsici* (05-06 and 98-93), *P. sojae*, *P. infestans* and *P. ramorum* were taken for this analysis. Proteins with signal peptides were identified by sorting out proteins without transmembrane domains and subcellular localization for further comparative genomics studies (Table 2).

Table 2: *In silico* prediction of proteins with signal peptides from whole genome data of *Phytophthora* spp.

Species	Total number of sequences	No of secretory proteins	% of secretory proteins
P. capsici 05-06	19805	3180	16.056
P. capsici 98-93	9831	2085	21.208
P. sojea	19027	2037	10.705
P. ramorum	15743	1739	11.0461
P. infestans	18140	1848	10.187
P. parasitica	18795	2001	10.646

ICAR-RESEARCH COMPLEX FOR NEH REGION, UMAIM

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A. DISEASE MANAGEMENT

In vitro bio-efficacy of native *Trichoderma* species against *Phytophthora* spp.

In vitro evaluation of *Trichoderma* spp. following dual culture technique showed antagonism against *Phytophthora*. Out of the seven species, the highest inhibition was noticed with *T. harzianum* (RCT5, 88.89%) followed by *T. brevicompactum* (RCT8, 86.30%) and *T. asperellum* (RCT1, 84.81%) (Fig. 1). The performance of these two potential bio-control agents is being evaluated under field condition.
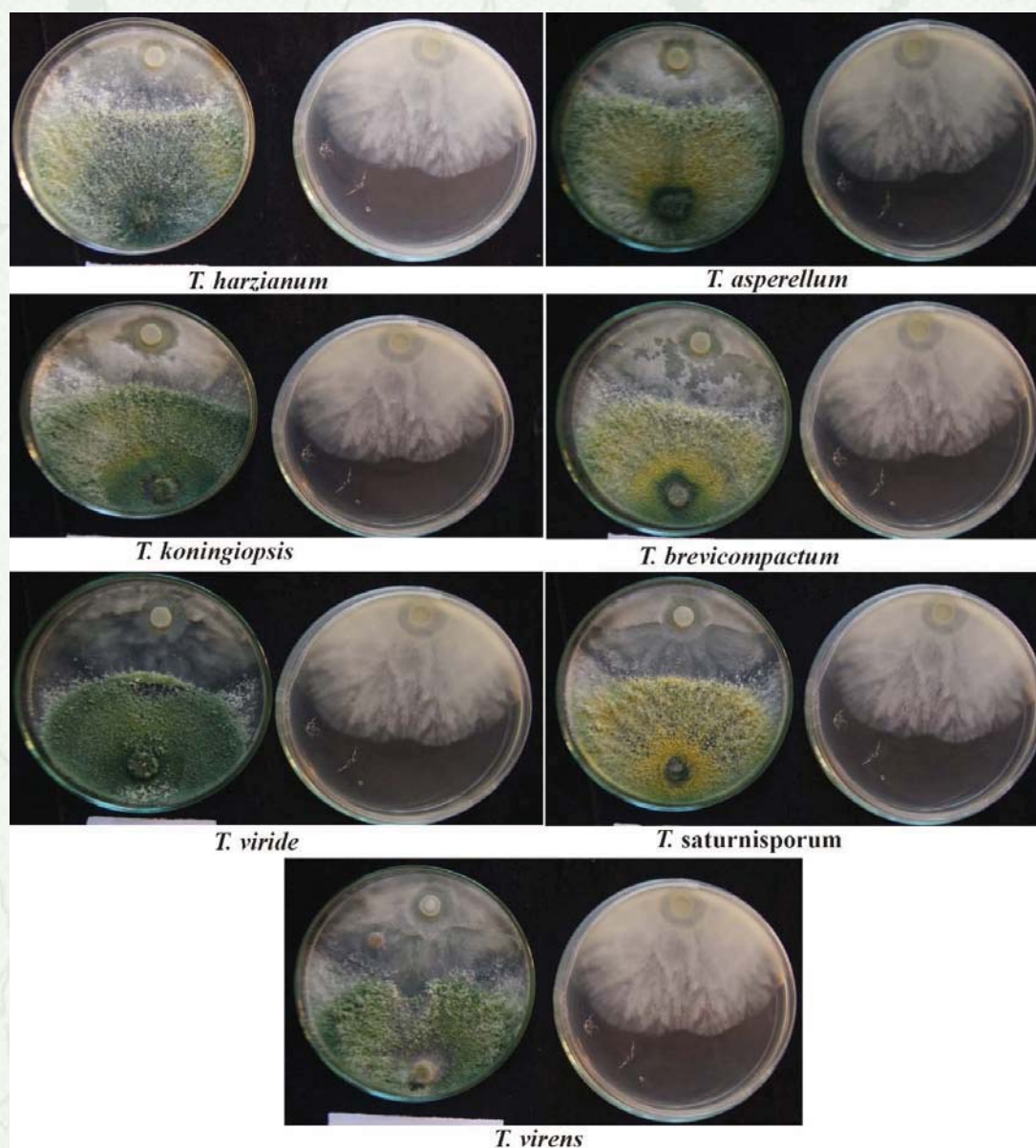
Fig. 1: *In vitro* evaluation of seven *Trichoderma* spp. against *Phytophthora* spp.
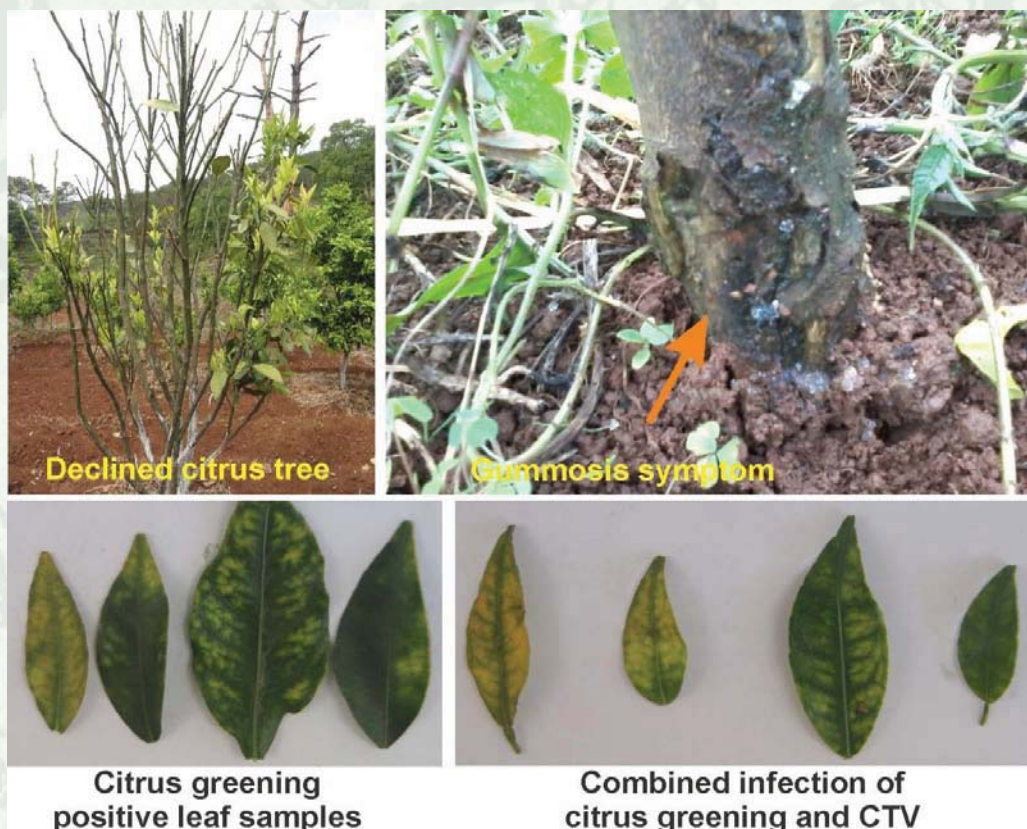
Fig. 2: The citrus plants showing combined symptoms of citrus gummosis, citrus greening and citrus tristeza virus (CTV).

Field evaluation of potential bio-control agents

The declined citrus orchard of Horticulture Farm, ICAR Research Complex for NEH Region, Umiam was selected for improvement by applying potential bio-control agents. In the orchard the combined infection of citrus gummosis, citrus greening and citrus tristeza virus (CTV) were detected (Fig. 2).

Management of citrus orchard was initiated with the application of biocontrol agents. The data on plant height, canopy height, canopy width, number of leaves/twig and percent yellowing of canopy has been recorded from 100 plants prior to the addition of biocontrol agents. The data on same characters will be generated after one year after application of bio-control agents.

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A. HOST-RESISTANCE

Transcriptome sequencing for gene discovery and gene-based marker generation

RRIM 600, a susceptible cultivated rubber clone and FX 516, an interspecific hybrid clone tolerant to *Phytophthora,* were challenged with fungus. Leaf samples from both control and treated plants at different time intervals (6, 12, 24 and 48 h) were collected following challenge inoculation.

Transcriptome sequencing of control and pooled challenged samples was performed on NGS platform (Next-Seq). In total 4.75 GB (17617074 PE reads) and 3.31 GB (12017116 PE reads) of filtered paired end sequence data were generated with FX 516 in control and *Phytophthora* challenged samples, respectively. Similarly for the susceptible clone RRIM 600, 2.86 GB (10295859 PE reads) and 3.96 GB (14859400 PE reads) RNASeq filtered sequence data were generated in control and treated samples, respectively. The read length ranged between 130-140 bp with a quality score above 32.

De novo assembly of RNASeq data was carried out using Velvet-Oases assembler across a range of kmers (39 to 51) in both control and treated samples for both the clones. The control transcriptome sequence of RRIM 600 at kmer 51 assembled 44831 transcripts with a maximum transcript size of 8483 bases and a read participation of 62.61%. Whereas, in the treated samples of RRIM 600, a total of 36522 transcripts were assembled with a maximum transcript size of 13678 bases and a read participation of 60.91%. In the control transcriptome of the tolerant clone FX 516, a total of 64151 transcripts were assembled. The maximum transcript size was 7392 at kmer 51 and the read participation was 76.57%. While in the treated samples a total of 41377 transcripts were assembled at kmer 51 and the maximum transcript size was 14968 bases with a read participation of 50.74%.

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A. DIVERSITY

Survey of apple orchards and incidence of collar rot disease

Survey of apple orchards indicated that degree of incidence of collar rot disease was between 2.5-20.1, 1.2-22.1 and 0.4-10.3 per cent. Maximum incidence (20.1%) was observed in apple orchard at Jattota in Shalooni block followed by Baragarh (10.9%) in Tisha block of Chamba District, Pulwahal (22.1%) in Sirmour and Lippa (10.3%) in Kinnaur District of Himachal Pradesh. Nine different isolates were morphologically characterized.

Virulence studies

Virulence of the above nine isolates of *Phytophthora* on susceptible rootstock MM106 by excised twig method indicated that isolate PC7 exhibited maximum lesion size (64.2 mm) followed by isolate 3 (63.2 mm) and 2 (61mm),

respectively. On the basis of lesion size (>50 mm) three were designated as highly virulent, two were virulent, three moderately virulent and one as less virulent. Virulent strain produced dark brown lesions whereas less virulent produced light brown lesions. Two isolates *P. ultimum* were found to be moderately virulent.

Monitoring and quantification of *P. cactorum* in apple orchards of Kullu and Mandi districts

In order to develop a geographic map of collar rot pathogen in apple growing districts of the state, soil samples from 30 different locations of each district of Kullu and Mandi were collected to monitor and quantify the collar rot pathogen by adopting modified SADAMCAP (Soil Air-Dried And Moistened Chilled And Plated and standardized baiting technique) using the sun hemp seeds (baits) and PARP medium (Table 1).

Table 1. Quantification of collar rot pathogen (*P. cactorum*) at different locations in Kullu District

Place		No. of positive plates/5		No. of positive baits/25		No. *P. cactorum* colonies/ plate*			
Kullu	Mandi	Kullu	Mandi	Kullu	Mandi	Kullu		Mandi	
						Mean	Range	Mean	Range
Barsaini	Chiuni	5/5	4/5	23	17	45.4	36- 70	28.6	18-34
Khanag	Panjain	5/5	4/5	24	18	52.4	36-84	40.4	36-56
Johal	Manjhali Dhar	5/5	5/5	25	25	60.6	39-86	45.6	39-68
Chohani	Manjholi	5/5	4/5	25	17	55.8	32-80	38.6	28-54
Kungus	Ruhanda	4/5	5/5	17		29.3	19-42	48.2	40-72
Kamand	Ruhmini	5/5	5/5	24	24	48.8	34-72	58.6	45-76
Bhaliayan i	Chhatri	4/5	5/5	20	25	46.8	32-65	45.8	40-68
Summa	Janjelli	4/5	4/5	19	22	46.8	32-53	38.2	32-54
Larakalo	Karsog	4/5	4/5	19	18	39.2	26-52	32.8	24-38
CD at 5% level						3.21		2.92	

The soil samples collected from Barsaini, Johal, Khanag, Chohani, Bhaliayani, Summa and Kamad in Kullu District showed highly positive response for the presence of *P. cactorum*. Highest number of colonies per plate was observed from the soil samples collected from Johal (60.6) followed by Chohani (55.8), Khanag (52.4), Kamad (48.8), Bhaliayani (48.8), Summa (46.8) and Barsaini (45.4), respectively.

B. EPIDEMIOLOGY

Studies on host-pathogen-environment interaction for developing forecasting module

Under nursery conditions: Data on disease incidence were recorded periodically under nursery conditions (sick plot) and final data were recorded when the disease

reached nearly up to 100 per cent. The disease first appeared in the first week of April with the occurrence of mean minimum and maximum soil temperature ranging between 7.7-9.2 and 16.0-19.7ºC accompanied with soil moisture of 66.3-84.1 per cent up to 5 cm depth accompanied with frequent rains (1-5 rainy days/ week) during two week prior to the appearance of disease. Similarly, mean minimum and maximum soil temperature of up to 10 cm depth varied between 7.1- 7.6ºC and 12.8-16.6ºC with soil moisture varying between 69.3-78.2 per cent two weeks prior to the appearance of disease. The disease increased with greater speed with the of mean soil temperature ranging between 18.8-26.9ºC accompanied with soil moisture varying between 62.8-78.8 per cent up to 10 cm depth during May – September, reached its maximum in the first week of September 2014 (Fig. 1).
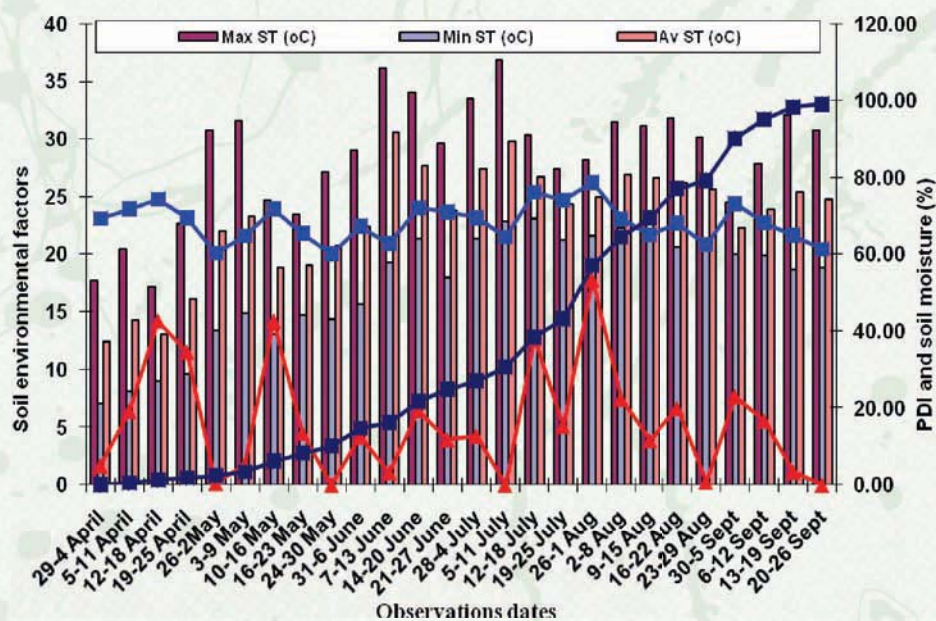

Fig. 1: Effect of soil environment at 10 cm depth on the development of collar rot in apple under nursery conditions
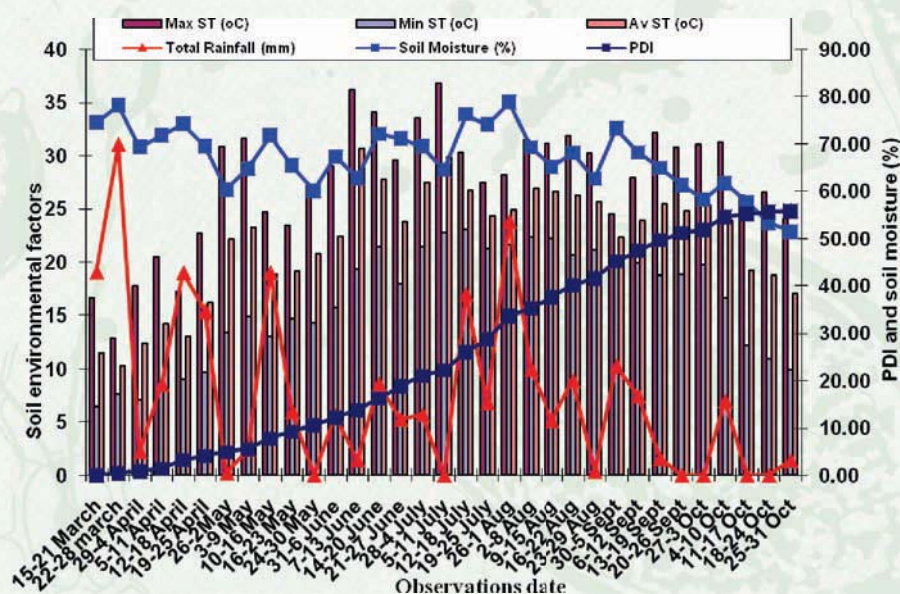

Fig. 2: Effect of soil environment at 10 cm depth on the development of collar rot in apple under orchard conditions

Under orchard conditions: Disease was first noticed on 22 March with the occurrence of mean minimum and maximum soil temperature ranging between 5.3-7.7ºC and 14.4 -19.7ºC accompanied with soil moisture of 73.2-76.2 per cent up to 5 cm depth accompanied with frequent rains (2-3 rainy days/wk) during two weeks prior to the appearance of disease. Further, the results indicated (Fig. 2) that the disease increased rapidly with the mean soil temperature up to 10 cm depth ranging between 16.1-26.9ºC and soil moisture varying between 60.1-73.2 per cent during 19 April- 19 September and reached its maximum in the first week of October 2014.

Correlation and regression studies: Correlation studies on environmental factors with collar disease (Table 2) indicated that the disease is positively correlated with rainfall, soil moisture and minimum soil temperature and was negatively correlated with soil temperature at 5 cm.

Table 2: Correlation of different environmental factors with collar disease severity in apple

Factors	Correlation under conditions	
	Nursery	Orchards
Rain fall x PDI	0.09	0.39
Max Soil Temp 5 cm x PDI	-0.34	-0.38
Min Soil Temp 5 cm x PDI	0.55*	0.63
Av. Soil Temp 5 cm x PDI	0.51	0.40
Soil Moisture 5 cm x PDI	0.10	0.53*
Max Soil Temp 10 cm x PDI	-0.33	-0.41
Min. Soil Temp 10 cm x PDI	0.65*	0.64
Av. Temp Soil 10 cm x PDI	0.49	0.42
Soil Moisture 10 cm x PDI	0.53*	0.51*
Max Soil Temp 20 cm x PDI	0.21	0.21
Min. Soil Temp 20 cm x PDI	0.61*	0.58*
Av. Soil Temp 20 cm x PDI	0.39	0.34
Soil Moisture 20 cm x PDI	0.08	0.52

*= Significant at 1% level

Correlation of weather factors with development of disease

In nursery condition

Y1 5 cm = 16.216 -5.668 X1 + 5.643 X2 – 2.151X3 – 3.656 X4 (R2 =0.6257)

Y110 cm= -254.38 -3.498X1+11.731X 2 + 8.577X3 – 3.2255X4 (R2=0.6177)

Y1 20 cm=-59.064 – 27.011X1-17.133X2 +4.738X3 – 1.747 X4 (R2= 0.5546)

In orchard condition

Y2 5 cm= 156.93 – 4.068X1+2.417X2+ 6.819X3 – 2.454 X4 (R2=0.6975)

Y2 10 cm=235.02 -1.704X1+ 6.752X2 + 3.963X3 – 3.281X4 (R2 =0.6848)

Y3 20 cm= 178.40 – 17.088 X1 - 11.919X2 + 4.981X4-3.109X4 (R2=0.7105)

Where X1 = Max soil temperature ºC, X2 =Min Soil temperature ºC, X3 = Rainfall (mm)

X4 = Soil moisture (%), Y1= Disease level under nursery conditions, Y2= Per cent disease index under orchard conditions.

Studies on rhizosphere micro-flora in *Phytophthora* sick and free soil

Fungal and bacterial microflora from soil samples collected from healthy and diseased areas of Chamba District were analyzed by using rose bengal supplemented PDA/malt extract and nutrient agar medium, respectively. *Phytophthora* containing soil sample had low population of *Trichoderma* species (0.32-0.49×10^3), *Penicillium* species (0.20-0.32×10^2) and bacterial species (0.62-0.89×10^6) whereas, *Aspergillus* species (3.64-4.56×10^2) and *Pythium* spp. (2.62-3.48×10^2) were present in higher level and reverse was noticed for soils without *Phytophthora*. There was no relation of *Fusarium* population in both conditions. *Trichoderma* species were identified as *T. viride, T. harzianum, T. koeningi, T. virens* and *T. hamatum*. The identified *Penicillium* species were *P. funiculosum, P. citricum, P.*

turbatum, P. expansum, and *P. pubercum*. Bacteria belonging to *Serratia* sp., *Bacillus* spp. (3 species), *Pseudomonas putida, P. aeruginosa* and *P. fluorescens* were the rhizosphere microflora.

C. HOST RESISTANCE

Evaluation of seedlings of pollinizer cultivars of apple for resistance under pot and nursery conditions

The experiment on evaluation of pollinizer cultivars for resistance was repeated to validate the results. The seeds of 27 pollinizer cultivars *viz.*, Golden Delicious, Red Gold, Red Fuji, Scarlet Gala, Tydeman,s Early Worcester, Gold spur, Mollies Delicious, Granny Smith, Commercial, winter Delicious, Gale gala, Royal Gala, Bray burn, Gloster, Black Ben Davis, McIntosh, Spartan, Summer Queen, Red June, Lord Lambourne, Golden Spur, Jonathan, Winter Banana, *Malus floribunda* and Star Crimson were collected, stratified and sown in the pot containing sterilized soil and also under nursery conditions. The inoculum of target pathogen was added @2% (w/w) at four leaf stage in the pots and 60 g/m^2 under nursery conditions. The results indicated that *Malus floribunda* showed maximum tolerance as minimum mortality of 11.1% and 9.4% was observed under pot and nursery conditions, respectively. It was followed by Stark Spur Golden (13.6, 10.1%), Bray Burn (13.9, 10.4), Star Crimson (18.3, 13.2%), Winter Delicious (18.5, 15.2%), Gloster (25.2, 20.3%) and Golden Delicious (27.6, 22.1%). Eight cultivars *viz.*, McIntosh, Granny Smith Red Fuji Red Gold, Scarlet Gala, Golden Spur, Gale Gala, and Spartan behaved highly susceptible (>70% mortality).

Adaptive trial on resistant rootstocks

Adaptive trials at Poojan, Summa and Sainj in Kullu District, Gihiri, Ruhanda and Chhatri in Mandi District and Dalgaon, Sewa and Kadei in Shimla District revealed that plants of resistant rootstocks (M9, M7 & MM111) are performing well, whereas plants of susceptible rootstocks (MM106) were dead due to disease. In addition, these resistant rootstocks survived in the sick soil under nursery conditions at Nauni. During the current year, 40 grafted plants of improved varieties on moderately resistant and most preferred (MM111 & M7) resistant (M9) rootstocks were distributed for planting at hot spot area at Ruhanda, Ruhmani in Mandi District, Summa, Sainj Poojan in Kullu District and Dalgaon in Shimla District.

Development of resistant rootstocks/ plants through somaclonal variations

Plant materials have been produced under *in vitro* shoot cultures of apple rootstocks MM106 and MM111. Callus induction, shoot regeneration and *in vitro* selection have been done. Pure cultures of *P. cactorum* collected from different areas were procured from Department of Mycology and Plant Pathology, UHF Nauni, Solan and maintained in tissue culture laboratory for further studies on somaclonal variations.

D. DISEASE MANAGEMENT

Evaluation of novel fungicides under nursery and orchard conditions

Curzate (cymoxanil 8% + mancozeb 64%), Melody Duo (iprovalicarb 5.5% + propineb 61.25%), Cabrio Top (pyraclostrobin 5% + metiram 55%), Sectin (fenamidone 10% + mancozeb 50%), Matco (metalaxyl 8% + mancozeb 64%), Acrobat 50WP (dimethomorph), Amistar 23% (azoxystrobin), Ergon 500SC (kresoxim methyl), Ridomil Gold 68WG (mefonoxam 4% mancozeb 64%), Infinito 68.75%SC (flupicolide 6.25% + propamacarb hydrochloride 62.5%) and four commonly used fungicides propineb, mancozeb, copper hydroxide and copper oxychloride during the last year were again evaluated under field conditions in the nursery and orchards. These fungicides were found to be effective under *in vitro* condition. The fungicides were added @ 5 l/m^2 under nursery conditions during April, June and August. Similarly these fungicides were drenched in soil @10 l/1.5 ft area around the stem of infected plants in the orchard. Disease mortality under nursery conditions were recorded periodically and final data on disease severity was recorded in the month of December 2014 by uprooting the plants. Under orchard conditions shoot growth (cm) and lesion recovery were recorded in the month of November 2014. Data obtained revealed that soil drenching with Curzate (0.3%) was highly effective both under nursery (97.2 PDC) and orchard conditions (increased shoot length 37.1 cm against 5.9 cm in control untreated). Fungicides Cabrio Top (0.25%), Ridomil Gold (0.3%), Sectin (0.25%) and Matco (0.3%), were effective both under nursery (92.1-95.5 PDC) and orchard (31.5-36.2 cm shoot length and lesion recovery 32.4-35.5%) conditions. Fungicide namely Melody Duo (0.25%) was the next best followed by Ergon 500SC (0.15%), Infinito (0.25%) and Amistar (0.15%) (85.3-89.9 PDC under nursery and increased shoot length 27-30.8 cm under orchard conditions). Other fungicides *viz.*, Polyram, Indofil M-45, Kocide and Blitox were comparatively less effective under both conditions. These effective novel fungicides also reduced the collar wound up to an extent of 32.4-36.3 per cent.

Mass multiplication of potential biocontrol agents

Talc formulation of five fungal (*Trichoderma harzianum* 5, *T. viride*-4, *T. virens*-2, *T. hamatum* 2 and *Penicillium funiculosum*) and three of bacterial (*Bacillus subtilis* 11,4 BS11, *P. fluorescens* 6 (KB6), were prepared by specific media; (molasses: soy flour medium for *Trichoderma* species, MEA medium for *P. funiculosum*, King's media for *Pseudomonas* and Nutrient broth for *Bacillus* spp.) in a fermenter. Maximum cfu/g of *Trichoderma, Pseudomonas* and *Bacillus* antagonist species were obtained at a temperature of 26ºC, pH 6.8, Dissolved Oxygen (DO) 80% and rotation 250 rpm; 28ºC, pH 7.0, DO 100% and rotation 250 rpm; and 30ºC, pH 7.0, DO 100% and rotation 250 rpm, respectively.

Evaluation of effective antagonists

Four fungal *Trichoderma* (*T. harzianum* -5, *T. hamatum* -2 *T. viride* 4, *T. virens*-2) and *Penicillium funiculosum*, two bacterial antagonists *Bacillus* sp. -4 and *Pseudomonas* sp. -3 and two mycorrhizae *Glomus mosseae* -2 GM 2, G. *heterosporum* GH1 isolated from soils of different locations in two apple growing districts (Sirmour and Chamba) were evaluated under field conditions in the nursery. Above mentioned BCAs were added initially (0 day), 20 days, 10 days before and 10 days after the planting of apple seedlings. The bran culture and coconut coir culture were added @150 g/m^2 plots along with 10 g talc based formulation of individual BCA. Fifteen apple seedlings were planted in each plot of 1 m^2 and each treatment was replicated three times. Perusal of data indicated that addition of BCAs 20 days prior to planting of apple seedlings was highly effective. Disease incidence was decreased in order when added 10 days prior, on the day of plantation and minimum when added only on 10 days after plantation. Among different BCAs *T. harzianum* -5 (79.8 PDC) was highly effective followed by *T. hamatum* -2 (78.4 PDC), *T. viride* 4 (76.6 PDC) and *Bacillus* sp. 4 (75.8 PDC), respectively. Addition of *P. funiculosum* and *Pseudomonas* sp. were almost equally effective (72.2-72.4%) when added 20 days prior to planting of apple seedlings.

Evaluation of frequency of applications of effective BCAs

Shortlisted effective BCAs were added in the form of above formulation 20 days prior to planting of seedlings and repeated doses were added at an interval of one month. Fifteen apple seedlings were planted in each plot of 1 m^2 and each treatment was replicated three times. Data on disease mortality was recorded periodically and final data on disease severity by following 0-5 disease rating scale was recorded in the month of December 2014 by uprooting the plants. Analysis of data indicated that as the number of application of BCAs increased the disease control also increased and the maximum disease control ranging between 95.8-98.8 per cent was recorded in the treatment wherein four consecutive applications were done followed by three (91.0-97.4%), two (81.4-87.9%) and single (72.2-79.8%) round of application, respectively. Addition of *Glomus mossae* provided 58.3, 65.4, 78.3 and 85.2 PDC for one to four rounds of applications, respectively.

Studies on compatibility of effective botanicals with selected BCAs under pot and field conditions

Under pot conditions: Two fungal biocontrol agents (BCAs) *viz.*, *Trichoderma harzianum* 5 (TTH5), *T. hamatum* 2 (THM2) and two bacterial antagonists *viz.*, *Bacillus* sp.-4 (BS-4) and *Pseudomonas fluorescens* KB6 were added individually as 2 per cent bran/coconut coir culture + 0.2% talc based formulation as well as in combination with *Murraya koeningi*, *Eucalyptus* and seeds of *M. azedarach* @ 3.0% (w/w) at 7 days prior to inoculation, simultaneously

at the time of inoculation, 7 and 12 days of inoculation of target organism for their compatibility. The plants were infected by adding the pathogen grown on maize grain + potato slices medium @ 2.0 (w/w). It contained both mycelium and sporangia (nearly 100). Data on seedling mortality were recorded periodically till 45 days of first appearance of symptoms and per cent disease control for each treatment was calculated. Data recorded indicated that pre-inoculation treatments with either BCA as well as their combination with botanicals exhibited more disease control in comparison to their post inoculation applications. Separate addition of TH5, THM2, *Bacillus* sp.-4 and KB6 at 7 days prior, simultaneously (0 days), 7 days and 12 days after inoculation with target organism provided, 80.4, 70.3, 55.2, 43.1, 78.1, 69.2, 60.6, 42.8, 82.5, 71.8, 57.2 , 48.0 and 80.2, 70.2, 61.0, 51.4, per cent disease control, respectively. Similar trial under field conditions and nursery yielded identical results but slightly less disease control. Pre-planting application of BCAs *viz.*, TH5, THM2, BS4 and KB6 with effective botanicals was highly effective to manage collar rot infection in apple. The combination with *Eucalyptus* leaf provided maximum disease control followed by *M. koeningi* leaf and *M. azedarach* seed, respectively.

Development of integrated disease management (IDM) strategy

Pot conditions: Effective BCAs (TH5, THM2 BS11, BS4 and KB6) were added @ 2% (w/w) bran/coconut coir culture + 0.2% talc based formulation), botanicals (mustard cakes, dried leaves of *M. koeningi* and *Eucalyptus*, seeds of *M. azedarach* @ 3 per cent (w/w), bio-fumigation with mustard plants @ 20% (w/w), cow urine decoction (7.5%) and fungicides Curzate and Cabrio Top (0.3%) were evaluated in 36 different combinations. These were added at seven days prior to inoculation, simultaneously at the time of inoculation and also 7 and 12 days after inoculation of target pathogen. Seedling mortality was recorded periodically with the first appearance of disease up to two months. Results indicated that pre-inoculation treatments with BCAs, fungicides and amendments exhibited enhanced disease control in comparison to their post-inoculation applications. Among 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. Further combined applications of fungicides along with bio-fumigation with mustard plant were also effective (>92%), whereas different combinations of bio-resources alone were less effective (73-82%).

Under field conditions: Similar experiments with most effective treatments were also laid out under nursery conditions (sick plot) and orchard conditions (2 hot spots). In a sick plot under nursery conditions 16 different combinations of above management inputs were added 20, 10 days before, simultaneously at the time of planting and 10 days after planting apple seedlings and grafted plants of cv. Vance Delicious on apple seedlings. Seedling mortality was recorded periodically with the first appearance of disease in the month of May 24, 2014 onwards. Final data was recorded in the month of December by following 0-5 disease rating scale. The results indicated that

pre-planting treatments were more effective in providing 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 (>99%) of disease when applied 20 days prior to planting of seedlings. Other combinations without fungicide were also effective (83.2-90.4% PDC). Combined application 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 highly effective and provided disease control between 91.0-100 per cent when applied at 10 days pre-inoculation/ pre-planting followed by 0 days (simultaneously) and after 10 days of inoculation.

Fifteen different combinations of above BCAs, bio-resources, botanical cow urine decoction, approach grafting, with fungicides were evaluated under orchard conditions against target disease in highly infested apple orchards at Poojan and Summa during 2014. Data on shoot growth were recorded in the month of September and November (after complete leaf fall). Data on lesion recovery were also recorded in the month of December 2014. Data (Table 3) indicated that addition of cow urine decoction (CUD) thrice @7.5% (10 l/tree) during March, June and August accompanied with approach grafting (AG) and two applications of Curzate (0.3%) during April and August was most effective and increased the shoot growth between 43.1 cm against 6.3 cm in untreated plants. CUD (thrice @7.5% (10 l/tree) during March, June and August and AG showed 39.9 cm shoot growth and lesion size recovery 37.6% followed by various other treatments (Table 3). The recovery in lesion size directly corresponds to the increase in shoot length recorded in different treatments.

Table 3: Effect of combined application of bio-control agents, bio-resources and fungicides against collar rot disease in apple during 2014

Treatment	Dose (g)/plant	Increase in shoot length (cm)#	lesion size (%)#
TH 5 + BS11 + Curzate	A+B+ 0.3%	32.4	26.3
TH5 + BS11 + MC + Curzate	A+B+0.5 Kg+0.3%	39.1	34.7
TH5 + BS11 + MA + Curzate	A+B+2 Kg+0.3%	35.2	28.9
THM + BS11 + Cabrio top	A+B+0.25%	32.0	26.3
THM + BS11 + MC + Cabrio top	A+B+ 0.5 Kg+0.25%	36.5	31.8
TH5 + BS11 + MA + Cabrio Top	A+B+2 Kg+0.25%	38.1	32.7
THM + BS11 + MPB + Curzate	A+B+ PB+0.3%	38.9	35.1
TH5 + BS11 + Murraya leaf + Curzate	A+B+3 kg+0.3%	35.5	30.6
TH5 + BS11 + CUD + Curzate	A+B+10 + 0.3%	36.4	32.3
TH5 + BS11 + MA + Murraya leaf + curzate	A+B+2 Kg+2 Kg+0.3%	39.9	34.6
MC + Murraya leaf + Curzate	0.5 Kg + 3 Kg +0.3%	31.3	25.8
AG + CUD + curzate	10 @7.5%+0.3%	43.1	38.6
Cow urine decoction (CUD)	3D**10 (7.5%)/tree	32.3	30.5
Cow urine	3D 10 (7.5%)/tree	23.1	16.2
CUD + AG	3DC+ AG	39.9	37.6
Control	-	6.3	60.3*
CD at 5% level		2.16	2.41

A = 200 g bran culture & 50 g talc formulation of THM2, TH5; B = 200 g coconut coir culture + 50 g talc formulation. BCA and fungicides were added in first week of April and last week of August while botanicals and cow urine decoction were added in the first week of March. ** = Three drenchings; AG = Approach grafting, CUD = Cow urine decoction, * = Increase in lesion size, # = Mean of two locations.

In the second experiment the soil around stem was removed up to 10 cm and the basins were drenched separately with Curzate (0.3%), cow urine decoction of three effective botanicals (10 l @ 7.5%), BCAs (TH5, BS11). Then red soil was spread around the stem up to 10 cm depth in the collar rot infected plants. These were later approach grafted. Results indicated that combined application of red soil + TH5+ BS11 + Curzate was highly effective in increasing the shoot length 41.9 cm as against 6.1 cm in control untreated plants but treatment red soil + cow urine decoction + approach grafting exhibited maximum recovery of lesion at the collar portion. Combined applications of red soil with other effective treatments were significantly more effective over its individual application.

Fusarium

ICAR-INDIAN INSTITUTE OF OILSEEDS RESEARCH, HYDERABAD

Principal Investigator:
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Co-investigators:
Dr. K Anjani, Dr. S Chander Rao &
Dr. V Dinesh Kumar

A. GENOMICS

Marker assisted selection and molecular breeding for *Fusarium* wilt resistance

F1 to F5 generations of the crosses between Nira x *C. oxyacantha, C. palaesntinus, C. creticus, C. lantaus, C. turkestanicus, C. glaucus* are being screened for wilt resistance. After five rows of test entries, one row each of two susceptible checks *viz.*, Nira and PBNS 12 were planted. Wilt incidence was recorded from 15 days after sowing and at 15 days intervals in all generations as well in susceptible checks. F6 generation of Nira x *C. oxyacantha*, BC1F3 of Nira x *C. oxyacantha*, F4 generation of A1- *C. tinctorius* x *C. oxyacantha*, F2 generations of *C. oxyacantha* x *C. palaestinus*, A1- *C. tinctorius* x *C. palaestinus* are being screened against wilt in a sick plot.

A total of 133 SSR markers were screened for wilt between interspecific crosses *viz.*, Nira x *C. oxyacantha*, and Nira x *C. palaestinus* using bulk segregant analysis (BAS) in F2 generations of these crosses. Of the 133 SSRs, eight were found polymorphic in between Nira x IP-16 (*C. oxyacantha*) and co-segregated with wilt resistance; and 20 SSRs were found polymorphic in between Nira x PI-2356632-2 (*C. palaestinus*) and co-segregated with wilt resistance.

The same markers were used to select wilt resistant progenies in F3-F4 generations of Nira (susceptible cultivated species parent) x *Carhaums oxyacantha* (resistant wild species) and Nira x *C. palaestinus* (resistant wild species). And thus marker-assisted selected progenies of each generation were further validated for their resistant reaction by screening in wilt sick plot. This has further confirmed and validated for the efficacy of identified markers in selecting the wilt resistant progenies.

B. INTEGRATED DISEASE MANAGEMENT

In vivo screening of biocontrol agents against *Fusarium* wilt in castor

In vivo screening of twenty *Trichoderma* isolates *viz.*, Phytofura 1 to 15, *Trichoderma harzianum* Th4d, *T. asperellum* TaDOR7316, *T. asperellum* TaDOR673, *T. asperellum* Tv5, *T. asperellum* N13 and *Pseudomonas fluorescens* Pf2 were screened against *Fusarium* wilt in castor. Six strains *viz., Trichoderma harzianum* Th4d, Phytofura 3, 12, 13 and 14 and Pf2 recorded significantly less disease incidence (30.8%, 33.3%, 31.0%, 34.6%, 34.6% and 32.0%, respectively) when compared to pathogen check (76.9%) (Fig. 1 & 2).
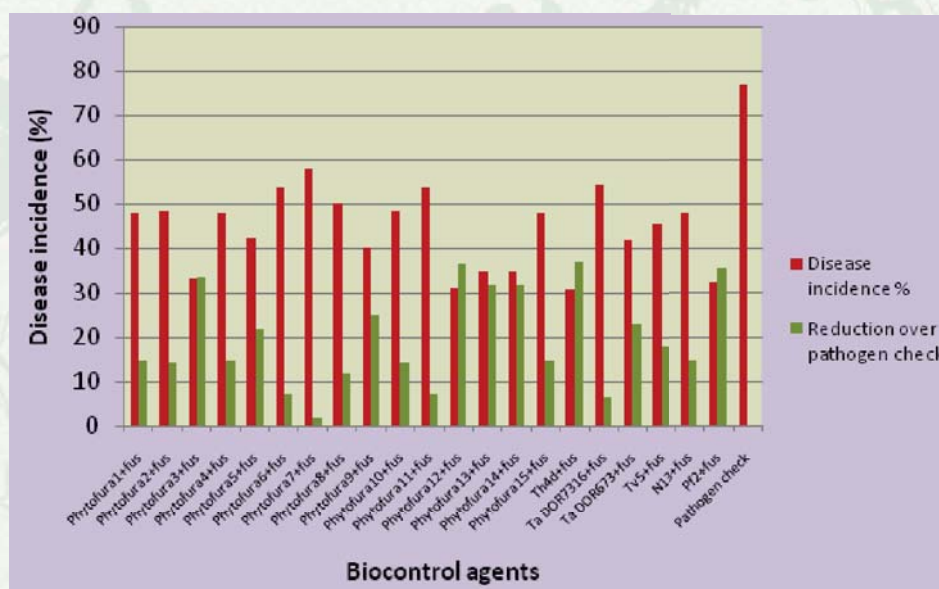

Fig. 1: Efficacy of biocontrol agents against *Fusarium* wilt in castor

T. harzianum   P. fluorescens   Phytofura 12 Pathogen check

Fig. 2: Effect of seed treatment with bioagents on castor

Effect of fungicides and biological agents on *Fusarium* wilt in safflower

A management trial on safflower crop using chemical fungicides, combination fungicides and biological control agents formulations was taken up during 2014-15.

Cymoxanil 8% + Mancozeb 64% (Curzate-M) @0.2% and *T. harzianum* Th4d SC @ 2 ml/kg were found to be the most effective (Fig. 3) as they recorded significantly low incidence of *Fusarium* wilt (10.3 & 11.3%) whereas control plots recorded high incidence of wilt (21.9%).

Cymoxanil 8% + mancozeb 64% @ 0.2%   *Trichoderma harzianum* Th4d SC@ 2 ml/kg

Carbendazim + mancozeb @ 0.2%   Control

Fig. 3: Effect of fungicides and biocontrol agents on *Fusarium* wilt in safflower under field conditions

ICAR-INDIAN AGRICULTURAL RESEARCH INSTITUTE, NEW DELHI

Principal Investigator:
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Dr. Parimal Sinha (Nov. 2014 onwards)

Co-investigator:
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(up to Nov. 2014)

A. DIVERSITY

Sequence-related amplified polymorphism-PCR analysis

Seventy one isolates of *Fusarium oxysporum* f. sp. *ciceris* (Foc) were used for the genetic diversity analysis using SRAP primers. These isolates are representatives of 14 different states and five pulse growing agro ecological zones, namely, North Eastern Plane Zones (NEPZ), North Western Plane Zones (NWPZ), North Hill Zones (NHZ), South Zones (SZ) and Central Zones (CZ) of India. These isolates were representing eight races of the pathogen. The primers that gave reproducible and scorable amplifications were used for the analysis. The SRAP amplified all the isolates of Foc and the isolates were highly variable in respect of banding profiles. The level of polymorphism on 228 DNA fragments amplified was 100%. The dendrogram derived from UPGMA analysis grouped the isolates into six clusters at 30% genetic similarity (Fig. 1). The groups generated by UPGMA analysis were partially corresponding to the state of origin/chickpea geographical region of the isolates.

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 *Foc* originating from 14 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 fragment in all the isolates of the pathogen. Fourteen isolates of the pathogen representing different races as well as places of origin were given for sequencing and the nucleotide sequences obtained were subjected to BLAST analysis. The nucleotide sequences were submitted to GenBank at NCBI and accession numbers (KJ881391-KJ881404) obtained. The gene specific sequences of *Fusarium* species available in NCBI database from elsewhere that showed more than 90% sequence similarity with the sequence generated in the present study were also used.
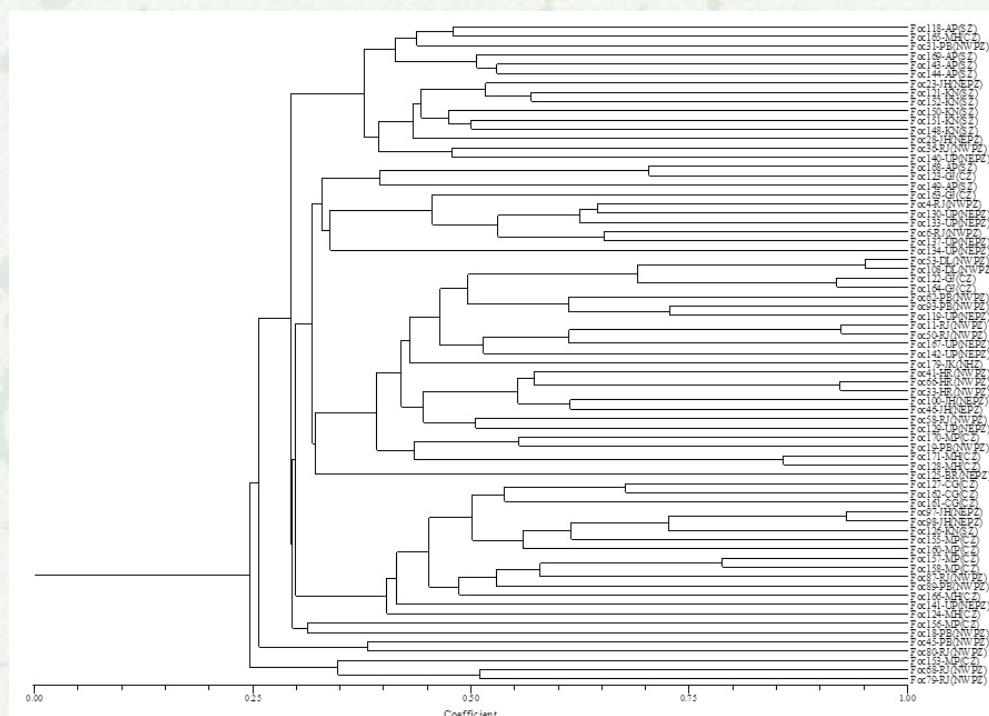

Fig. 1: Dendrogram of 71 isolates of *Fusarium oxysporum* f. sp. *ciceris* with 20 SRAP primers. The bottom scale is the percentage of Jaccard's similarity coefficient.

The phylogenetic tree constructed using bootstrap neighbor-joining analysis of 14 isolates of the present study along with eight foreign isolates grouped them into two major clusters. The first major cluster had all 14 isolates of the present study along with seven isolates from different countries. The first major cluster was further sub-divided into two sub-clusters. The first sub-cluster consisted of 14 isolates of the present study representing nine states and six races along with four isolates from other countries. The second sub-cluster had total of seven isolates, of which, four isolates from the present study and remaining three from other countries. The remaining one foreign isolate was grouped into the second major cluster (Fig. 2). The phylogenetic analysis of 28S nuclear large rDNA region of Foc isolates did not reveal any clear correlations among state of origin and races of the isolates.
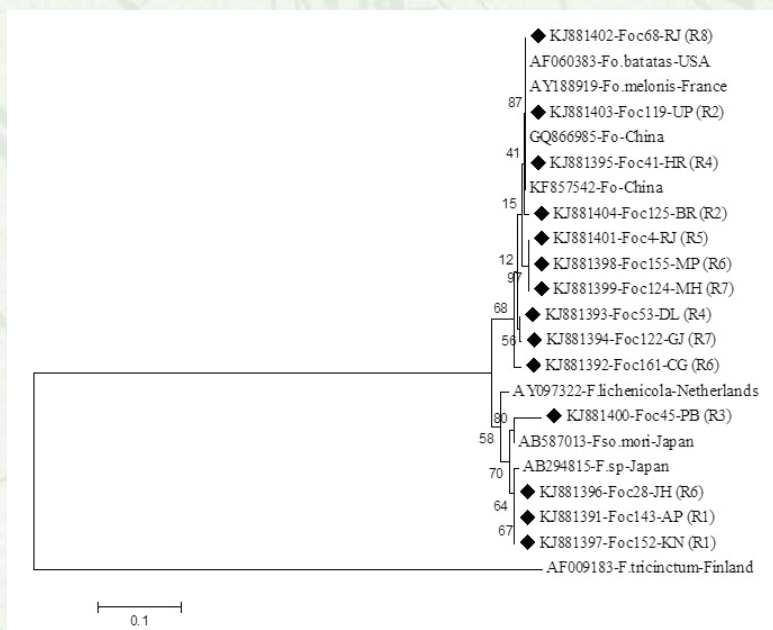

Fig. 2: Phylogenetic relationships among isolates of *F. oxysporum* based on their 28S ribosomal DNA sequences. The sequences generated during this study were diamond labeled.

B. HOST PATHOGEN INTERACTION

Differential expression pattern of MAP kinase gene family

Conventional and real time PCR assays were used to determine differential expression pattern in the mitogen-activated protein (MAP) kinase gene family of Foc infected resistant (GPF 2) and susceptible (JG 62) chickpea plants. 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 different treatment combinations for each chickpea variety were used for RNA isolation and cDNA preparation at 1-4 days after inoculation (dai). Sequence data of MAP kinase region obtained from NCBI GenBank database were used for primer designing. To determine the expression levels of MAPK and MAPKKK genes, PCR was performed. Strong and weak expression of the genes were presented by presence of thick band and faint band respectively, due to differences in the transcript levels in the samples.

The results of the expression analysis of MAP genes at 1, 2, 3 and 4 days after inoculation (dai) between treated and untreated susceptible (JG 62) and resistant (GPF 2) cultivars of chickpea performed by using conventional PCR showed the presence of expected amplicon size for MAP genes, namely, MAPK- F1R1 (248 bp) and MAPKKK-F2R2 (196 bp) .

Real-time PCR clearly showed that MAP kinase (MAPK) gene was differentially expressed at different time intervals in both treated (*T. harzianum*) and untreated plant samples. The expression analysis indicated that Foc infection up-regulated MAPK gene in susceptible variety, whereas in resistant variety the gene is down-regulated as compared to control (Un-inoculated). The treatment of *T. harzianum* + Foc also showed gene up-regulation in susceptible variety (Fig. 3) but in case of resistant cultivar, the gene was down regulated except 1 dai as compared to control (Fig. 3). Considering all the treatments with Foc and *T. harzianum*, the MAPK gene was up-regulated in susceptible variety whereas, it was down-regulated in resistant variety. The different treatments of chemicals also indicated that MAPK gene was up-regulated in susceptible as well as resistant variety as compared to un-inoculated control. The highest expression was observed with the treatment of salicylic acid + Foc in susceptible as well as resistant variety (Fig. 4).
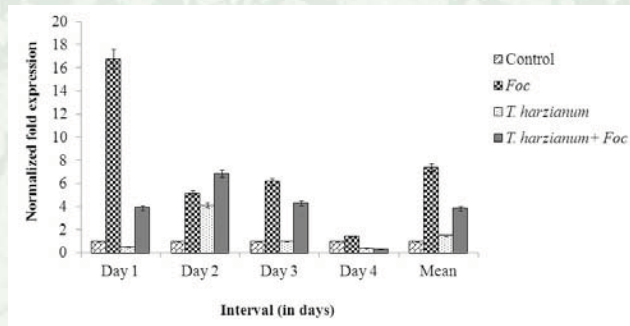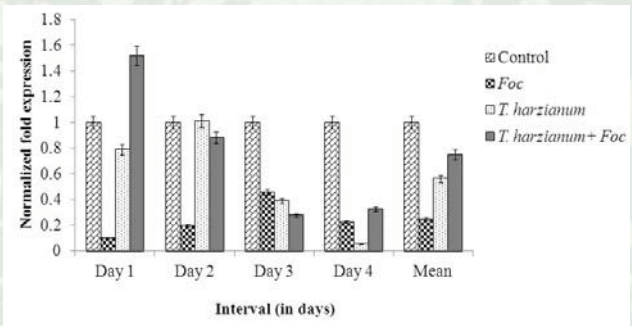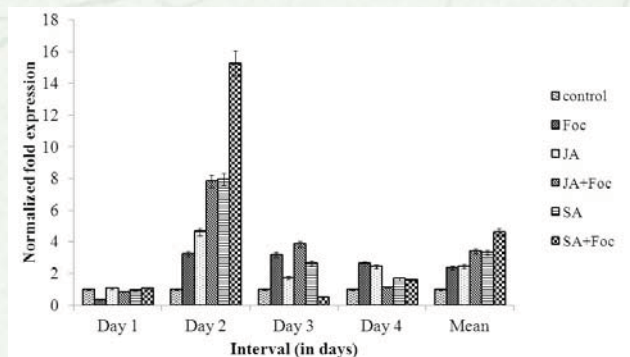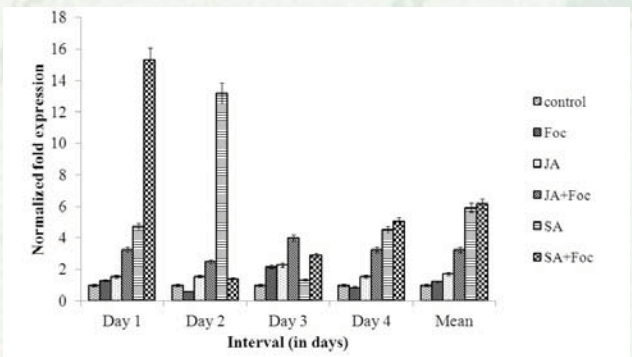
Susceptible chickpea variety JG62   Resistant chickpea variety GPF2

Fig. 3: Expression profiling of MAPK gene on inoculation with bioagents by using primer MAPK- F1R1

Susceptible chickpea variety JG62   Resistant chickpea variety GPF2

Fig. 4: Expression profiling of MAPK gene on treating with chemicals by using primer MAPK- F1R1

The MAP kinase kinase kinase (MAPKKK) gene was also differentially expressed at different time intervals in both treated and untreated plant samples. The gene was up-regulated with treatment of *T. harzianum* + Foc only at 2 dai, whereas in the case of Foc inoculated sample the gene was up-regulated (except 1 dai) as compared to the control in susceptible variety (Fig. 5). The MAPKKK gene of resistant variety showed up-regulation with the treatment of *T. harzianum* + Foc and the highest expression was found at 3 dai (Fig. 5). The plants treated with different chemicals indicate that MAPKKK gene was up-regulated in susceptible as well as resistant variety as compared to un-inoculated control. As in the case of MAPK gene, the highest expression was noticed in the treatment of salicylic acid + Foc in both susceptible and resistant varieties (Fig. 6). The average expression pattern of MAPK and MAPKKK genes were similar in both varieties against different chemical treatments.
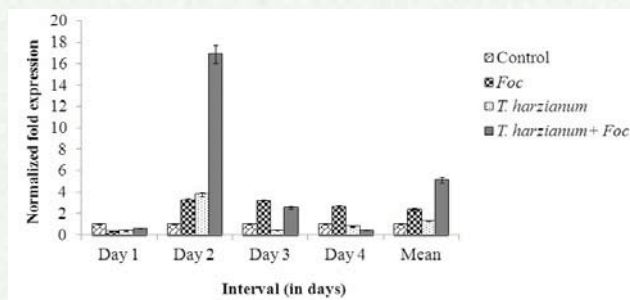
Susceptible chickpea variety JG62
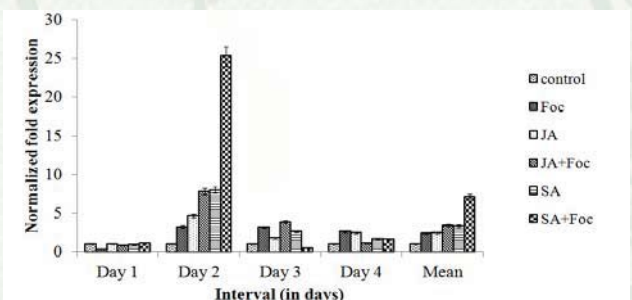
Resistant chickpea variety GPF2
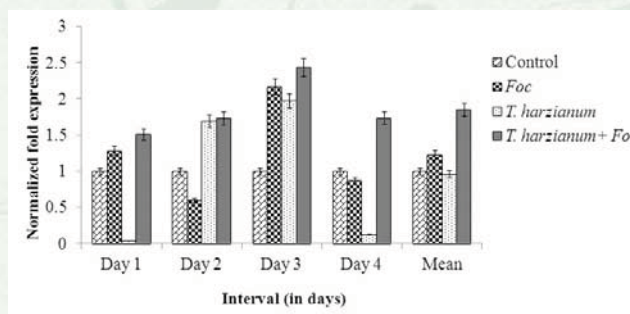
Resistant chickpea variety GPF2
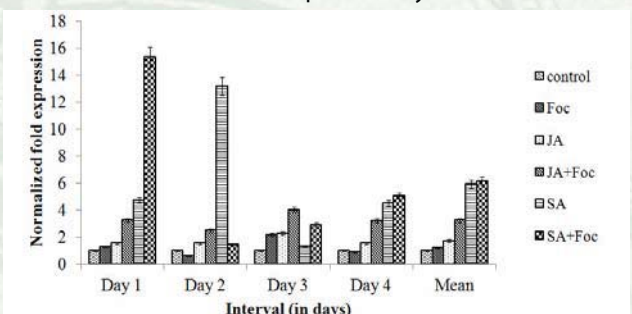
Susceptible chickpea variety JG62

Fig. 5: Expression profiling of MAP KKK gene on treating with bioagents by using primer MAP KKK- F2R2

Fig. 6: Expression profiling of MAP KKK gene on treating with chemicals by using primer MAP KKK- F2R2

ICAR-INDIAN INSTITUTE OF HORTICULTURAL RESEARCH, BENGALURU

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Co-investigator:
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A. DIVERSITY

Collection of wilt pathogens of gladiolus and carnation

Fusarium oxysporum f. sp. *gladioli* and *Fusarium oxysporum* f. sp. *dianthi* isolates were obtained from corms of wilt affected gladiolus and carnation plant. A total of 40 isolates of *F. oxysporum* f. sp. *gladioli* and 20 isolates of *F. oxysproum* f. sp. *dianthi* are maintained. The pathogenicity test was carried for these isolates to prove Koch's postulates. The morphological characters of these isolates like growth on potato dextrose agar (PDA), conidia characters (length and breadth of macro conidia, micro conidia, and shape), colony morphology on PDA, discoloration on the reverse side and colony growth were recorded for each isolate. Nine new isolates of *F. oxysproum* f. sp. *dianthi* were added to the culture collections (Table 1, Fig. 1).

Table 1: Morphological characterization of *F. oxysproum* f. sp. *dianthi* isolates

| Isolate name	Place	Culture plate morphology
Fod-1a	Kundha taluk -Ooty	Irregular margin, raised fluffy mycelium, brown pigmentation
Fod-2b	Kundha taluk -Ooty	Irregular margin, raised mycelium
Fod-3a	Udagamandalam taluk- Ooty	Irregular margin, Raised mycelium
Fod-4a	Udagamandalam taluk- Ooty	Irregular margin, flat mycelium
Fod-4b	Udagamandalam taluk- Ooty	Irregular margin, raised fluffy mycelium
Fod-5a	Coonoor taluk	Irregular margin, raised yellowish mycelium, No pigmentation
Fod-5b	Coonoor taluk	Irregular margin, Flat creamish white mycelium with pale violet pigmentation.
Fod-5c	Coonoor taluk	Irregular margin, Flat mycelium, concentric ring formation with pale violet pigmentation
Fod-5d	Coonoor taluk	Irregular margin, Raised fluffy mycelium, no pigmentation
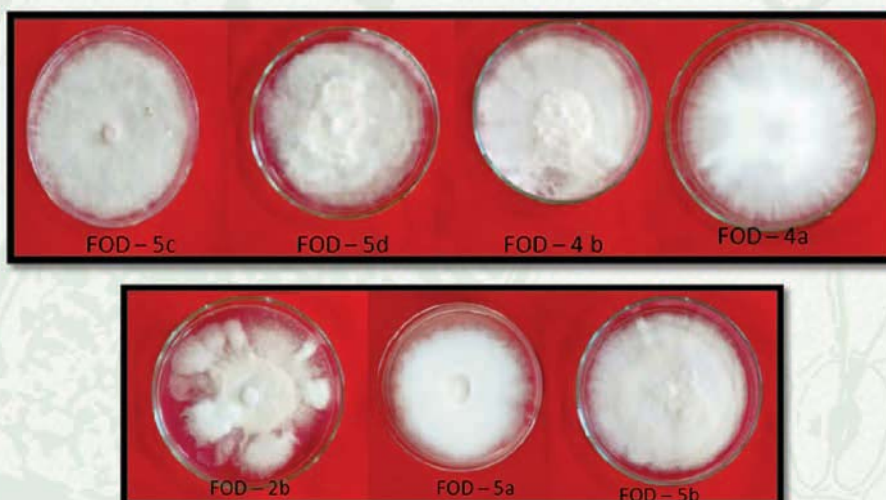
Fig. 1: Colony morphology of *F. oxysproum* f. sp. *dianthi* isolates

Pathogenicity screening for isolates

Morphological and molecular characterized FOG and FOD isolates were screened for their pathogenicity to check whether the cultures are virulent or non virulent. Results showed that FOD IARI C4 and IIHR 007 culture were able to induce wilt in carnation (Fig. 2). These isolates were re-isolated from the infected plant.

Fig. 2: Pathogenicity test FOD IARI C4 and control on carnation

Fungicide resistance in *Fusarium oxysproum* f. sp. *gladioli* and *F. oxysporum* f. sp. *dianthi*

As carbendazim has been used extensively for wilt management and at times not giving expected results, we tested the isolates of wilt pathogens of carnation and gladiolus for resistance to carbendazim and found that at least five isolates were resistant to this fungicide upto 2000 ppm concentration.

Molecular identification of *F. oxysproum* f. sp. *gladioli* and *F. oxysproum* f. sp. *dianthi* isolates

F. oxysproum f. sp. *gladioli* and *F. oxysproum* f. sp. *dianthi* isolates were characterized by sequencing ITS and tef regions (Fig. 3 - 6).

Table 2: Inhibition of mycelial growth of *Fusarium* isolates around the carbendazim discs (cm)

Isolate	100 ppm			200 ppm			500 ppm		
	3rd day	5th day	7th day	3rd day	5th day	7th day	3rd day	5th day	7th day
Fog4(11)	0.49	0.45	0.41	0.60	0.58	0.55	0.94	0.90	0.84
FogL3	0.83	0.71	0.61	1.09	0.94	0.78	1.1.2	0.94	0.78
FOGF9	0.21	0.0	0.0	0.25	0.0	0.0	0.27	0.0	0.0
Fog f11	0.19	0.61	0.0	0.26	0.23	0.0	0.27	0.24	0.0
F. solani	1.3	1.22	1.03	1.55	1.36	1.36	1.80	1.75	1.70
C$ IARI FoD	1.0	0.8	0.7	1.3	1.2	1.1	1.50	1.40	1.35
FOD13*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
IIHR 07b FOD*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
NRPC FOD *	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fog Solan*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fog M1*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
FOD KC*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

* isolates were tested at higher concentration of 2000 ppm and found resistant.
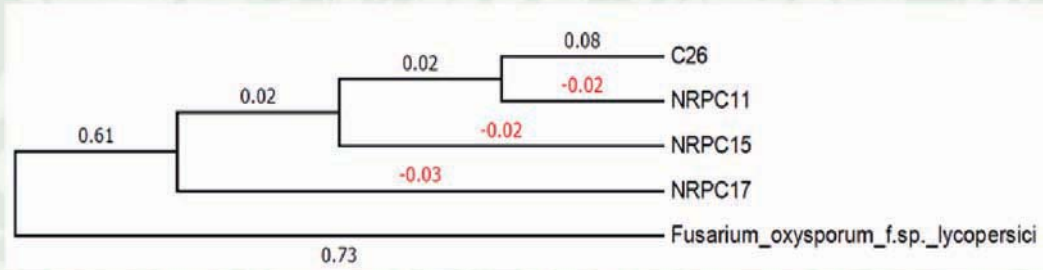
Fig. 3: Phylogenetic analysis of ITS sequences of *Fusarium oxysproum* f. sp. *dianthi* isolates
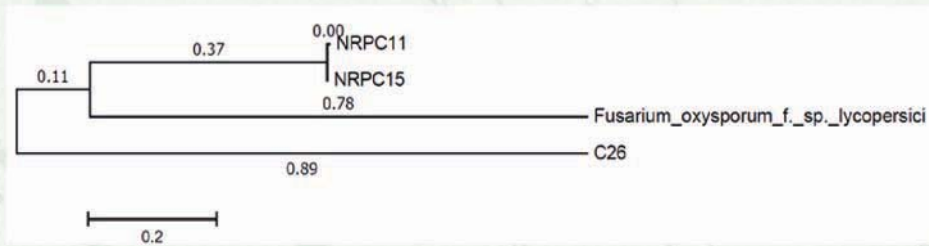
Fig. 4: Phylogenetic analysis of TEF region of *Fusarium oxysproum* f. sp. *dianthi* isolates
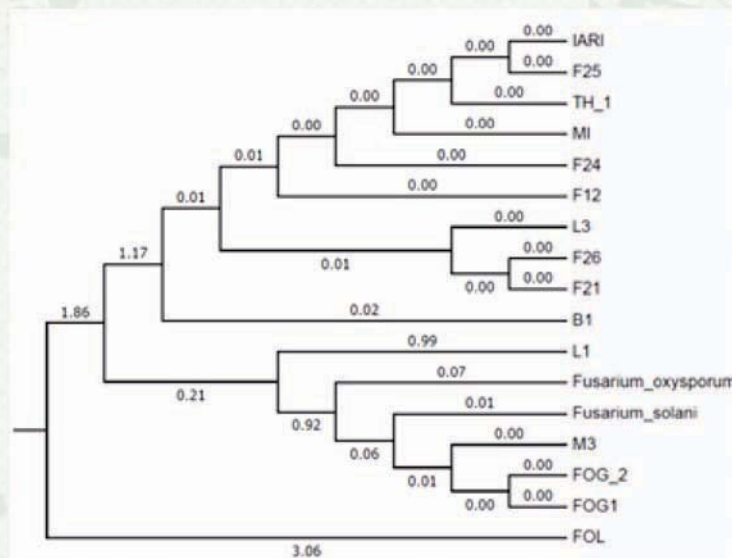
Fig. 5: Phylogenetic analysis of ITS sequences of *F. oxysproum* f. sp. *gladioli* isolates
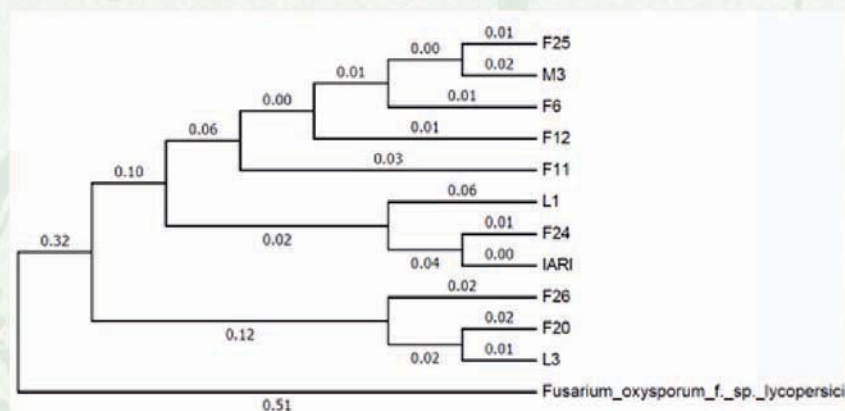
Fig. 6: Phylogenetic analysis of TEF region of *F. oxysproum* f. sp. *gladioli* isolates

B. HOST PATHOGEN INTERACTION

Production of fusaric acid from selected FOD and FOG isolates

FOG-Solan, FOD-IARI C4 and FOD-007 cultures were inoculated in PDB for isolation of fusaric acid and incubated for 21 days for fusaric acid production in culture. Fusaric acid was isolated from FOD solan, FOD IIHR07, FOD IARI C4 and FOG solan isolates mainly for HPLC, GC-MS studies and varietal screening.

C. HOST PLANT RESISTANCE

Molecular characterization of resistant gene analogs from carnation against *Fusarium oxysporum* f. sp. *dianthi*

Degenerative oligonucleotide primers were used for the characterization of genes conferring resistance to *Fusarium* wilt in carnation. Three varieties namely Arka Flame, Arka Tejus and H13 were used for the disease resistance screening.

D. INTEGRATED DISEASE MANAGEMENT

Preliminary trial on the management of gladiolus wilt with carbendazim tolerant *Trichoderma harzianum* isolates

A preliminary trial with carbendazim tolerant *Trichoderma harzianum* isolate (NBAII GJ16B) on the management of gladiolus wilt was carried out. The isolate was developed at NBAII and the same was tested as soil application and corm treatment. The wilt due to *Fusarium* could be reduced upto 4 per cent from 18 per cent under field condition in the preliminary trial.

ICAR-INDIAN INSTITUTE OF PULSES RESEARCH, KANPUR

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Co-investigators:
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A. DIVERSITY

Diversity in *Fusarium udum*

Fifty isolates of *Fusarium udum*, the causal agent of pigeon pea wilt, were rejuvenated by subjecting them to pathogenicity on wilt susceptible genotype of pigeon pea-Bahar. They were inoculated on to seven pigeon pea differentials (*viz.,* BAHAR, C 11, ICP 8863, ICP 7035, BDN 1, KPL 44, and ICP 9174). It was found that majority (34 nos.) of isolates could be grouped into five major pathogenic group named as variant I, II, III, IV and V (Table 1). Sixteen isolates could not be differentiated as all the differentials were susceptible to them.

Table 1: Reaction of pigeon pea differentials to *Fusarium udum* isolates

Variant/Group	Isolates	Number of isolates
Variant I	NF17, NF 133, , NF 117, Fu 49, Fu 61,P 3, KA 11, KA 14	8
Variant II	MP 137, HF 1, AP 1, AP 7, MSF 12	5
Variant III	F 8, NF 15, NF 16, NF 20, NF- 55, DF 3, AP 5, RF 3, KA 15 , MSF 4, Fu 12, Fu 24, Fu 88, KA 15	14
Variant IV	F 3, I 9, KA 8, NF 59, KA 1, HF 23	6
Variant V	NF 36	1
Un- grouped	ICRI 1, I 3, I 8, NF 3, NF 27, NF 72, NF 81, NF 98 , NF 134, NF 142, FU 37, FU 43, H1, RF 6, AKOLA1, MSF 14	16

Molecular identification of *Fusarium udum* and *F. oxysporum* f.sp. *ciceri* isolates

To classify the *Fusarium* species, ITS fragments (680-800 bp) of 34 isolates representing *F. udum* as well as *F. oxysporum* f. sp. *ciceri* were amplified and sequenced by outsourcing (Fig. 1).
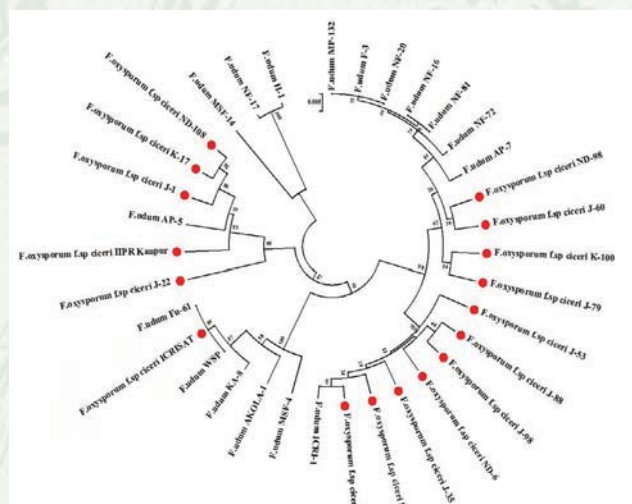
Fig. 2: Phylogenetic relationship of *Fusarium udum* and *F. oxysporum* f.sp. *ciceri* isolates

B. DIAGNOSTICS

Molecular detection of *Fusarium udum* and *F. oxysporum* f.sp. *ciceri* from soil

A simple and rapid procedure was used for efficient isolation of DNA from soil and for screening filamentous fungi (*Fusarium* sp.) using PCR detection. For this, four soil samples (1-2 g) were randomly collected from sick plot of *Fusarium* sp. and used for DNA extraction using CTAB method with some minor modifications. The quality and quantity were estimated by Biophotometer plus (Eppendorf) and were also confirmed by restriction digestion using Hind III and Hae III enzyme (Fig. 3).

Isolation and characterization of avirulence gene (AVR) in chickpea races (*F. oxysporum* f. sp. *ciceri*)

Based on the knowledge of AVR genes, seven *Fusarium* avirulence (AVR) gene specific primer combinations were synthesized and used for amplifying AVR genes in six chickpea race isolates. Out of them, only two primers (SIX-3 and SIX-5) amplified and gave repeatable bands (420 bp and 480 bp in size, respectively) for each race.
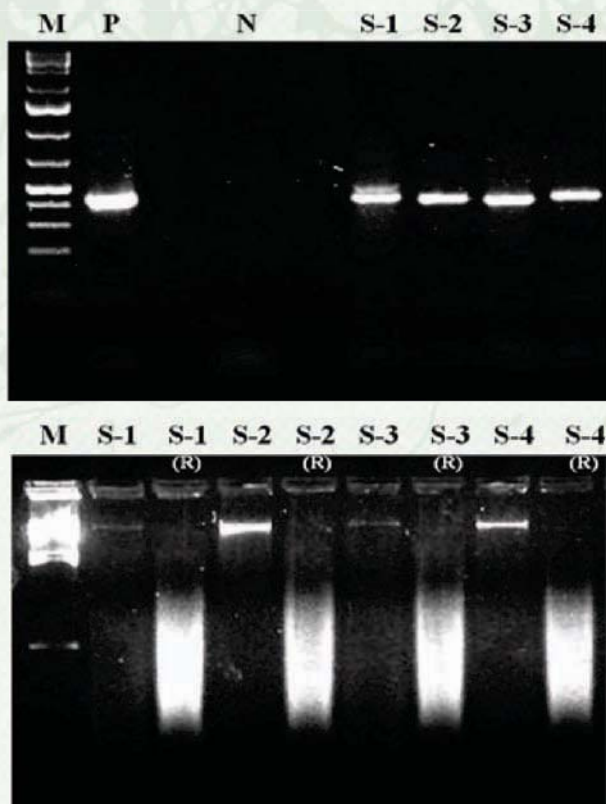
Fig. 3: (A) Restriction result using soil DNA samples of *Fusarium* sp.; (B) ITS primer profile of soil samples. M - 1 Kb plus ladder; P - Positive control (fungal mycelium); N - Negative control (healthy plant DNA); S1 to S4 - PCR product of soil sample DNA

C. HOST PLANT RESISTANCE

Pigeon pea: Seventeen genotypes of pigeon pea which are reported to be promising against wilt disease were planted for working out their reaction against *F. udum* variants 1-6. Based on the observations recorded so far, genotypes ICP 8862, ICP 89049, ICP 8858, BDN 1 appear to be resistant to all the six variants.

Twenty nine genotypes of pigeonpea that are used as donors in resistance breeding programmes all over the country were planted in the sick plot (variant 2). Based on the observations recorded so far, all the 14 IIPR promising lines are showing resistant reaction. Reaction to wilt is likely to change as the crop age increases. Eighty four genotypes received from AICRP on pigeonpea were planted in the wilt sick plot. Based on the observations recorded so far, genotypes BDN 2004-1, BDN 2011-1, BRG3, Mabeej 105, WRP223, PT257, IPA8F, GRG 140, GRG 160 appear to be resistant.

Chickpea: Of the chickpea lines planted in the wilt sick field, desi chickpea lines IPC 10-128, IPC 10-78, IPC2007-28, IPC 2007-04, IPC 07-36, IPC07-98, IPC 09-66, IPC09-153, IPC09-43, IPC10-3, IPC 10-41, IPC 10-173, IPC 10-71, IPC 10-146, IPC 10-121, IPC 2005-45, IPC 2005-26, IPC 2005-41, IPC 2005-19 and Kabuli chickpea genotypes IPCK12-306, IPCK12-258, IPCK12-310, IPC12-278IPCK12-94 were found resistant to race 2 of *F. oxysporum* f. sp. *ciceri*.

D. INTEGRATED DISEASE MANAGEMENT

The efficacy of 15 isolates of *Trichoderma* against pigeonpea wilt (*F. udum* variant 2) was worked out under pot conditions. The experiment was conducted in pots using wilt susceptible variety of pigeon pea (Bahar). Inoculum of *F. udum* was raised on pigeonpea seeds and added to the steam sterilized soil @ 5% (w/w). Seeds were treated with formulations of *Trichoderma* isolates @ 5 g/kg. Nine of the 15 isolates of *Trichoderma* (coded as isolate 1, 2, 3, 13, 7, 8, 9, 5, and 12) were found effective in reducing plant mortality by > 50% (range 53.57- 85.71%). Rest six isolates were relatively less effective in reducing plant mortality (range 14.28-46.42%). Overall, *Trichoderma* isolates 1, 2, 3, 5 and 7 were superior to others in reducing the plant mortality due to *F. udum* and also in increasing the root and shoot length.

ICAR-INDIAN INSTITUTE OF VEGETABLE RESEARCH, VARANASI

Principal Investigator:
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Co-investigators:
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A. DIVERSITY

Study on pathogenic variation in *Fusarium* isolates through amplification and sequencing of endo polygalacturonase gene: Seventeen isolates of FOL confirmed by ITS sequences were subjected to PCR with endo polygalacturonase gene specific primer PGLENDO-F (CCAGACTGCGCATACCGATT) and PGLENDO-R (AAGTGTTGGTAGGATAGTTG) amplification. Out of the 17 isolates of FOL, 13 showed positive amplification with *pgl* (endo polygalacturonase gene) primer and yielded 1.5 kb gene fragment. To compare the evolutionary relationship among the *pgl* gene of *F. oxysporum* f.sp. *lycopersici* a phy-

logenetic tree was constructed (Fig. 1). The tree showed major two clusters, in which one *Fusarium* isolate (FWT 77) was in one group and rest of the isolates were in another major cluster. The latter one was having two sub clusters comprising two isolates FWT71 and FWT5 in one and rest all in another.

B. HOST RESISTANCE

Grafting with resistance root stock is an easy technique to import resistance in desirable cultivars. Hence a brinjal root stock (EG219) resistant to *Fusarium* wilt, bacterial wilt and nematode was imported from AVRDC, Taiwan. Grafting technique to establish seedlings with brinjal as root stock and tomato (cv. Kashiamman) as scion was standardized (Fig. 2).

C. DISEASE MANAGEMENT

Different components such as talc based formulation of *Trichoderma* isolates (Phyto 1-15), two fungicides and botanicals (Datura and Garlic extracts) have been evaluated against *Fusarium* wilt of chilli and tomato under field conditions. In tomato, all the *Trichoderma* isolates showed significant reduction of wilt severity, however, Phyto 13 followed by Phyto1, Phyto3, Phyto 7, Phyto4 showed significantly higher yield than other treatments.

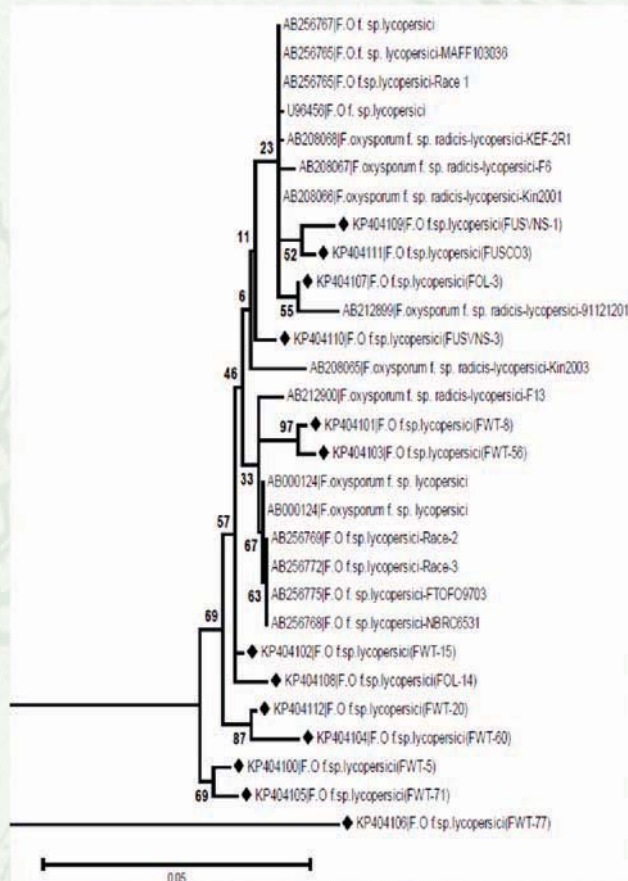Fig. 1: A neighbor-joining phylogenetic tree obtained from the nucleotide sequences of *pgl* gene of *F. oxysporum* f.sp. *lycopersici*. Vertical distances are arbitrary and the horizontal distances are proportional to the calculated mutation distances. Tree was created by MEGA 5.0 tool. The percentage of trees having the given branch is shown below the horizontal line.

Fig. 2: Tomato seedlings grafted with brinjal as root stock

ICAR-NATIONAL BUREAU OF AGRICULTURALLY IMPORTANT MICROORGANISMS, MAU

Principal Investigator:
Dr. Prem Lal Kashyap

Co-investigator:
Dr. Alok Kumar Srivastava

A. DIVERSITY

Characterization of *Fusarium solani* isolates from chilli based on multi-gene analysis

Molecular diversity and phylogenetic analysis of 31 isolates of *Fusarium solani* causing wilt of chilli, from various regions of India was analyzed using translation elongation factor-1α (TEF-1α), β-tubulin, mitochondrial gene sequences and internal transcribed spacer (ITS) gene regions. TEF-1α, β-tubulin, mitochondrial and ITS gene-specific markers produced ~350, ~680, ~600-700 and ~570 bp amplicons, respectively, in all the isolates of the pathogen. Most of the isolates of the present study showed unique grouping pattern in case of the four gene sequences. β-tubulin nucleotide sequences of 31 isolates ranged from 650 to 700 bp. The β-tubulin gene sequences of the isolates shared more than 90% nucleotide sequence similarity with β-tubulin sequences of *F. solani* available in NCBI Gen Bank. The phylogenetic tree constructed using bootstrap neighbor-joining analysis of 31 isolates grouped them into two major clusters. The first major cluster had 30 isolates while the remaining one isolate (FS 8) was grouped into the second major cluster. Similarly, in case of nucleotide sequences of ITS region, the amplicon size ranged from 500 to 600 bp and all the 31 isolates shared more than 90% nucleotide sequence similarity with ITS gene sequences of *F. solani* available in NCBI Gen-Bank. The phylogenetic tree constructed using bootstrap neighbor-joining analysis of 31 isolates grouped them into two major clusters. The first major cluster had 24 isolates while the remaining seven isolates (FS5, FS9, FS12, FS14, FS15, FS16 and FS24) were grouped into the second major cluster.

In case of translation elongation factor-1α (TEF-1α) gene analysis, all the 31 isolates shared more than 90% nucleotide sequence similarity with TEF-1α gene sequences of *F. solani* available in NCBI Gen Bank. The phylogenetic tree constructed using bootstrap neighbor-joining analysis of 31 isolates of grouped them into two major clusters. The first major cluster had 28 isolates. The remaining three isolates (FS6, FS17 and FS19) were grouped into the second major cluster. The phylogenetic tree analysis of mitochondrial gene sequences of 31 isolates of *F. solani* grouped all the 31 isolates into two major clusters, which indicates considerable variability among isolates. The first major cluster had 29 isolates. The remaining three isolates (FS28 and FS32) were grouped into the second major cluster.

Genetic diversity analysis of *F. oxysporum* f. sp. *lycopersici* by RAPD-PCR analysis

Among the ten RAPD primers, three primers *viz.,* OPA-2, OPA-3 and OPA-13 and OPA-18 were chosen based on their capacity to reveal polymorphisms among isolates (Fig. 1). RAPD analysis of genomic DNA from the pathogenic isolates revealed the presence of 12 clusters at the arbitrary level of 50% similarity. Maximum isolates were clustered in group I followed by group IV (Fig. 1).
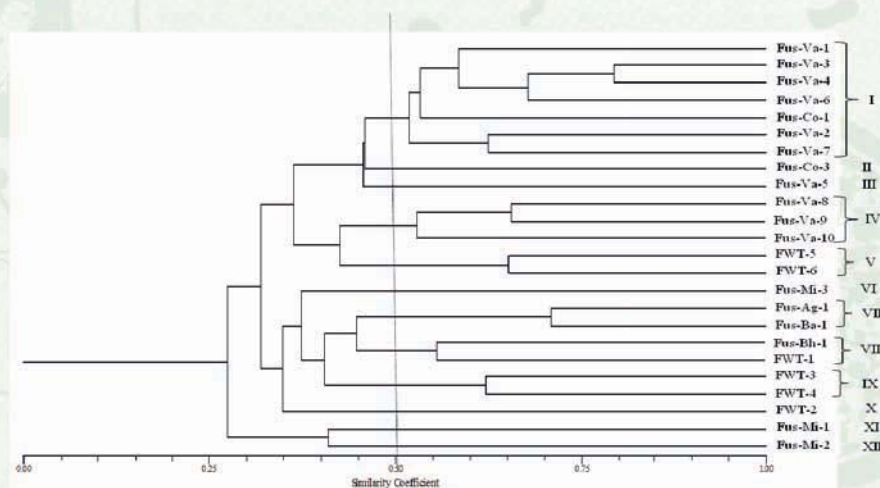
Fig.1: Dendrogram based on banding pattern of 24 Fol isolates obtained from RAPD markers

Genetic diversity analysis by BOX-PCR analysis

Analysis of BOX-PCR banding pattern (Fig. 2) showed that the Fu isolates were clustered into four clusters, sharing 50-100% similarity. A perusal of the dendrogram revealed that the seventeen isolates formed a major cluster (Cluster I), while only five isolates (Cluster -II) and isolates Fus-Va-2 and FWT-5 formed independent clusters III and IV, respectively (Fig. 2).
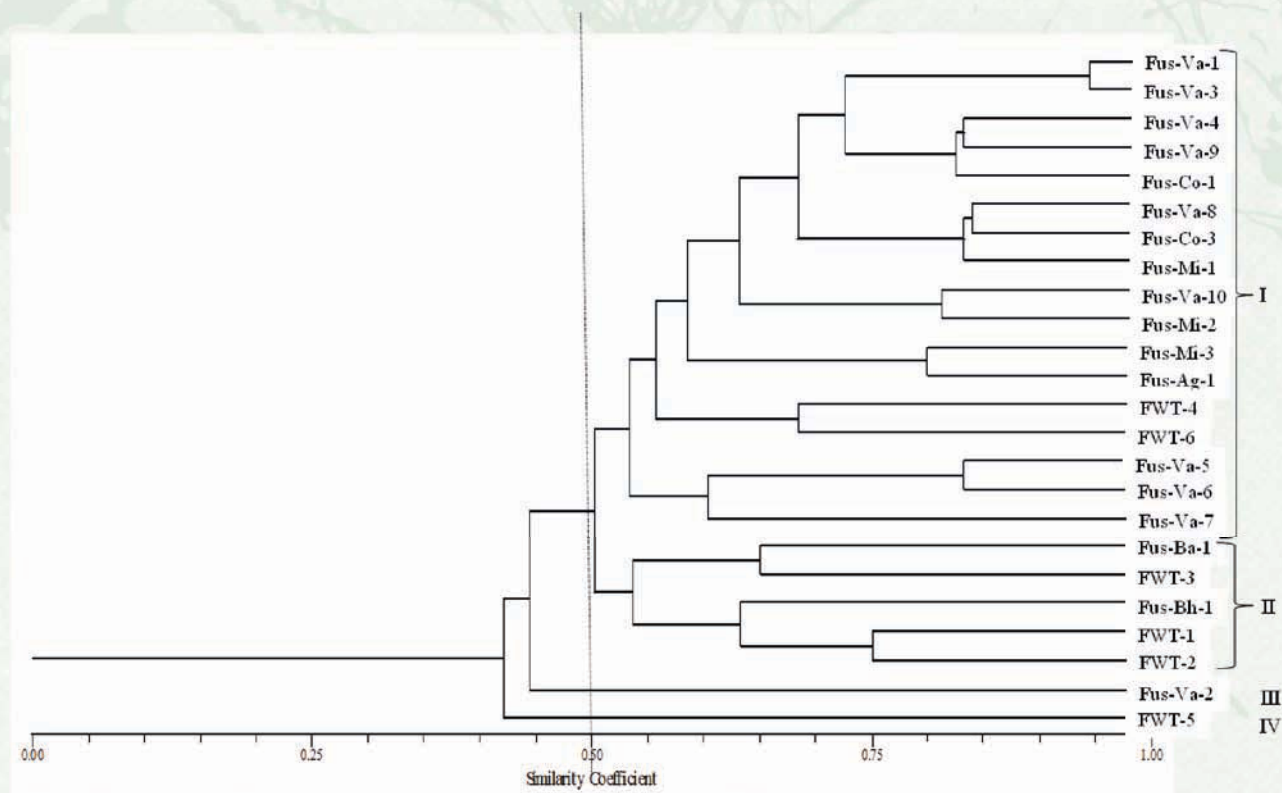
Fig. 2: Dendrogram based on banding pattern of 24 Fol isolates obtained from BOX-PCR

Genetic diversity analysis by mating type sequences

PCR assay was performed to assign mating types (MAT1 and MAT2) for 24 different isolates of *F. oxysporum* f. sp. *lycopersici*. A single product was generated by PCR from each isolate using primer pairs complementary to the alpha domain and HMG domain genes, respectively. A ~300 bp portion of the alpha domain (MAT1) was obtained from 13 isolates. Similarly, a 650 bp portion of HMG domain (MAT2) was detected in ten isolates. The presence of MAT-1 was detected in 12 isolates of FOC, while rest of the isolates showed the presence of MAT-2 gene.

ICAR-NATIONAL RESEARCH CENTRE FOR BANANA, TIRUCHIRAPPALLI

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Co-investigator:
Dr. S Backiyarani

A. DIVERSITY

Role of zimmu planting in reducing the Foc inoculum in soil

The role of intercropping of zimmu plants in reducing the *Fusarium oxysporum* f. sp. *cubense* (Foc) inoculum was studied under pot culture conditions. The results indicated that zimmu planting reduced the initial soil population of 5.60×10^5 to 6.6×10^2, which is approximately 1000 times less than the initial population. The study showed that zimmu planting can effectively suppress the survival of Foc inoculum in soil.

Management of nematodes in the *Fusarium* wilt and nematode complex system

A pot culture experiment was carried out to find out the effect of *Fusarium* wilt suppressive biocontrol agents on the control of root knot nematode (*Meloidogyne incognita*) in cv. Grand Naine (AAA). The results indicated that among the bioagents evaluated, the soil application of wild endophytic *Trichoderma asperellum* recorded significant decrease in the percent of infected roots, number of galls/plant (79%) and nematode population both in roots (96%) and soil (49%) compared to nematode alone inoculated control plants (Fig.1& 2) .
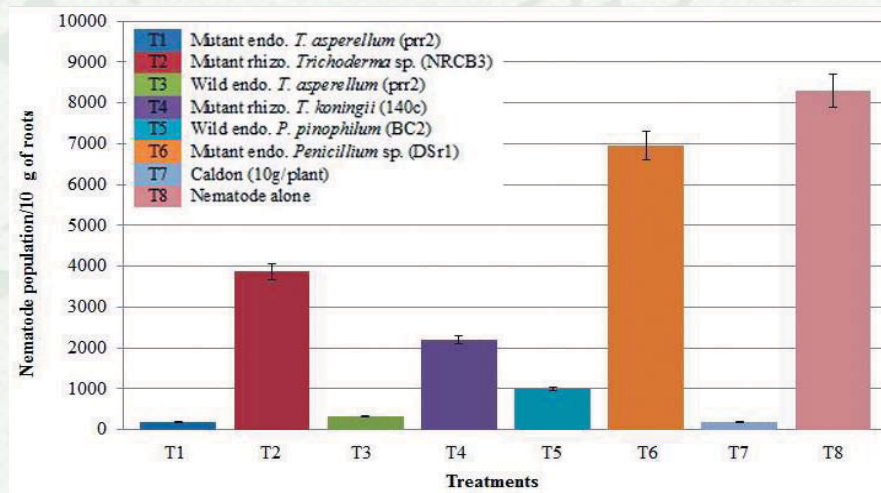
Fig. 1: Effect of biocontrol agents on the nematode population in roots of banana plants
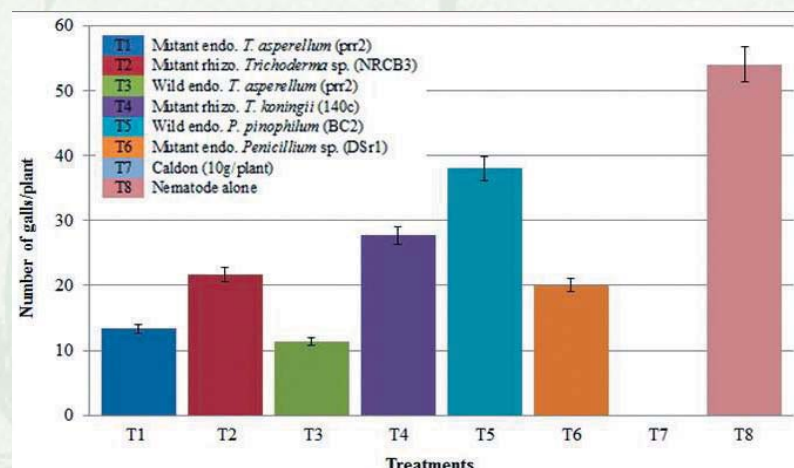
Fig. 2: Effect of biocontrol agents on the number of galls per plant in the roots of banana plants

B. GENOMICS

Differential expression of genes in banana by SSH approach

To identify the differentially expressed genes due to the interaction of Foc pathogen and effective biocontrol agent in banana, suppressive subtractive hybridization (SSH) was carried out in cv. Grand Naine. The sequences obtained were pre-processed to remove low-quality sequences, contaminant sequences (from vector to any other artifacts), and special features (like Poly-A tail or adaptors). TopBlast analysis of 300 sequences retrieved 28 contigs and 44 single sequences with various functions. Three contigs (contig 6, 7 and 10) were identified to contain defence related function (Table 1) and are also involved in phenylalanine and phenylpropanoid biosynthesis based on KEGG analysis.

Table 1: TopBlast analysis of three contigs with defence related functions

Sequence name	Sequence description	Sequence length	Hit ACC	E-Value	Role
Contig 6	catalase	1034	Q59296	1.45E-12	Lactoperoxidase in phenylpropanoid biosynthesis phenylalanine metabolism
Contig 7	PREDICTED: pleiotropic drug resistance protein 1-like	759	XP_004506879	1.75E-26	Cell rescue and defence through PDR-type multidrug transporter
Contig 10	pleiotropic drug resistance protein 1	823	Q949G3	1.78E-14	Lactoperoxidase in phenylpropanoid biosynthesis phenylalanine metabolism

Functional annotation of a hypothetical protein derived from SSH analysis

A total of 300 clones were sequenced, processed and 45 contigs were obtained. BLAST2GO analysis revealed the presence of contig 21 as among hypothetical proteins from SSH analysis. Functional annotation of hypothetical proteins yielded contig 21 as endoribonuclease. Sequential docking scores with miRNA>Phytoalexins>defense proteins show that it has a role in defense pathway. Atomic contact energies affirm that sequential docking can be phenomenal in elucidating a role of protein in a pathway. miRNA binding properties and phytoalexin binding confirm that contig 21 is a NPR1 homolog. Polyubiquitination supports additionally that differentially derived hypothetical protein from SSH analysis is NPR1 homolog.

Profiling of non-volatile metabolites from *Trichoderma asperellum*

The extracellular and intracellular metabolites of *T. asperellum* (Prr2) were extracted using ethyl acetate, butanol and hexane. The final extracts were re-suspended in methanol and subjected to antifungal assay against *F. oxysporum* f.sp. *cubense*. The results showed that only the methanol extract was found inhibiting the mycelial growth of the pathogen. Out of 12 fractions separated by column chromatography from *T. asperellum* extracts, fraction 12 recorded significantly higher inhibition of mycelial and spore germination under *in vitro* conditions.

ICAR-NATIONAL RESEARCH CENTRE ON SEED SPICES, AJMER

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Co-investigators:
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A. DIVERSITY

A survey was conducted in Rajasthan (Districts: Ajmer, Bhilwara, Chittorgarh) and Madhya Pradesh (Districts: Mandsaur, Ratlam, Ujjain, Neemach) for incidence of cumin wilt and diseased samples were collected for isolating *Fusarium oxysporum* f. sp. *cubense* (foc) (Table 1).

Table 1: Details of survey conducted in Rajasthan and Madhya Pradesh for incidence of cumin wilt

States surveyed	District	Location (village)	Geographical location	No. of samples	Date of collection
Rajasthan	Ajmer	Kaleshra	N 26º20.684' E 74 º 26.417'	02	4.2.2015
		Jethana	N 26º16.289' E 74º 26.627'	01	4.2.2015
			N 26º15.707' E 74º 27.154'	03	
		Nayagaon	N 26º15.478' E 74º 25.321'	04	4.2.2015
Madhya Pradesh	Neemach	Malkheda	N 24º28.892' E 74º 54.875'	01	29.1.2015
	Mandsuar	Narayangarh	N 24º52.310' E 74º 75.219'	01	29.1.2015
	Ratlam	Minawada	N 23º33.123' E 75º29.825'	01	30.1.2015
		Kasari	N 23º33.152' E 75º29.251'	02	30.1.2015

ICAR-SUGARCANE BREEDING INSTITUTE, COIMBATORE

Principal Investigator:
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Co-investigators:
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Dr. ML Chhabra and Dr. B Parameswari

A. DIVERSITY

Wilt suspected sugarcane varieties *viz.*, Co 2001-15-15, Co95020-21, Co 419-23, Co 86002, Co 419-22, Co 6304-23, Co 419-26, CoC 671-24, Co 419-24, CoSe 95422-26, Co 6304-24 were collected from the institute field. Surveys have been conducted in different states in both tropical and sub-tropical regions for wilt and pokkah boeng (PB) infections individually or in combination. About 46 wilt infected samples were subjected to pathogen isolation, in which 22 isolates were recovered. They were sub cul-

tured and added to the existing *Fusarium* culture collections in the lab.

National Hybridization Garden (NHG) houses more than 500 parental materials and being used by sugarcane breeders from all the sugarcane research centers in the country. Surveys were conducted for severity of wilt and PB symptoms during 12-13 months stage of 2014-15 season. For each clone, infections due to PB and wilt were recorded on clump basis. Based on the expression of disease severity, the clones were categorized as low, moderate and severe based on infections up to 40%, 41-70% and more than 71%, respectively (Table 1).

Table 1: Details of wilt suspected samples collected from National Hybridization Garden, SBI, Coimbatore

SL.No	Variety	Wilt	Sl.No	Variety	Wilt
1	ISH 41	Severe	33	CoH 133	Low
2	CoH 48	Moderate	34	CoJ 70	Moderate
3	CoSe 92423	Moderate	35	CoA 90081	Moderate
4	CoT 8201	Moderate to Severe	36	Co Or 01352	Severe
5	ISH 307	Moderate	37	CoS 87216	Moderate
6	Co 775	Moderate to Severe	38	CoS 97261	Moderate
7	LG 99122	Low	39	CoC 671	Low
8	Se / 922/ 98	Moderate	40	CoA 7602	Low to moderate
9	CoV 92102	Moderate	41	Co 88025	Low
10	Co 7915	Moderate	42	CoC 671	Low
11	Co 419	Low	43	CoC 90063	Low
12	Co 88025	Moderate	44	Co 740	Moderate
13	LG 06604	Moderate	45	Co 86002	Severe
14	UP – 2	Low	46	CoSe 92423	Moderate
15	CoH 13	Moderate	47	CoJaw 270	Low
16	CoJn 86141	Low	48	Cov 94101	Low
17	CoJ 85	Moderate	49	Co 99006	Moderate
18	CoTl 85116	Low	50	CoH 114	Moderate
19	Madhurima	Low to severe	51	C79218	Severe
20	2002V48	Moderate	52	CoH 110	Low
21	MS 901	Severe	53	CoJ 83	Moderate
22	Q70	Moderate to Severe	54	Co 94012	Moderate
23	CoS 90269	Moderate	55	Co 0238	Low
24	70A5	Moderate	56	Co 98010	Moderate
25	ISH128	Moderate	57	BO 91	Moderate
26	Coc 8001	Moderate	58	ISH 100	Severe
27	CoH 7802	Low	59	ISH 12	Moderate
28	CoH 112	Low	60	CoH 48	Moderate
29	Co 95005	Moderate	61	Co 86011	Moderate
30	97R383	Moderate	62	CoM 0265	Moderate
31	CoPant 97222	Low	63	CoM88121	Severe
32	CoH 128	Moderate			

Ralstonia
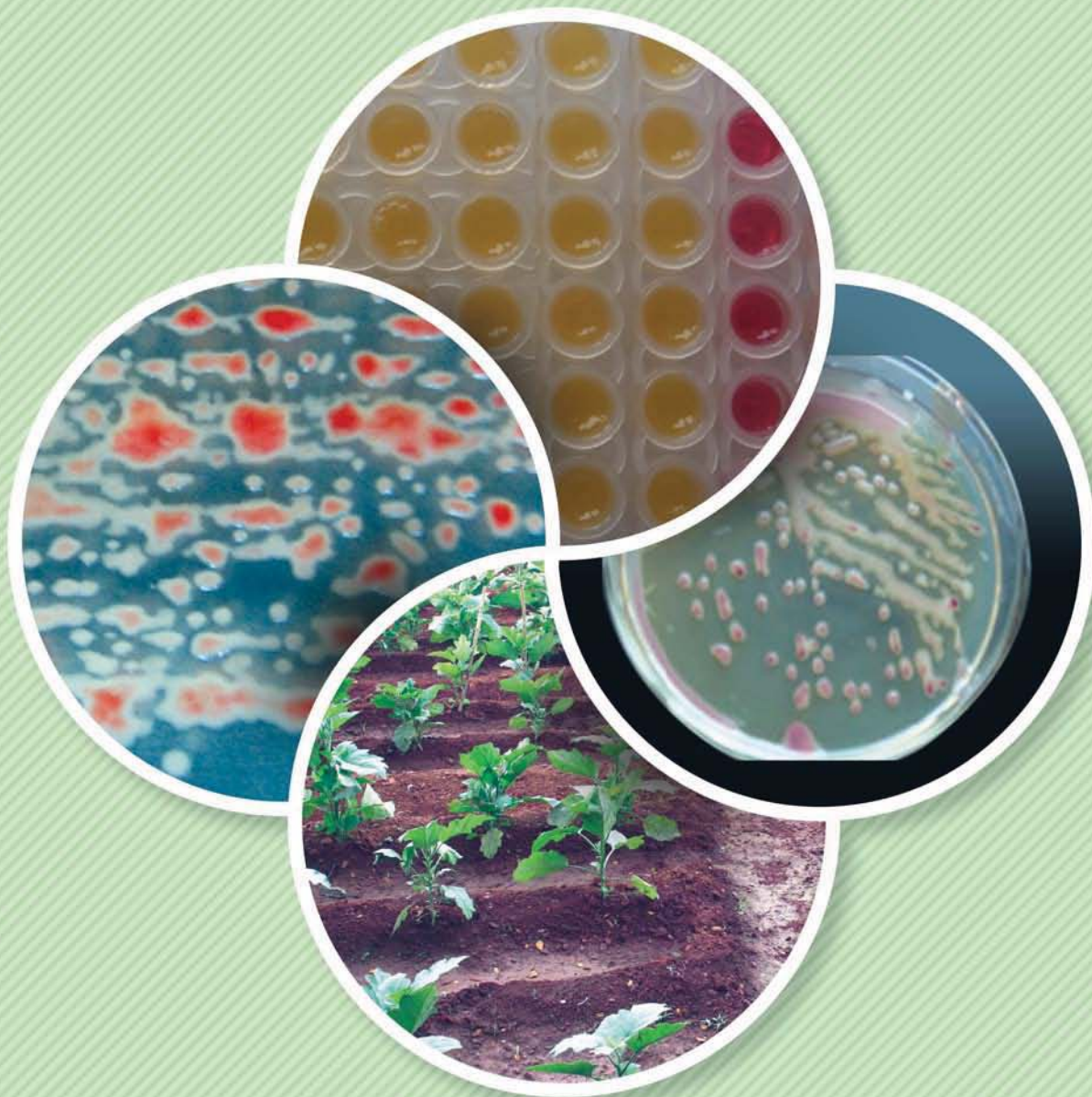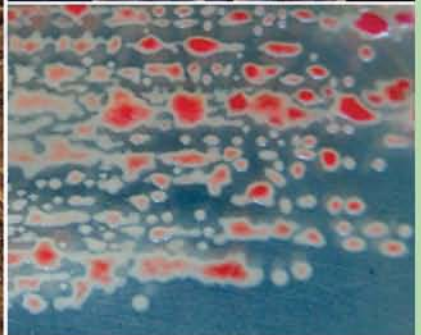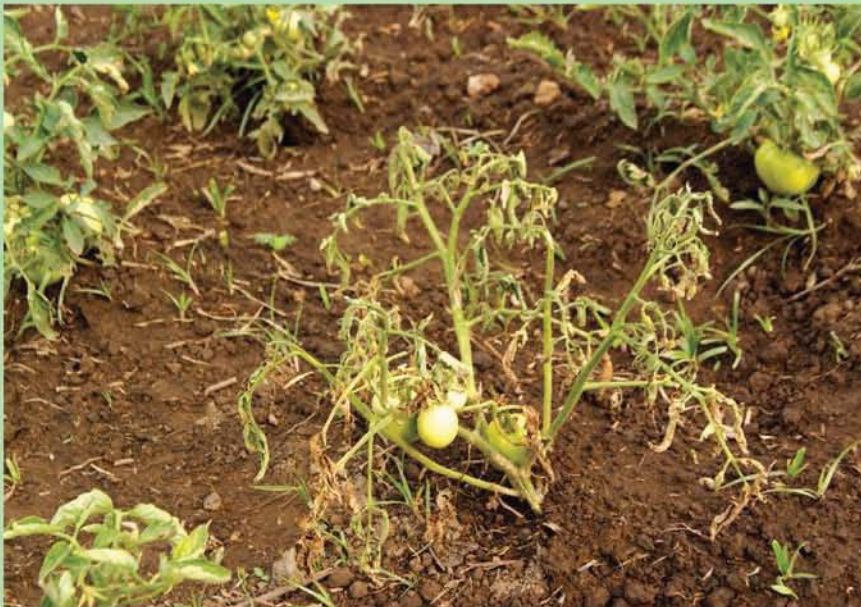
ICAR-CENTRAL COASTAL AGRICULTURAL RESEARCH INSTITUTE, OLD GOA

Principal Investigator:

Dr. R Ramesh

Co-investigator:

Dr. M Thangam

A. DIVERSITY

Collection of *Ralstonia solanacearum* isolates

Nineteen *R. solanacearum* have been deposited at NBAIM, Mau. The accession numbers are given below. The whole genome sequenced strains *viz.* Rs-09-161 and Rs-10-244 are deposited at MTCC, Chandigarh.

Analysis of genetic diversity of *R. solanacearum*

To study the diversity within a small geographical region, 10 *R. solanacearum* isolates from Goa and adjoining region of Maharashtra were selected. Six genes (*mutS, ppsA, adk, gapA, egl & fliC*) were aligned based on their alignment in GMI1000 and concatenated sequences were analyzed. The phylogenetic relationships of these 10 isolates were compared with the whole genome sequenced Phylotype I strains Rs-09-161 and Rs-10-244 along with GMI1000. The results indicated that Rs-08-44, Rs-09-172, Rs-11-344, Rs-10-278, Rs-11-376 were 100% identical with the Indian strain Rs-09-161 isolated from brinjal. Three isolates Rs-10-332, Rs-10-325 and Rs-09-83 were closely related to the other reference strain Rs-10-244.

MLST analysis of the selected 10 isolates was carried out. Nucleotide diversity was in the range of 0.0041-0.00207 across all the genes. It was highest in case of *mutS* gene (0.0041) and lowest in case of *ppsA* gene (0.00207). There was no diversity when *fliC* gene sequences were compared (Pi=0). Maximum number of polymorphic sites were present in *egl* sequences (6 sites), *gapA* (5 sites) and three sites each for *adk* and *ppsA* genes sequences. Only one site was detected for *mutS* gene. Haplotype numbers for each of the six genes ranged from 2 (*mutS*) to 6 (*gapA*). Five isolates *viz.* Rs-08-44, Rs-09-172, Rs-10-278, Rs-11-344 and Rs-11-376 were 100% identical to each other and belonged to haplotype1 and all were from brinjal and their sequevars are unknown. The frequency of mutations was more in *egl* as compared to other genes. Only one recombination event was detected in *gapA* gene sequences between 63 and 663 sites. Remaining genes appeared to be recombination free.

B. DIAGNOSTICS

Loop-Mediated Isothermal Amplification (LAMP) PCR for detection of *R. solanacearum*

Primers were designed for LAMP PCR. Several parameters of reaction mix and PCR cycle were optimized to obtain discrete ladder type banding pattern. PCR conditions were isothermal and maintained at 65°C. Using the optimized reaction mix and cycle conditions, ladder pattern with 6 to 7 bands was observed indicating positive detection of *R. solanacearum*.

To determine specificity of primers, LAMP PCR was performed using DNA from eight different commonly found soil and plant associated bacteria *viz. Pseudomonas aeruginosa, Agrobacterium* sp., *Bacillus cereus, Streptomyces* sp., *Burkholderia* sp., *Flavobacterium* sp., *Pseudomonas stutzeri, Enterobacter* sp. DNA of *R. solanacearum* isolates Rs-10-244 and Rs-09-161 served as positive control. Amplification was observed only with *R. solanacearum* DNA and no amplification was observed from DNA of other bacteria (Fig. 1).
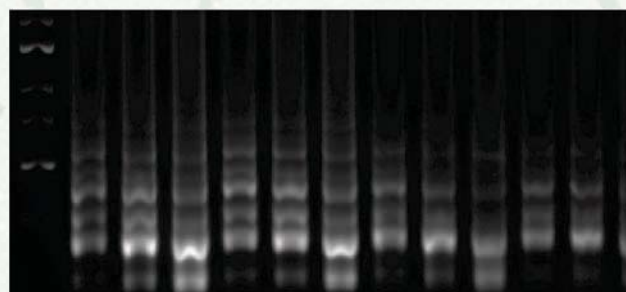
Fig.1: Amplification of DNA specific to *Ralstonia solanacearum* using LAMP primers

LAMP PCR was standardized to detect the bacterium directly from soil and the minimum threshold of detection from soil was 70 CFU. Using water bath similar pattern of amplification and intensity of bands as obtained using PCR machine was observed from positive samples. Water bath was found suitable for maintaining the isothermal temperature of 65°C required for LAMP PCR detection.

Suitability of several dyes for visual observation of positive LAMP reaction for detection of *R. solanacearum* under field conditions without the need for agarose gel electrophoresis was also standardized. Results of visual obser-

vation using dyes were comparable to those obtained by agarose gel electrophoresis. The results indicate that using water bath and a dye, *R. solanacearum* could be detected under field conditions using LAMP PCR.

C. HOST-PATHOGEN INTERACTION

To study the effectors of *R. solanacearum*, mutants of *hrcV* and *hrpB* are necessary. *hrpB* mutant of *R. solanacearum* was confirmed. *hrcV* mutant is being developed by insertional mutagenesis. Deletion mutagenesis of *hrcV* and *hrpB* was not successful and the process is in progress. Based on the whole genome sequences, two type 3 effector genes of *R. solanacearum* were shortlisted to study their role in pathogenesis. Cloning of these genes into destination vector to develop reporter based delivery system is in progress.

D. HOST RESISTANCE

Development of suitable mapping population

Fifty five lines of F4 were raised and seeds were collected from different plants which vary in fruit colour, fruit shape, colour pattern and bearing habit. Further 21 F4 lines are in the field for seed collection. A total of 100 F5 lines are in the field for evaluation.

E. DISEASE MANAGEMENT

Grafting of cultivated brinjal on wild brinjal

As grafting of cultivated brinjal on wild type controlled bacterial wilt, grafting technique was standardized. Grafts are planted in the field and are being evaluated for wilt management (Fig. 2).

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

Validation experiments on the effect of lime application to soil on the bacterial wilt incidence are repeated and evaluation is in progress.

Colonization of xylem residing bacteria (XRB) in brinjal

Antagonistic XRB of genera *Agrobacterium* sp. (XB1, XB86 and XB165), *Enterobacter* sp.(XB99 and XB123), *Pseudomona s aeruginosa* (XB7 and XB122) and *Bacillus cereus* (XB177) which prevented wilt were found to re-colonize brinjal stem and rhizosphere. Studies using GFP tagged XB177 proved that bacteria isolated from xylem could re-colonize xylem vessels of stem and also rhizoplane of brinjal, tomato and chilli when tested under greenhouse conditions. Further, pre-treatment of brinjal is an important pre-requisite for effective wilt prevention by the antagonistic bacteria.

Management of bacterial wilt using biocontrol agents

Six strains of biocontrol agents able to prevent wilt in brinjal in the greenhouse conditions are being evaluated for wilt prevention under field conditions. Four strains of xylem residing bacteria (XB86, XB122, XB102 and XB177) and two strains of endophytic bacteria (EB69 and RP7) were applied as talc formulations in the nursery and also during planting. Colonization of brinjal seedlings in the nursery by the introduced strains was determined before transplantation.

Fig. 2: Field evaluation of cultivated brinjal grafted on wild type for control of bacterial wilt

F. GENOMICS

Degradation of 3OH-PAME

Eight strains out of 167 XRB degraded 3OH-PAME, a quorum sensing molecule of *R. solanacearum*. Two of the 3OH-PAME degrading XRB (XB7 and XB122) were an-tagonistic to *R. solanacearum*. Degradation of 3OH-PAME to 3OH-palmitic acid was confirmed by HPLC-MS. Crude quorum quenching molecule from six XRB had high specific activity towards 3OH-PAME degradation. Five XRB (XB7, XB102, XB109, XB115 and XB122) which degrade 3OH-PAME were able to re-colonize and prevent wilt in brinjal.

ICAR-CENTRAL POTATO RESEARCH INSTITUTE, SHIMLA

Principal Investigator:
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Co-investigators:
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  Dr. S Sundaresha & Dr. MS Gujjar

A. DIVERSITY

Collection, isolation and maintenance of *Ralstonia solanacearum* isolates

Symptomatic plants/ tubers of potato were collected/ obtained from potato growing areas of East Khasi hills (Meghalaya), Raipur (Chhattisgarh) and Hooghly (West Bengal) (Fig. 1). Tubers/ stem pieces were washed thoroughly to remove soil adhered to their surfaces and air dried. The samples were then surface disinfected with 70% ethanol, peeled, subsampled and made to ooze bacterial cells in sterile distilled water (1 ml). The bacterial suspensions were streaked on Kelman' s triphenyl tetrazolium chloride (TZC) agar medium (Peptone, 10 g; glucose, 2.5 g; Casamino acid, 1 g; agar, 18 g; TZC, 50 mg litre-1; pH 7.0-7.1). Plates were incubated at 28±2ºC for 48 to 72 h. Bacterial colonies developing the typical irregular mucoid colonies were again streaked onto fresh TZC medium for further purification. Well separated typical wild type *R. solanacerarum* colonies were further transferred to medium modified by exclusion of TZC for multiplication of inoculum. Two loops full of bacterial culture were then transferred in 2 ml of double distilled sterile water and the cultures were stored at 20±2ºC for further studies.

Fig. 1: Bacterial wilt infected potato tubers (brown rot symptoms); left: dirty white slimy bacterial mass oozing from infected tuber's eye, right: vascular browning of tubers with tiny drops of slimy bacterial mass

Initially, a total of 45 isolates were recovered from symptomatic plants/ tubers collected from East Khasi hills (Meghalaya), Raipur (Chhattisgarh), Hooghly (West Bengal). Out of these, 30 isolates (15 from East Khasi Hills, 10 from West Bengal and five from Chhattisgarh) were confirmed as *R. solanacearum*. As expected a single 280 bp fragment was observed in these strains on PCR amplification using the *R. solanacearum* specific universal primer pair 759/760. The isolates were purified and maintained in distilled water at 20ºC for further studies.

Biovars determination

Strains were classified into biovars using a variation of the physiological test developed by Hayward (1964) which assays ability to oxidize a set of sugars and sugar alcohols. The basal medium used for biovar identification constituted $NH_4H_2PO_4$, 1.0 g; KCl, 0.2 g; $MgSO_4.7H_2O$, 0.2 g; Peptone, 1.0 g; 1% (wv^{-1}) aqueous solution of bromothymol blue, 0.3 ml; agar, 1.5 g; distilled water, 1litre. The pH of the medium was adjusted to 7.1 with 40% (wv^{-1}) NaOH

solution before addition of the agar. Five millilitres of a 10% (wv^{-1}) pre-sterilized solution of the sugars (sucrose, lactose, maltose, cellobiose, trehalose) and sugar alcohols (mannitol, sorbitol and dulcitol) were added to 45 ml of molten cooled basal medium separately. Two hundred µl of these media were then dispensed into each well of 96-well micro-titre plates. Hayward's medium without a carbon source and un-inoculated wells served as control. Each well was inoculated with 3 µl of a 2 x 10^9 CFU ml^{-1} cell suspension prepared from overnight CPG broth culture. The cultures were incubated at 28±2ºC for three weeks and colour was recorded every two days. Positive cultures change the culture medium from green to yellow. Each test was replicated three times.

The biovar analysis revealed that out of 15 isolates from East Khasi hills of Meghalaya, 11 (73.3%) belonged to biovar 2 and 4 (26.7%) to biovar 2T of the pathogen. However, all the 10 isolates from West Bengal and five from Chhattisgarh belonged to biovar 2 of the pathogen (Table 1).

Table 1: Characterization of 30 *R. solanacearum* isolates from Meghalaya, West Bengal and Chhatisgarh into biovars based on the ability to utilize disaccharides and oxidize hexose alcohols

District	Number of isolates						Total no. of isolates
	Biovar						
	1	2	2T	3	4	5	
East Khasi hills	0	11 (73.3%)	04 (26.7%)	0	0	0	15
West Bengal	0	10 (100%)	0	0	0	0	10
Chhattisgarh	0	05 (100%)	0	0	0	0	05
Total no of strains	0	26 (86.7%)	04 (13.3%)				15

Phylotype determination

Total genomic DNA was extracted and phylotype identification of each isolate was done. Phylotype specific multiplex PCR (Pmx-PCR) was carried out in 25 µl final volume of reaction mixture, containing 1×Taq Master Mix (PCR buffer, 1.5 mM MgCl2, 200 µM of each dNTP, 50 mMKCl, 10 mMTris-HCl and 1.25U of Taq DNA polymerase.), 6 pmoles of the primers Nmult:21:1F, Nmult:21:2F, Nmult:22:InF, 18 pmoles of the primer Nmult:23:AF and 4 pmoles of the primers 759 and 760. The following cycling programme was used in a thermal cycler (Gen-AmpR PCR System 9700 of M/S Applied Biosystem): 96°C for 5 min and then cycled through 30 cycles of 94°C for 15 s, 59°C for 30 s and 72°C for 30 s, followed by a final extension period of 10 min at 72°C. A 5 µl aliquot of each amplified PCR product was subjected to electrophoresis on 2% agarose gel, stained with ethidium bromide and bands were visualized on a UV-trans-illuminator. This Pmx-PCR amplifies the 280-bp "universal" *R. solanacearum* specific reference band plus following phylotype-specific PCR products: a 144-bp amplicon from phylotype I strains; a 372- bp amplicon from phylotype II strains; a 91-bp amplicon from phylotype III strains; and a 213-bp amplicon from phylotype IV strains. Strains that were classified as phylotype II were further tested using the PCR primer pair 630-F and 631-R, which specifically amplifies a 278-bp fragment from strains in phylotype IIB, sequevar 1, historically known as r3bv2.

The multiplex PCR (Pmx-PCR) confirmed all the 30 isolates(15 from East Khasi Hills, 10 from West Bengal and 5 from Chhattisgarh) to be *R. solanacearum* as expected 280 bp fragment resulted in all the isolates following PCR amplification using the *R. solanacearum* specific universal primer 759/760. Of the 15 isolates from East Khasi hills, 11 (73.3%) belonged to phylotype II and 4 (26.7%) to phylotype IV. Whereas, all the 10 isolates from West Bengal and 5 from Chhattisgarh belonged to phylotype II of the pathogen (Fig. 2, Table 2).

Fig. 2: PCR based identification and phylotype affiliation of representative *R. solanacearum* strains from Meghalaya

Table 2: Phylotype distribution of 30 *R. solanacearum* isolates from Meghalaya, West Bengal and Chhatisgarh based upon DNA typing using phylotype specific multiplex–polymerase chain reaction (Pmx-PCR)

District	No. of strains					Total no. of strains
	Phylotype I	Phylotype II Subcluster/sequevar		Phylotype III	Phylotype IV	
		A/non-R3bv2	B/R3bv2			
East Khasi hills	0 0	0 0	11 (73.3%)	0 (26.7%)	04	15
West Bengal	0 (6.4%)	0	10 (100%)	0	0	10
Chhattisgarh	0	0	05 (100%)	0	0	05
Total no. of strains	0	0	26 (86.7%)	0	04 (13.3%)	30

Phylogenetic analysis of *egl* gene sequences of *R. solanacearum* strains

PCR amplifications of a 750-bp region of the *egl* gene were performed using the primer pair Endo-F and Endo-R. The reaction mixture (50 µl) contained 1× PCR buffer, 1.5 mM MgCl^2, 200 µM of each dNTP, 0.25 µM of each primer, 50 ng of template DNA and 1.25U of Taq DNA polymerase. PCR amplifications were carried out in a thermo-cycler programmed for an initial denaturation step of 96°C for 9 min followed by 30 cycles of 1 min at 95°C, 1 min at 70°C, 2 min at 72°C, with a 10 min final extension at 72°C, and a final hold at 4°C. Samples (5 µl) of reaction mixtures were examined by electrophoresis through 1.2% agarose gels stained with ethidium bromide in TAE buffer and bands were visualized on a UV-trans-illuminator.

Amplified PCR products were further purified using Qiagen Minielute PCR purification kit following manufacturer's guidelines. The concentration of each product was estimated by gel electrophoresis with a low DNA mass ladder (Fermentas) and diluted with ultra-pure water to give a final concentration of 10-20 ng µl^{-1} for sequencing. DNA samples were cycle sequenced using forward primer in thermal cycler (Gen-AmpR PCR System 9700 of M/S Applied Biosystem). Cycle sequenced products were further purified. Sequencing is in progress.

Maintenance and preservation of isolates

All the 30 *R. solanacearum* strains, isolated this year, have been maintained in double distilled sterile water in 2 ml vials stored at 20±2°C for further studies.

B. DISEASE MANAGEMENT

Management of *Ralstonia solanacearum* through biotechnological approaches

To analyze the molecular mechanisms of disease susceptibility, we screened cv. Kufri Jyoti showing susceptibility to *R. solanacearum* for the expression of lipid signaling pathway gene (PAP) (inhibitor for accumulation of reactive oxygen molecule and hypersensitive reaction) and cDNA was cloned from the infected tissues and confirmed their sequence homology (100%) by PCR cycle sequencing and NCBI BLAST analysis (Fig. 3). Real time PCR analysis showed 2.5 fold higher expression of PAP transcript compared with non-infected tissues (Fig. 4). This confirmed that expression of PAP might contribute to disease susceptibility by inhibiting the accumulation of reactive oxygen molecules (this need to be validated). Silencing of genes needs to be performed through virus induced gene silencing (VIGS) technique to validate/confirm the genes involved in susceptible reaction. Simultaneously, virulence gene of the pathogen, *hrpB* is also being silenced through VIGS for initial confirmation. Based on these proofs of concept, RNAi based transgenics will be developed to build tolerance against bacterial wilt disease in potato.

C. GENOMICS & BIOINFORMATICS

Genome sequencing of four strains of *Ralstonia solanacearum* that cause potato bacterial wilt in India

We sequenced the genomes of all four strains using mas-

```
PP       ------------------------------ATGGCGGATTTACTCAGAAATGGAGATGCTGAG  33
Lipase   GGATTAAATTCGCGATGCATCTAGATTATGGCGGATTTACTCAGAAATGGAGATGCTGAG  60
                                     *****************************************

PP       GATGTTGCTGTAAAGCTTGGAGCTTTTGAACAACAAGCTCTCATTCATGACTTCTCAACT  93
Lipase   GATGTTGCTGTAAAGCTTGGAGCTTTTGAACAACAAGCTCTCATTCATGACTTCTCAACT  120
         ************************************************************

PP       TCTGCTTCTGCTCGATTCAACGCCACTCTCAATAGCCTGAGTAAGTGGCTGGTGGCTGCA  153
Lipase   TCTGCTTCTGCTCGATTCAACGCCACTCTCAATAGCCTGAGTAAGTGGCTGGTGGCTGCA  180
         ************************************************************

PP       GTTTTTGTTATAATCTTCCTTTGGAGGCATGACACTGAAGCACTTTGGGCAGCTTCTGGT  213
Lipase   GTTTTTGTTATAATCTTCCTTTGGAGGCATGACACACAGAAGCACTTTGGGCAGCTTCTGGT  240
         **********************************    *************************

PP       TCTATTCTGAATGCTAGTCTCTCAACTGTATTGAAGAGAATACTAAACCAGGAGCGACCT  273
Lipase   TCTATTCTGAATGCTGGGCTCTCAACTGTATTGAAGAGAATACTAAACCAGGAGCGACCT  300
         ***************  *  *****************************************

PP       GTTTCTGCGATAAGATCAGACCCGGCAATGCCATCTGCTCATGCACAGTCAATTTTCTAT  333
Lipase   GTTTCTGCGATAAGATCAGACCCGGGAATGCCATCTACTCATGCACAGTCAATTTTCTAT  360
         *************************  *********  *******************************

PP       ACAGTGATCTTCTGTATTGTCTCAATGGTAGAATATTCCGGTTAAATGCAGTCACTGCA  393
Lipase   ACAGTGATCTTCTGTATTGTCTCAATGGTAGAATATTCCGGTTAAATGGAGTCACTGCA  420
         ****************************************************** ********

PP       GTCATTAGTGCACTTATCTTTGCAATTGGCTCCTACTTGTCATGGCTACGGGTTTCACAA  453
Lipase   GTCATTAGTGCACTTATCTTTGCAATTGGCTCCTACTTGTCATGGCTACGGGTTTCACAA  480
         ************************************************************

PP       CAACTCCACACGACCAGTCAAGTAGCCGTAGGTGCTGCACTGGGATTTTCTTTCTCCGTT  513
Lipase   CAACTCCACACGACCAGTCAAGTTGCCGTAGGTGCTGCACTGGGATTCTCTTTCTCCGTT  540
         ***********************  ***********************  ************

PP       TTCTGGTTTTGGTTGTGGGATGCAATAGTCCTTGATGCATTTATATCCCACTTGTGGATT  573
Lipase   TTCTGGTTTTGGTTGTGGGATGCAATAGTCCTTGATGCATTTATATCCCACTTGTGGATT  600
         ************************************************************

PP       CGGATTCTTGTTGTTCTTGGCACGGTGGCCATTTGTGTTAGCTTTCTCCTTTATATGTTC  633
Lipase   CGGATTCTTGTTGTTCTTGGCACGGTGGCCATTTGTGTTGGCTTTCTCCTTTATATGTTC  660
         ***************************************  **********************

PP       CGATACTGGGTTCTTGAGAGAGACTGA-----------------------------  660
Lipase   CGATACTGGGTTCTTGAGAGAGACTGAAATCGGATCCCGGGGCCCGTCGACTGCAGAGGC  720
         **************************

PP       --------------------------------------------------------
Lipase   CTGCATGCAAGCTTTCCCTATAGTGAGTCGTATTAGAGCTTGGCGTAATCATGGTTCATA  780

PP       --------------------------------------------------------
Lipase   GCTGTTTCCTGTGTGAATGTATCCGGCTCACATCCACACAACATACGAGCGGAGCATAAA  840

PP       -------------------------------------------------------
Lipase   GTGTAAGGCCTTGCCGACCTATGAGTGAGCTTACTCACAATTAATTG  888
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Fig. 3: Multiple sequence alignment of T/A-PAP clone using clustalW2 multiple sequence alignment tool to confirm the integration of PAP gene in T/A vector and sequence homology with the reference gene
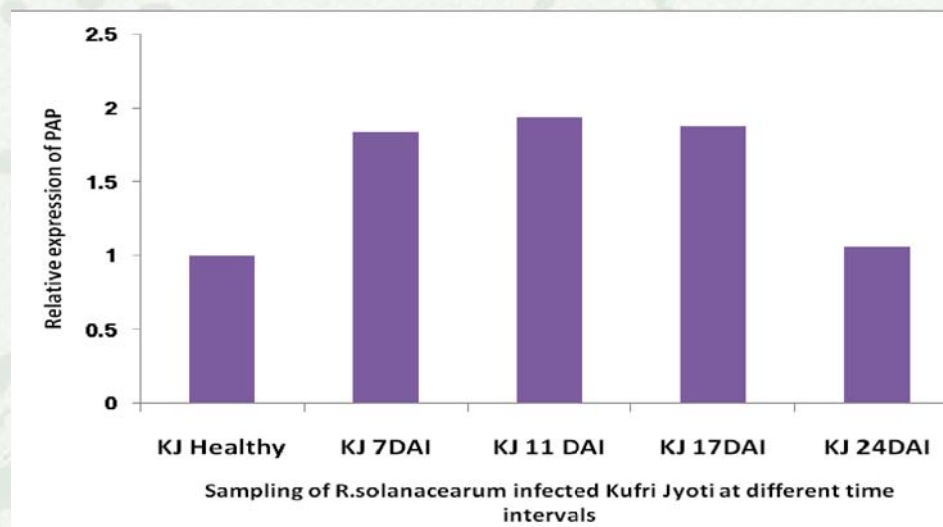
Fig. 4: Relative expression of PAP gene in *Ralstonia solanacearum* infected Kufri Jyoti potato cultivars

sive-parallel pyrosequencing technology (Roche-454, GSflx-Titanium). Shotgun sequencing runs were carried out which yielded apprx. 2.88 million reads (>500 bp) of which nearly 99.5% reads were of high quality. The genome coverage ranged from 18X (RS48) to 76X (RS75). The filtration of raw reads was performed using Prinse-qlite.pl program with optimized parameters. High quality reads were mapped on to publically available reference genomes, GMI1000 (RS25 & RS48), Po82 (RS2) and PSI07 (RS75) using gsMapper with optimized mapping parameters and variants were called. SAM tool was used to optimize call consensus from the BAM file generated from gsMapper and obtained total genome coverage and per cent GC content for all four strains. The total protein coding regions, rRNA and tRNA coding, regulatory and pathogenicity genes including the Type III secretary genes

were obtained from the consensus using the .gff (from public database) file with the help of in-house perl scripts (Table 3). The presence of repetitive elements were analyzed using MISA and was observed that nearly 90% of the elements were of di or tri and 7.3% hexanucleaotide repeats. The chromosomes carried higher portion (60% to 70%) of the repeat elements than megaplasmids in all the four strains. Initial studies of whole genome alignment with GMI1000 indicated rearrangements at whole genome level (Fig. 5).

Table 3: General features and distribution of CDS, tRNA, rRNA, regulatory genes and pathogenic genes between chromosome and megaplasmid of *R. solanacearum* strains Rs2, Rs25, Rs48 and Rs75

Strain id	Phylotype/ Biovar	Raw reads	HQ reads	% HQ reads	Total Data (Mb)	(%) GC Content	Ref strain	CDSa		tRNA		rRNA operons		Genes	
								CHR b	MLP c	CHR	MLP	CHR	MPL	REG d	PAT e
RS2	Phylotype IIB biovar 2	729,477	724,092	99.26	186	57.36	Po82	3004	1354	53	0	2	0	149	41
RS25	Phylotype I, biovar 4	566,754	561,461	99.07	223	60.11	GMI1000	3124	1432	54	3	2	1	361	82
RS48	Phylotype I, biovar 3	320,126	317,068	99.04	107	60.10	GMI1000	3127	1430	54	3	2	1	359	84
RS75	Phylotype IV, biovar 2T	1,265,491	1,264,138	99.89	459	60.06	PSI07	3089	1549	53	1	2	0	157	58

aCDS – Protein coding sequences,
bCHR – Bacterial chromosome,
cMLP – Bacterial mega plasmid
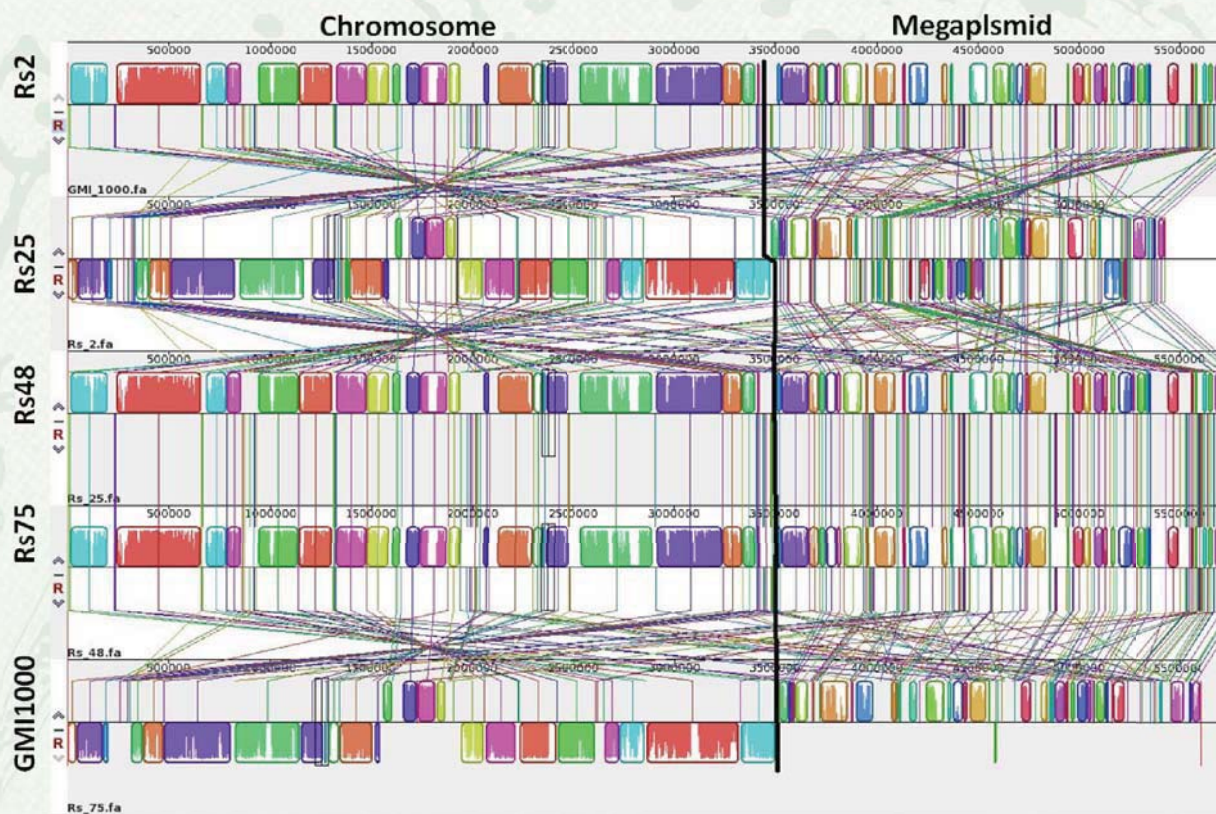dREG – Regulatory genes
ePAT – Pathogenicity genes

Fig 5: Multiple genome alignment of both chromosome and megaplsmid of RS 2, RS 25, RS 48 and RS 75 along with GMI1000 (as reference) representing the rearrangements or inversions relative to GMI1000 genome

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Co-investigator:
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A. DIVERSITY

Collection of *Ralstonia solanacearum* isolates

A total 175 isolates of *R. solancearum* were collected from tomato, potato, brinjal, chilli and capsicum during 2009-2015.

Whole-genome of *Ralstonia solanacearum*

The whole-genome of *R. solanacearum* strain UTT-25 isolated from wilted tomato from foot hill area Chorgaliya, Nainital, Uttarakhand was sequenced using paired end IlluminaMiSeq with an average insert size 145 to 155 bp. Based on 16 S rRNA sequence analysis it has maximum similarity with *R. solanacearum* GMI1000 and belongs to the Phylotype I, race I, which represents the *R. solanacearum* population infecting vegetables of solanaceous crops. Contigs were annotated using MaSuRCAgenome assembler NCBI bioinformatics. The trimmed reads were *de novo* assembled into contigs and the meta-assembly was done with *R. solanacearum* strain GMI1000 as the reference genome. The chromosome is represented by 3.6 Mbp and the megaplasmid by 1.9 Mbp. The overall length is approximately 5.5 Mbp (Including unassigned contigs). Biological functions were assigned to 99% of the predicted proteins. The whole genome project has been deposited at GenBank under the Project IDPRJNA258488.

Expression induction of epsBgene *in vitro*

In vitro induced expression analysis of *epsB* gene was done using SDS-PAGE. PCR of *epsB* gene was performed using a thermal cycler (BIORAD C-1000TM Gradient thermal cycler). A 709 bp band was detected after the amplification of DNA by PCR analysis. The amplification bands were cut and eluted from gel for purification. Expression vector digestion was done with vector pSMART. DNA ligation was performed and transformation was done by using electroporation method. Colony PCR was done for the confirmation of cloned product. A colony was picked from the LA plate and transferred in LB medium supplemented with kanamycin (25 mg/ml). Cells were grown under 37°C conditions of temperature and isopropyl-beta-D-thiogalactopyranoside (IPTG) concen-

tration (1 mM) to optimize protein expression. At 37°C, some protein was localized in the inclusion body fraction while almost all *epsB* protein was obtained in the pellet fraction in cultures grown and induced by adding IPTG to a final concentration of 1 mM. Cells were harvested by centrifugation. As shown in Fig. 1, an obvious band about 30 kD of *epsB* was visualized in the pellet. In control, IPTG was not incorporated. The aliquots were withdrawn at different time intervals (2h, 3h, 4h and 5h). After centrifugation, the supernatant was decanted and the pellet obtained was used for subsequent analysis of expressed protein and quantified under nanodrop spectrophotometer (O.D600) = 1.95 mg/ml. According to this study the *in vitro* expression of *epsB* protein has increased after inducing with IPTG compared to control (without IPTG).
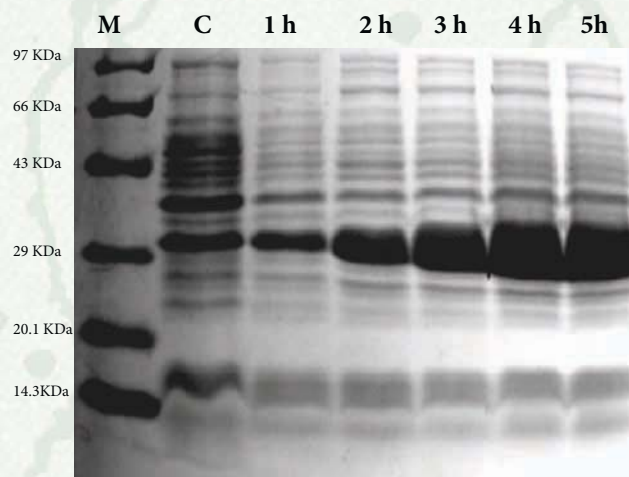
Fig. 1: *In vitro* expression of epsB protein in *E. coli* after five hours of induction with IPTG

Development of *epsB* gene del- mutant of *Ralstonia solanacearum*

epsB gene of this bacterium UTT-25 was amplified with pfu taq polymerase (high fidelity enzyme) using conventional PCR. An approximately 709 bp internal DNA fragment of the *epsB* gene of *R. solanacearum* strain UTT-25 was amplified. To delete *epsB* gene, this amplicon was digested with EcoR1 and XbaI and ligated into the pSMART blunt-end vector (suicide vector) to create DELEPSB. This construct was moved into UTT-25 by conjugation with

selection for kanamycin resistance. Construction of *epsB* gene del- mutant was developed by site-directed mutagenesis technique. Correct deletion was verified by colony PCR of wild-type and mutant and amplified product was separated on 4% metaphor agarose (high resolution). According to gel analysis the wild type was amplified at 709 bp whereas the mutant DEL type was down amplified at lower 109 bp.

In vitro growth of *epsB* DEL-mutant

To study extracellular polysaccharide production of *epsB*

DEL-mutant, *epsB* DEL-mutant and parent *R. solanacearum* UTT-25 strain were grown alone or in combination. They were inoculated in CPG broth medium and incubated at 28°C and 120 rpm till 96 h. Bacterial growth of *R. solanacearum* was measured at optical density (OD 600) using a spectrophotometer. Growth of *R. solanacearum* was considerably reduced by combination of *epsB* DEL-mutant+ *R. solanacearum* compared to control at different time intervals (Fig. 2). Results from this study indicate that production of extracellular polysaccharide was significantly reduced in *epsB* DEL- mutant leading to inhibition of *R. solanacearum* growth.
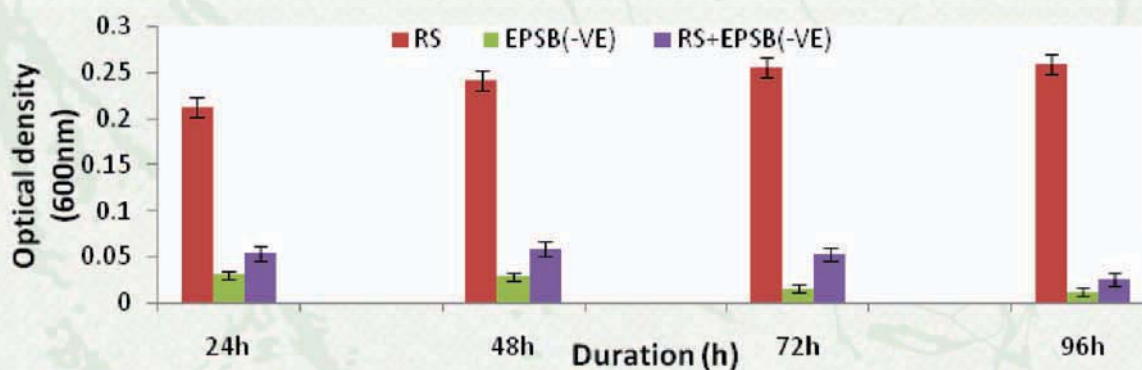

Fig. 2: *In vitro* growth of races of *R. solanacearum* in CPG medium at different intervals. RS: *R. solanacearum*, EPSB (-ve): Mutant of *epsB* gene of *R. solanacearum*, RS + EPSB (-ve): Mixed culture of *R. solanacearum* and mutant of *epsB* gene of *R. solanacearum*

B. HOST – PATHOGEN INTERACTION

Production of extracellular polysaccharide-expression of *R. solanacearum* in tomato cultivars with *epsB* gene

Extracellular polysaccharide (EPS), a major virulence factor, was extracted from *R. solanacearum* strain UTT-25 and purified using standard procedure. The purified EPS was air-dried, dissolved in 250 ml distilled water and quantified by nanodrop spectrophotometer (OD600). *EpsB* gene expression in response to purified EPS(EPS directly injected into the vascular system through the cut petiole) was determined having three treatments *i.e.*T1: control, T2: *R. solanacearum*, T3: purified extrapolysaccharide in tomato cv. Hawaii-7996 (resistant) and Pusa Ruby (susceptible) plants at different time intervals (6, 24, 48 and 96 h). Expression analysis was done using real time PCR. Expression of *epsB* gene was up-regulated in resistant cv. Hawaii-7996 and down regulated in susceptible cv. Pusa Ruby of tomato plant after 96 h irrespective of treatments. *R. solanacearum* showed up-regulation at 96 h after infection in susceptible cultivar rather than in resistant cultivar as compared to control; whereas purified extracellular polysaccharide (T3) was up-regulated in resistant cultivar than susceptible tomato plant (Fig. 3). It was noticed that EPS increased disease incidence more in susceptible cultivar Pusa Ruby (64.8%) than resistant cultivar Hawaii 7996 (27%).

Reactive oxygen species (ROS) detection in tomato plants

Reactive oxygen species (ROS) detection in tomato leaves and stems in host tissue was detected by endogenous peroxidase-dependent *in situ* histochemical staining with 3, 39- diaminobenzidine (DAB, Sigma, USA) using a standard protocol. Oxidation of DAB by ROS creates a visible brown precipitate in the host tissue. Leaves from 28-day old tomato plants of susceptible cv. Pusa Ruby and resistant Hawaii-7996 were infused with 109 CFU/ml *R. solanacearum* cells or water as control. The oxidative burst of cells was found more in *R. solanacearum* inoculated plants as compared to EPS and uninoculated treatments in both the cultivars. However, ROS activity was detected more in resistant than susceptible cultivars in all the treatments. ROS activity varied in different parts of the plant as it was noted that stems cells showed more number of cell death than leaf cells (Fig. 4).

C. DISEASE MANAGEMENT

Detection of antibiotics iturinA gene of *Bacillus amyloliquefaciens*

Iturin is a large family of cyclic lipopeptides produced by *Bacillus* sp. The members of iturin family exhibit heterogeneity at 1, 4, 5, 6 and 7 amino acid position/s in the peptide moiety as well as in the ßAA length, which var-
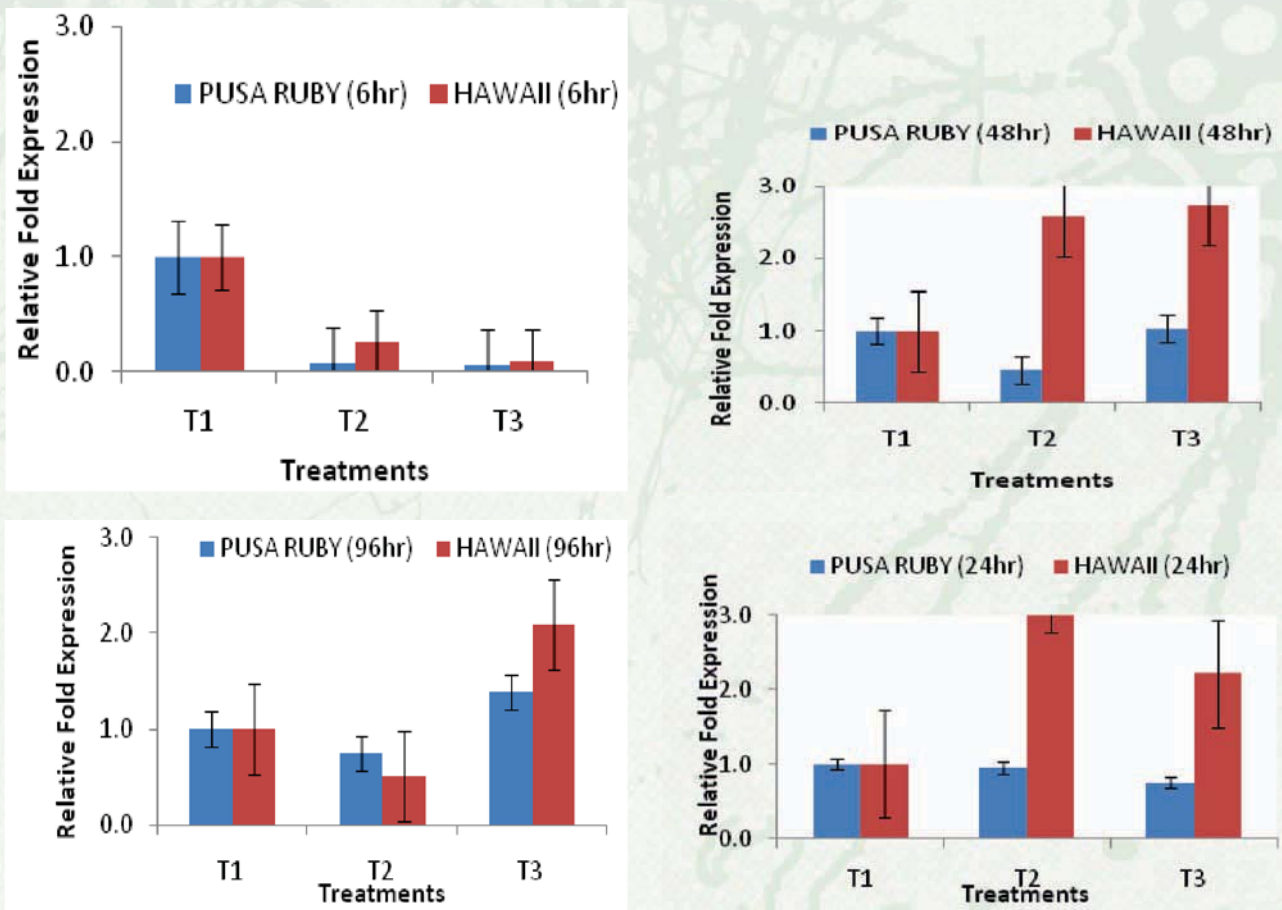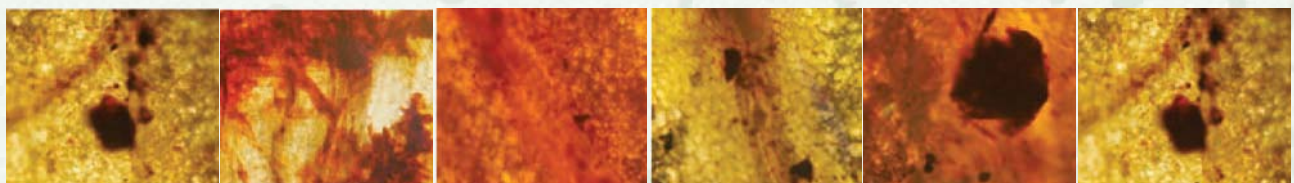
Fig. 3: Expression epsB gene in Pusa Ruby (Susceptible) and Hawaii 7996 (Resistant) cvs. of tomato treated with T1 (control), T2 (*Ralstonia solanacearum*), T3 (extracellular polysaccharide) at different intervals using RT- PCR.

A. Leaf

A. Leaf
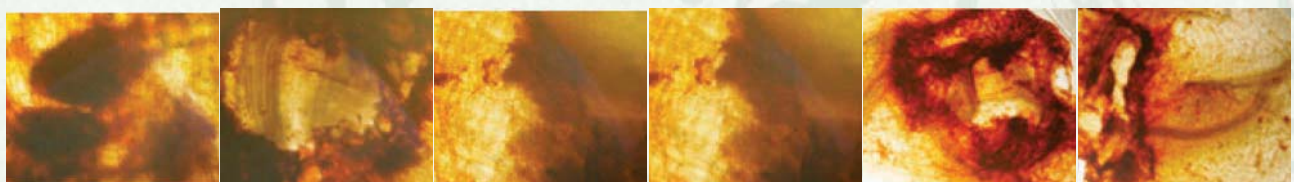
Fig. 4: Reactive oxygen species (ROS) accumulation in tomato leaves. ROS were determined in susceptible cv. Pusa Ruby (P) and resistant cv. Hawaii-7996 (H) 24 h after infusion with 109 CFU/ml *R. solanacearum* strain UTT-25. PL1, HL1, PS1 and HS1= uninocu-lated; PL2, HL2, PS2 and HS2= treated with *R. solanacearum*; PL3, HL3, PS3 and HS3=treated with eps

ies from 14 to 17 carbons. Iturin A play important role in disease suppression of plant pathogenic bacteria and fungi. In this study, expression of iturinA gene in different species of *Bacillus* was done by using set of primers ItuA forward and ItuA reverse for amplification at 617 bp. *B. amyloliquifaciens* (DSBA-11 and DSBA-12), *B. subtilis* (DSBA-5), *B. licheniformis* and *B. cereus* were used. *B. amyloliquefaciens* (DSBA-11 & DSBA-12), *B. licheniformis* and *B. subtilis* showed positive to amplify at 617 bp, while *B.*

cereus did not amplify. It shows that *B. cereus* did not contain iturin A gene and not able to produce this antibiotic (Fig. 5).

Cloning of iturinA gene of *Bacillus amyloliquefaciens* (DSBA-11)

To study the role iturinA gene of *Bacillus amyloliquefaciens* (DSBA-11) for suppression of growth of *R. solan-*

Fig. 5: The amplified DNA fragments of 617 bp produced by *Bacillus* spp. using Iturin A primer. Lane M: 1kb DNA Ladder, lane 1: *B. amyloliquifaciens* DSBA-11, lane 2: *B. amyloliquifaciens* DSBA-12, lane 3: *B. subtilis*, lane 4: *B. licheniformis*, lane 5: *B. cereus*, lane 6: -ve control

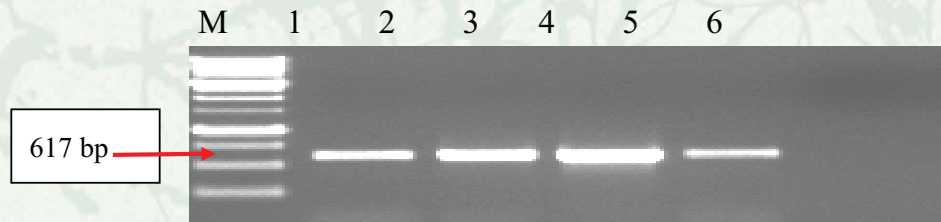*acearum*, cloning of Iturin A was done and PCR product of this gene was purified by using RBC T & A cloning vector (2.7 kb) and competent cell of *E. coli* strain DH5α by following standard procedure. The suspension was spread at 1X gal transformation plate with selective medium 100 ml of Luria agar medium containing 100 µl of 50 mg/ml Ampicilin, 20 µl of IPTG and 200 µl of X-gal. The plates were incubated at 37ºC for overnight and blue and white colonies appeared which were used for colony PCR for confirmation of cloned product. Only white colonies contained the 617 bp Iturin A gene (Fig. 6).
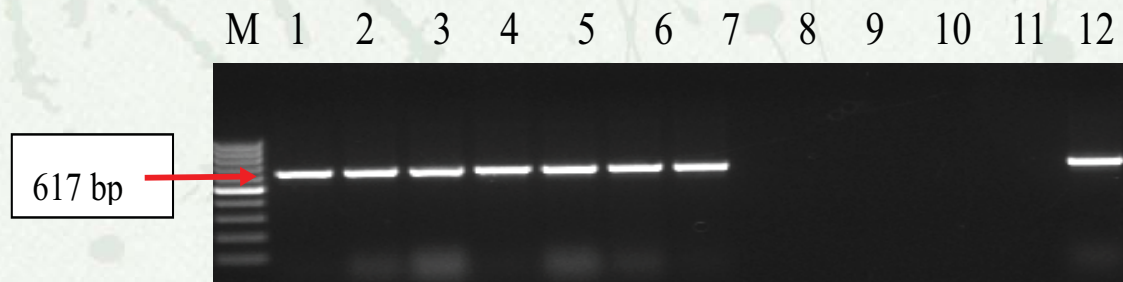

Fig. 6: Colony PCR confirming the amplification of Iturin A gene from *Bacillus amyloliquefaciens*. Lane M: 100 bp DNA Ladder, lane 1- 7: Cloned white colonies; Lane: 8-11: blue colonies. Lane 12: negative control, Lane 13: *B. amyloliquefaciens* (DSBA-11)

To study the mechanism of suppression of *R. solanacearum*, cloned product of Iturin gene (white colonies), parent *B. amyloliquefaciens* (DSBA-11) and non - cloned product (blue colonies) were tested against *R. solanacearum* under *in vitro* conditions. Maximum inhibition of *R. solanacearum* was found in parent strain *B. amyloliquefaciens* (DSBA-11) (2.0 cm diameter of inhibition zone) followed by cloned product of iturin A gene (1.7 cm diameter of inhibition zone). When cloned product of iturin gene along with parent were inoculated to 21 days seedlings of tomato cv. Pusa Ruby, 40% of disease incidence was recorded in parent *B. amyloliquefaciens* (DSBA-11) treated plants, while cloned colony showed 52% wilt disease incidence, which was lower than the in control (68%). The non-cloned colony of gene did not show activity to control the bacterial wilt.

Detection of phenazine-1-carboxylic acid (PCA) gene of *Pseudomonas fluorescens*

A total 17 strains of *Pseudomonas* spp. were screened for the amplification of antibiotic gene phenazine-1-carboxylic acid (PCA). Out of 17 strains isolated from rhizosphere and tissues of tomato plants, only 12 strains of *Pseudomonas* spp. such as DTPF-2, DTPA-11, DTPF-3, PSE-20, PSE-21, PSE-4, PSE-6, PSE-10, PSE-12, PSE-13, PSE-14 and PSE-15 amplified a 767 bp fragment, indicating the presence of PCA gene (Fig. 7).

Fig. 7: Amplification of PCA gene (767 bp) by *Pseudomonas* spp. Lane: 1M: 1kb DNA Ladder, lane 1: DTPF-2. Lane 2: DTPA-11, lane 3: DTPF-3, lane 4: PSE-20, Lane 5: PSE-21, Lane 6: PSE-4, Lane 7: PSE-6, Lane 8: PSE-10, Lane 9: PSE-12, Lane 10: PSE-13, Lane 11: PSE-14, Lane 12: PSE-15, Lane 13: PSP-7, Lane 14: PSP-10, Lane 15: PSP-27, Lane 16: PSE-27 and Lane 17: PSE-29

Cloning of PCA gene of *Pseudomonas fluorescens* (DTPF-3)

PCA gene of *P. fluorescens* was cloned and the product was purified by using RBC T & A cloning vector (2.7 kb) and competent cell of *E. coli* strain DH5α by using standard procedure. The suspension was spread at 1X gal transformation plate containing 100 ml selective medium of Luria agar medium (100 μl of 50 mg/ml Ampicilin, 20 μl of IPTG and 200 μl of X-gal). The plates were incubated at 37°C for overnight and blue and white colonies appeared which were used for colony PCR for confirmation. Only white colonies carried 767 bp amplicon for the PCA gene. The blue and white colonies along with the parent culture of *P. fluorescens* (DTPF-3) were tested for their antagonism against *R. solanacearum* by dual culture technique.However, PCA gene alone made lower inhibition zone as compared to their respective parent. When the cloned product along with the parent were inoculated to 21 days old tomato seedlings (cv. Pusa Ruby), 32% of disease incidence was recorded in parent *P. fluorescens* (DTPF-3) treated plants, while cloned colony showed 47.7% incidence, compared to 68% in control.

Management of bacterial wilt of tomato

An experiment was conducted at Chorgaliya village, Haldwani, Uttarakhand, for the management of bacterial wilt disease in tomato caused by *R. solanacearum* using biocontrol agents *P. fluorescens* (DTPF-3) and *T. harzianum* (Th3). Hybrid seeds of tomato (Unik 038 F1, moderately resistant to bacterial wilt) - Rashi seeds were used for this experiment. Bio-agents were applied @ 5 g/ litre of water as drenching during transplanting. There were four replications and 90 plants per replication. After 60 days of transplanting under field conditions the disease incidence was recorded. The minimum wilt disease incidence (22.13%) was recorded in combination with *P. fluorescens* (DTPF-3) + *T. harzianum* followed by *P. fluorescens* (DTPF-3) (28.73%) alone as compared to control 48.80% (Fig. 8).
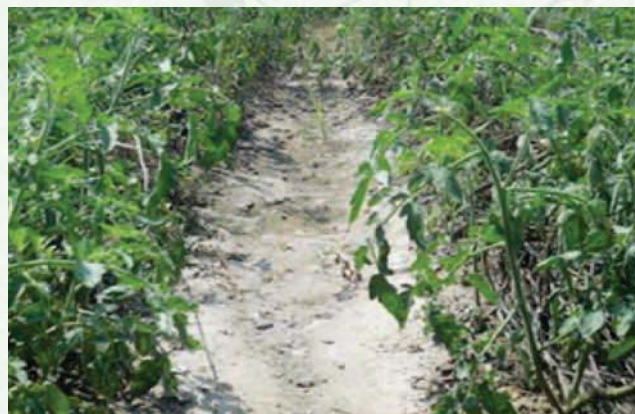

Fig. 8: Reduction of bacterial wilt disease incidence in tomato crops treated with *P. fluorescens* DTPF-3 + *T. harzianum* Th3. (a) *P. fluorescens* + *T. harzianum* (b) Control

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A. DIAGNOSIS

Biochemical characterization of bioagents

Biochemical characterization of different isolates of *Pseudomonas fluorescens* and *Bacillus subtilis* was carried out. For each test 24 to 48 h old cultures were used. *P. fluorescens* isolates gave positive result to KOH test, arginine dihydrolase, gelatin liquefaction, growth at 4°C and in producing fluorescent pigment. The isolates gave negative result to levan formation from sucrose, starch hydrolysis, gram staining and growth at 45°C in all the isolates tested (Table 1). Whereas, *B. subtilis* isolates showed positive reaction to starch hydrolysis, gelatin liquefaction, utilization of citrate, catalase test, levan production, gram staining, growth in 3.0% NaCl and negative reaction to KOH, growth at 4°C and anaerobic growth (Table 1). Molecular characterization of *P. fluorescens* and *B. subtilis* using specific primers is in progress.

Table 1: Biochemical characterization of potential bioagents of *Ralstonia solanacearum*

Biochemical test	Antagonist isolate					
	IHRBs18	IHRBs23	IHRBs39	IHRPf1	IHRPf13	IHRPf24
KOH	-	-	-	+	+	+
Gram stain	+	+	+	-	-	-
Catalase	+	+	+	+	+	+
Indole	-	-	-	-	-	-
Methyl red	-	-	-	-	-	-
Vogesproskaur	+	+	+	-	-	-
Oxidase	+	+	+			
Starch hydrolysis	+	+	+	-	-	-
Gelatin liquefaction	+	+	+	+	+	+
Urea hydrolysis	-	-	-	+	+	+
Levan formation	+	+	+	-	-	-
Arginine dihydrolase	-	-	-	+	+	+
Citrate utilization	+	+	+	+	+	+
Growth in NaCl 3%	+	+	+	+	+	+
Growth at 4°C	-	-	-	+	+	+
Growth at 45°C	+	+	+	-	-	-
Fluorescent pigment	-	-	-	+	+	+
Anaerobic growth	-	-	-	-	-	-

+ indicates presence and – indicates absence

B. HOST-PLANT RESISTANCE

Studies on induction of resistance by *Pseudomonas fluorescens* and *Bacillus subtilis* in tomato

The potential bioagents of *R. solanacearum, viz. P. fluorescens* (IHRPf-24) (Pf) and *B. subtilis* (IHRBs-39) (Bs) were used alone and in combination in the induction of defense reactions in tomato in a glasshouse. The six treatments were i) seeds treated with Bs, ii) seeds treated with Pf, iii) seeds treated with Bs and challenge inoculation with *R. solanacearum* (Rs) 15 days after sowing (DAS), vi) seeds treated with Pf and challenge inoculation with Rs 15 DAS, v) plants inoculated with the pathogen 15 days after sowing and vi) untreated control check. Seeds were sown in earthen pots (25 seeds per pot) filled with sterilized potting mixture. There were four replications for each treatment and each replication consisted of eight pots. The plants were sampled at 0, 1, 2, 3, 4, 5, 7 and 10

days after inoculation and assayed for PAL, PO and PPO activities. Results showed that, seed treatment with *P. fluorescens* induced the plants to synthesize PAL, whereas an additional increase in the synthesis was observed in *P. fluorescens* pretreated plants challenge inoculated with *R. solanacearum*. The activity reached the maximum level on the third day after pathogen challenge and thereafter the activity remained at higher levels throughout the experimental period of 10 days. In plants treated with the pathogen alone, increased activity of PAL was observed for a period of 2–4 days and thereafter declined drastically (Table 2). Increased activities of PPO and PO were observed in *P. fluorescens* pretreated tomato plants challenge inoculated with the pathogen, which remained at higher levels throughout the experimental period (Table 2). The activity of PPO and PO reached maximum levels of 3.12 and 2.58 OD/min/mg of protein, respectively, on the third day of challenge inoculation with the pathogen (Table 2). The maximum phenolic content was observed in *P. fluorescens*, pretreated plants challenge inoculated with the pathogen and the higher amount of phenolics (88.0 µg of catechol/mg of protein) were noticed even on the 10th day after challenge inoculation. In plants inoculated with pathogen alone, the phenolic content declined to the initial level on the 10th day after inoculation. Plants treated with *P. fluorescens* alone also increased phenolic contents as compared to untreated control plants (Table 2).

Table 2: Changes in defense enzymes and phenolics in roots of tomato pre-treated with bioagents and challenge inoculated with *R. solanacearum*

Treatment	Days after inoculation							
	0	1	2	3	4	5	7	10
Phenyl alanine ammonia lyase (nmol transcinnamic acid/min/mg protein)								
Bs (IHRBs39)	11.9	11.3*	11.1	10.5	10.2	10.4	10.6	10.9
Bs+Rs	11.9	16.0	20.1	27.0	25.0	25.2	26.1	26.5
Pf (IHRPf24)	12.5	12.0	11.9	11.5	11.0	11.2	11.4	12.0
IHRPf24+Rs	12.5	19.0	24.1	29.0	26.0	27.0	26.5	25.0
Rs (IHRTRs20)	9.2	9.3	9.5	13.5	14.5	13.5	10.0	9.0
Control	9.2	9.3	9.1	9.1	8.9	9.0	9.2	8.8
CD (P=0.05)	1.23	1.18	1.22	1.18	1.17	1.20	1.20	1.23
CV%	7.39	6.20	5.75	4.73	4.96	5.05	5.17	5.36
Polyphenol activity (Changes in absorbance/min/mg of protein)								
Bs (IHRBs39)	2.00	1.89	1.89	2.00	2.10	2.00	2.20	2.10
Bs+Rs	1.90	2.10	2.50	2.90	2.80	2.50	2.50	2.50
Pf (IHRPf24)	2.10	2.12	2.15	2.10	1.93	1.90	1.88	2.00
IHRPf24+Rs	2.20	2.30	2.80	3.12	3.00	2.70	2.80	2.60
Rs (IHRTRs20)	1.80	1.90	2.10	2.35	2.30	2.25	2.0	1.70
Control	1.90	1.80	1.80	1.70	1.60	1.60	1.50	1.50
CD (P=0.05)	0.11	0.12	0.10	0.11	0.10	0.11	0.11	0.11
CV%	3.65	3.94	3.06	3.01	2.93	3.34	3.44	3.60
Peroxidase activity (Changes in absorbance/min/mg of protein)								
Bs (IHRBs39)	1.72	1.73	1.76	1.73	1.74	1.73	1.73	1.72
Bs+Rs	1.72	1.73	1.92	2.00	2.00	1.90	1.83	1.80
Pf (IHRPf24)	1.81	1.83	1.85	1.80	1.81	1.82	1.82	1.81
IHRPf24+Rs	1.81	1.83	2.20	2.58	2.50	2.38	2.24	2.22
Rs (IHRTRs20)	1.69	1.70	1.72	2.01	2.05	1.95	1.82	1.60
Control	1.68	1.69	1.68	1.67	1.66	1.67	1.67	1.69
CD (P=0.05)	0.09	0.11	0.11	0.10	0.09	0.09	0.09	0.10
CV%	3.38	4.09	3.96	3.53	3.40	3.24	3.47	4.47
Phenolic content (Microgram catechol per milligram of protein)								
Bs (IHRBs39)	46.0	46.0	-	48.0	-	49.0	49.0	49.0
Bs+Rs	51.0	64.0	-	70.0	-	83.0	79.0	79.0
Pf (IHRPf24)	50.0	50.0	-	51.5	-	52.0	52.0	52.0
IHRPf24+Rs	54.0	68.0	-	74.0	-	88.0	86.0	85.0
Rs (IHRTRs20)	42.0	46.0	-	54.0	-	62.0	55.0	40.0
Control	44.0	44.0	-	43.0	-	42.0	42.0	42.0
CD (P=0.05)	5.05	2.31	-	3.07	-	3.37	3.20	4.44
CV%	7.10	2.93	-	3.65	-	3.61	3.55	5.17

*Mean of four replications

C. DISEASE MANAGEMENT

Evaluation of bioagents against *R. solanacearum*

The potential isolates of *P. fluorescens* (IHRPf-24), *B. subtilis* (IHRBs-39) and bactericide were evaluated alone and in combinations on suppression of bacterial wilt of tomato and enhancement of per cent seed germination under glasshouse conditions using bacterial wilt susceptible variety, ArkaVikas. The experiment included a total of seven treatments including control with three replications each in a completely randomized block design.   Among the treatments, the T1 treatment, where the seeds were treated with *P. fluorescens*, recorded significantly highest percent seed germination of 80.0 with lowest wilt incidence of 22.0 per cent (Table 6) followed by T2 (seeds treated with *B. subtilis* IHRBs39), which showed per cent germination and bacterial wilt incidence of 80.0 and 24.01 per cent, respectively. The combined treatments, viz., T4 (Pf+Bs)and T5 (Pf+Bs +COC) recorded per cent seed germination of 76.0 and 62.0 and bacterial wilt incidence of 26.36 and 26.66 per cent, respectively. The untreated control (T7), however, recorded lowest per cent seed germination and highest bacterial wilt incidence of 55.0 and 85.0, respectively (Table 3).

Table 3: Effect of *P. fluorescens* and *B. subtilis* isolates and bactericides against *R. solanacearum* under glasshouse conditions

Treatment	Germination* (%)	Bacterial Wilt * (%)
Sick soil+IHRPf-24 treated seeds	80.0 (63.5)a	22.00 (27.9)
Sick soil+IHRBs-39 treated seeds	80.0 (63.4)a	24.01 (29.3)
Sick soil+COC (0.2%) treated seeds	60.0 (50.8)	30.00 (33.1)
Sick soil+IHRPf-24 & IHRBs-39 treated seeds	76.0 (60.8)a	26.36 (30.8)
Sick soil+IHRPf-24 & IHRBs-39 treated seeds + COC @ 0.2%	62.0 (51.9)	26.66 (31.0)
Sick soil+ untreated seeds	55.0 (47.9)	85.00 (67.8)
Healthy soil+ untreated seeds	76.0 (60.7)a	00.00 (00.0)

*Mean of three replications. Figures in parentheses are arc sine transformed values

4. Formulation of potential bioagents of *Ralstonia solanacearum*

Formulation of potential strains of *P. fluorescens* (IHRPf24) and *B. subtilis* (IHRBs39) were prepared in the laboratory using talc, farmyard manure and cocoa peat. *P. fluorescens* and *B. subtilis* cultures were grown separately in nutrient broth for 48 h as shake culture in a shaking incubator at temperature 28 ±2°C at 150 rpm. Ten grams of carboxymethyl cellulose was added to 1 kg of sterile talc, FYM and cocoa peat as carrier material separately and mixed well. About 400 ml of bacterial suspension containing 2.5 x 10^8cfu/ml was added to the carrier material and mixed well under sterile condition. Calcium carbonate (15 g) was added to the formulations to adjust the pH to 7.0. Further study on rhizosphere colonization of the bioagents through seed priming, seedling root dip and efficacy against *R. solanacearum* under glass house condition are under progress.

Development of integrated disease management strategy for bacterial wilt in tomato

A field trial is being carried out for the management of bacterial wilt in tomato variety Shivam (susceptible) with thirteen treatments and four replications each in a randomized block design. The treatments imposed were, i) farm yard manure (FYM) @ 20 t/ha, ii) FYM + Green manure – sunnhemp (GM), iii) Green Manure @ 25 kg/ha, iv) *P. fluorescens* (Pf) – seed treatment & soil application @ 1.0 x 10^8 cfu/ml , v) *B. subtilis* (Bs) – seed treatment & soil application @ 1.0 x 10^8 cfu/ml vi) Neem cake @ 150 kg/ha, vii) Pongamia cake @ 150 kg/ha, viii) FYM + GM + Pf (seed treatment & soil application) + COC 0.2%, ix) FYM + GM + Bs (seed treatment & soil application) + COC 0.2%, x) FYM + GM + Pf + BS (soil application), xi) Streptocycline 250 ppm + COC 0.2%, xii) COC 0.2% and xiii) untreated control. Results presented in Table 4 indicated that all the treatments were effective in reducing the bacterial wilt incidence in tomato and increasing the yield as compared to untreated control check.   Among the treatments, the bacterial wilt incidence was significantly lowest as 10.20% with increased yield of 39.60 t/ha in plots where combined treatment of FYM + GM + Pf + COC (T8) was imposed. This was followed by the treatments, FYM + GM + Bs + COC (T9) and FYM + GM + Pf + Bs (T10), which recorded 10.90% and 12.0% wilt incidence and yield of 37.82 and 35.20 t/ha, respectively, and they

were on par with treatment T8. The next best treatments were *P. fluorescens* (T4) and *B. subtilis* (T5) imposed alone, as seed treatment at the time of sowing and two soil drenching at fortnightly intervals, which recorded 15.0 and 15.5 per cent wilt incidence and yield of 26.0 and 25.78 t/ha, respectively. The treatments, soil incorporation of FYM alone (T1), GM alone (T3), FYM in combination with GM (T2), soil application of neem cake alone (T6), pongamia cake alone (T7), foliar application of COC alone (T12), and COC in combination with streptocycline (T11) recorded 20.0 to 25.68% wilt incidence and tomato yield of 14.60 to 18.92 t/ha. However, the untreated control plot recorded highest bacterial wilt incidence of 65.00% and lowest yield of 9.5 t/ha (Table 4).

Table 4: Effect of various treatments on bacterial wilt incidence and yield of tomato

Treatment	Mean wilt incidence (%)	Decrease over control (%)	Yield (t/ha)*	Increase over control (%)
FYM @ 20 t/ha	22.84 (28.45) ef	64.86	14.60 c	34.93
FYM + GM	20.00 (26.50) d	69.23	18.92 c	49.79
Green manure (sunhemp) 25 kg/ha	20.40 (26.77) e	68.62	18.00 c	47.22
Pseudomonas fluorescens (seed treatment 10 g/kg & Soil application @ 5 kg/ha)	15.00 (20.06) bc	76.92	26.00 b	63.46
Bacillus subtilis (seed treatment 10 g/kg & Soil application @ 5 kg/ha)	15.50 (20.64) c	76.15	25.78 b	63.15
Neem cake @ 150 kg/ha	23.25 (28.76) ef	64.23	18.80 c	49.47
Pongamia cake @ 150 kg/ha	25.68 (30.40) f	60.49	15.60 c	39.10
FYM + GM + Pf + COC	10.20 (18.52) a	84.31	39.60 a	76.01
FYM + GM + Bs + COC	10.90 (19.06) ab	83.23	37.82 a	74.88
FYM + GM + Pf + Bs	12.00 (20.00) abc	81.54	35.20 a	73.11
Streptocycline 250 ppm+COC 0.2 %	20.00 (26.39) d	69.23	18.90 c	49.74
COC 0.2%	20.48 (26,81) e	68.49	17.90 c	46.92
Control	65.00 (53.72) g	--	9.50 d	--
CD (P=0.05)	4.29	-	4.48	-
CV%	7.64	-	13.97	-

* Mean of four replications; Figures in parentheses are arc sine transformed values; Means followed by common letters are not significantly different at 5 % level by DMRT

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A. DIVERSITY

Five new isolates of *Ralstonia solanacearum* were collected from different bacterial wilt infected fields of ginger and tomato and tested for cross infectivity of the isolates to ginger and vice versa. None of the isolates from tomato were found infecting ginger. But ginger isolates infect tomato and brinjal.

B. DIAGNOSTICS

Development of LAMP (Loop Mediated Isothermal Amplification) *R. solanacearum* biovar 3 infecting ginger

A set of six primers were designed using the software LAMP Designer 1.12 from http://www.premierbiosoft.com. This was validated with ginger *R. solanacearum* isolates as well as *R. solanacearum* isolates from solanaceous crops like tomato, brinjal and potato (Fig. 1A, B; Table 1). Very specific amplification was obtained for only ginger *R. solanacearum* showing the high specificity of the LAMP primer. Further validation was done with soil DNA extracted from *R. solanacerum* sick soil and also with genomic DNA from the same. The same set of primers were used for detection of *R. solanacearum* of ginger using Real Time LAMP. In RT LAMP also the primers detected only *Ralstonia* strain of ginger. Analyzing the annealing curve and Ta value (92ºC) confirmed the amplification of the correct product. The sensitivity of the method was found to be 1 pg of pathogen DNA. To standardize the sensitivity, different quantities of genomic DNA were amplified and the tests revealed that in Real Time LAMP even 50 pg of the DNA can be amplified. This can be used as a diagnostic kit for testing ginger *Ralstonia* in the field before planting as well as for seed testing.

Standardization of Real Time LAMP

Real Time LAMP was standardized and validated with ginger *Ralstonia* isolates along with *Ralstonia* isolates from solanaceous crops (Fig. 2A and B; Table 1). Further validation was done with genomic DNA and soil DNA from artificially inoculated soil (Table 2) and also with different quantities of genomic DNA to find out the specificity (Table 3).
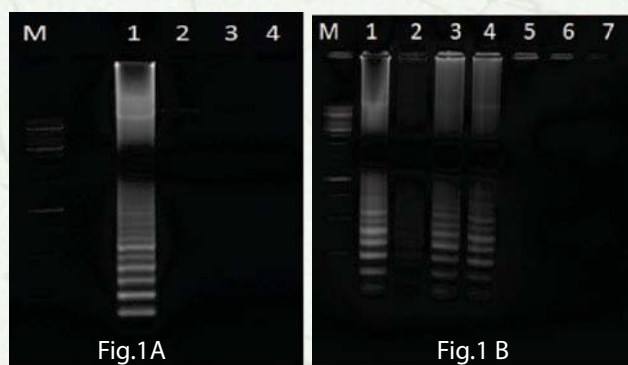

Fig.1 A: Amplification of DNA specific to *Ralstonia solanacearum* using LAMP primers. M. 1 Kb ladder, 1 - GRs Sik, 2-TRs Klm, 3 - PRs Pun, 4 - Water control, 1 B: Specificity of LAMP primers to amplify ginger *R. solanacearum*. M - 1kb ladder,1 - GRs Sik, 2 - GRS Mnt2,3 - GRs Vyt, 4 - GRs Ktm, 5 - ORB-3 BrinjalRs, 6 - ORP-1 Potato Rs, 7 - water control
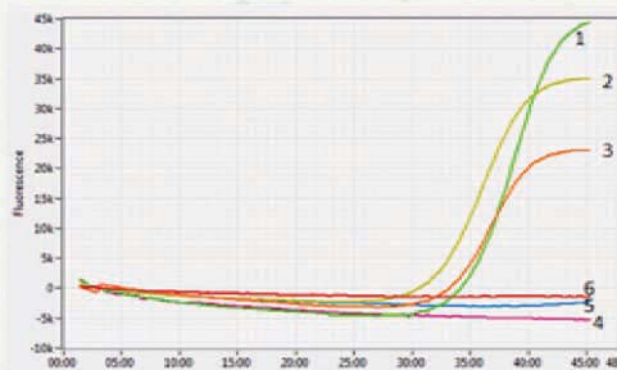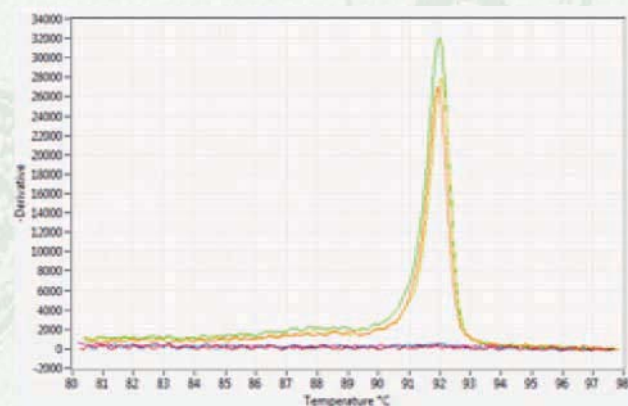

Fig. A

Fig. B

Fig. 2: Real time LAMP amplification of *Ralstonia solanacearum*. A: Amplification curve B: Annealing curve

Table 1: Validation of Real Time LAMP protocol for detection of *Ralstonia solanacearum*

Sample	Amp.time (mm:ss)	Ann.tem P. (°C)
GRs Sik	36:31	91.97
GRs Vyt	35:31	92.07
GRs Mnt 2	38:01	91.97
ORB-3	-	-
ORP-1	-	-
Water control	-	-

Table 2: Validation of Real time LAMP with soil and genomic DNA of *Ralstonia solanacearum*

Sample	Amp. time (mm:ss)	Ann. Temp (°C)
GRs Mep3 (soil DNA)	22:05	91.73
GRs Sik (50ng -gDNA)	21:35	92.02
GRs Sik (150ng)(gDNA)	19:35	92.02
GRs Sik (250 ng)(gDNA)	18:20	92.02

Table 3: Validation of Real Time LAMP with different quantities of genomic DNA of *Ralstonia solanacearum*

| Sample	Amp. time (mm:ss)	Ann. Temp (°C)
5×10^1	22:31	92.02
5×10^0	25:15	92.07
5×10^{-1}	29:15	92.07
5×10^{-2}	32:30	92.07
5×10^{-3}	34:31	92.03
5×10^{-4}		
5×10^{-5}		
5×10^{-6}		

C. HOST – PATHOGEN INTERACTION

Understanding the genetic diversity of *Curcuma amada* in response to *R. solanacearum*

Different accessions of *C. amada* were screened against *R. solanacearum* using pseudostem and soil inoculation methods. Only two accessions were found resistant under the two methods of screening.

Pathway analysis of transcriptomes of ginger and mango ginger and tissue specific expression analysis of shortlisted genes/ESTs in ginger using qPCR

NBS-LRR, ABC transporters, 4-coumarate: coenzyme A ligase (4-CL), WRKY transcription factor 8 and callose synthase were studied for their expression level in ginger and mango ginger at different time intervals (0, 1, 4, 8, 16, 24, 48, 72, 96 and 120 hpi) in leaves and pseudostem. The expression of 4-CL was higher in *C. amada* than all the transcripts studied and its expression peaked up 48 hpi. Increase in the expression of PR genes such as 4 CL might help in forming physical barriers (lignin) to prevent bacterial movement and proliferation (Fig. 3 a-d).
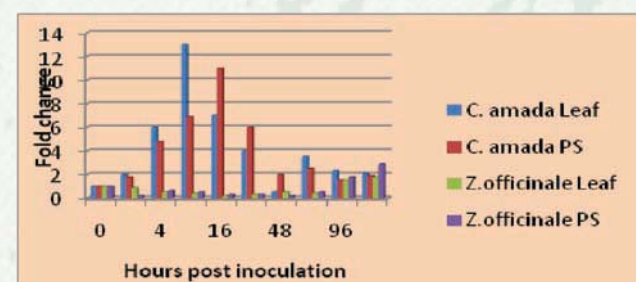
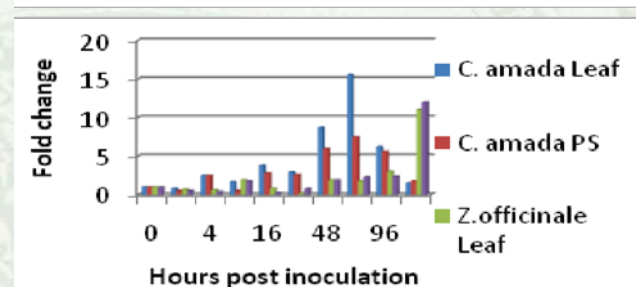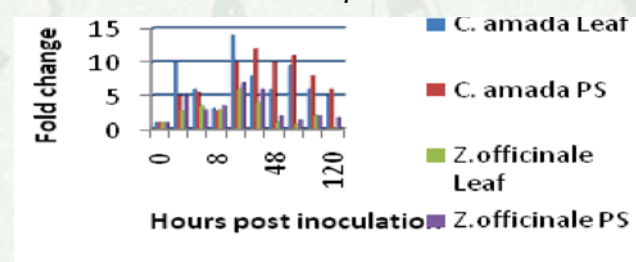*NBS-LRR genes*
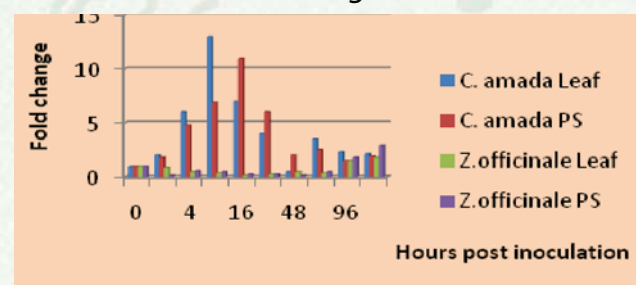
ABC transporters

Fig. 3: Tissue specific expression analysis of genes/ESTs in ginger and *Curcuma amada* using qPCR. A - NBS LRR genes; B – ABC transporters; C - Callose synthase and D - Expression of WRKY transcription factor

Light and fluorescent microscopic studies

No difference was noticed between the inoculated and uninoculated sections of the root of *C. amada* in light microscopy. In ginger there appeared to be the presence of gaps in cortical regions which can lead to the assumption that they could be bacterial pockets. When the unstained sections of *C. amada* and *Z. officinale* were subjected to fluoresence under microscope, the stele of *C. amada* showed thick casparian thickenings compared to *Z. officinale*. Bright field microscopic observation of the sections of ginger showed large intercellular pockets (bacterial pockets) in the root inner cortex. The cortical cells next to them showed the symptoms of degeneration. Bacteria advance from the cortex to vascular cylinder through vascular parenchyma crossing the endodermis (a barrier of lingo-suberized cells and phenolic compounds, thought to be a barrier for vascular pathogens) (Fig. 4 and Fig. 5).
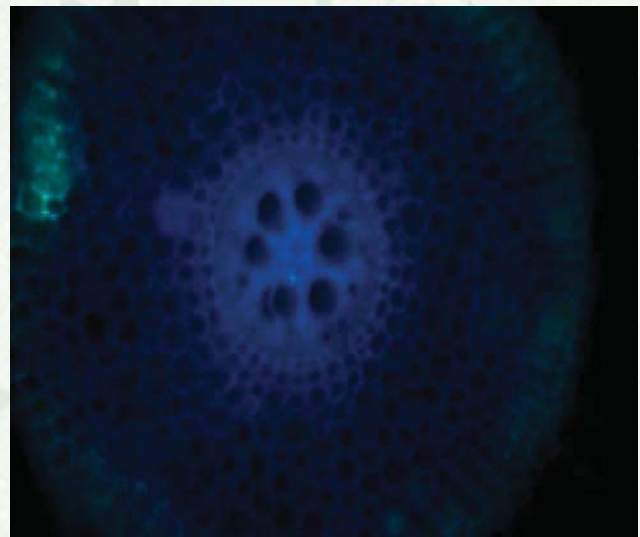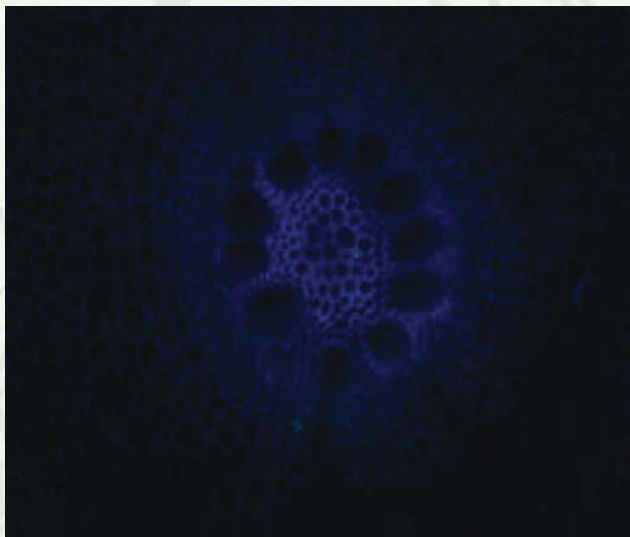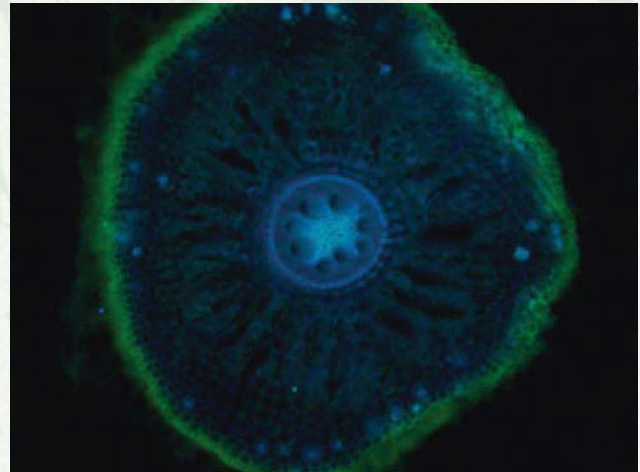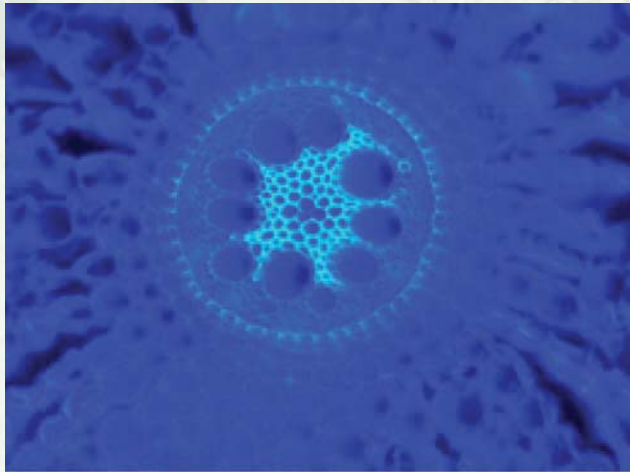

Fig. 4: Fluorescence microscopy of transverse sections of roots of *Zingiber officinale* and *Curcuma amada*. (Clockwise from top left) Stelar portion of *C. amada*; Strong casparian bands in *C. amada*; Stelar portion of *C. amada*, and *Z. officinale*
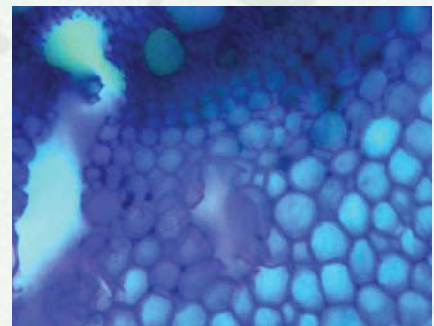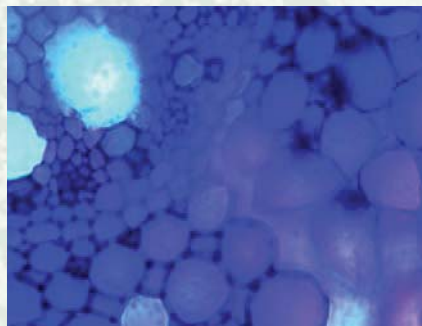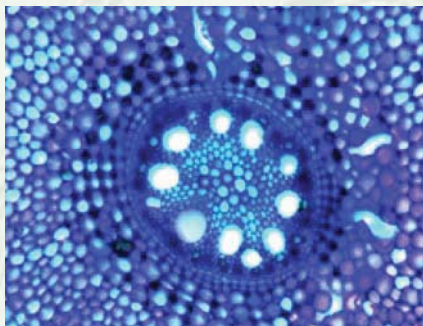

Fig. 5: Transverse sections of *Ralstonia* inoculated *Zingiber officinale* roots showing large intercellular pockets in cortical region

D. DISEASE MANAGEMENT

Evaluation of apoplastic microbes against bacterial wilt

Around 150 bacteria were isolated from ginger apoplastic fluid. Based on *in vitro* and *in planta* evaluation against *R. solanacearum*, six isolates *viz.*, GAB5, 24, 43, 48,107 and 148 were shortlisted. The biocontrol and growth promoting traits of the shortlisted apoplastic bacteria were tested *in vitro*. All the isolates showed siderophore and ammonia production. Two isolates *viz.*, GAB48 and GAB107 were found showing amylase, protease and cellulose activities (Table 4). Based on enzyme production and growth promoting as well as biocontrol traits, four apoplastic bacterial isolates were shortlisted and were evaluated under greenhouse and field conditions (Table 5). Under greenhouse condition, almost 50% reduction in disease incidence was noticed with GAB 43 in the initial stages of infection when compared to positive control and chemical treatment (Table 6). In field no bacterial wilt or soft rot incidence was observed during the period. However, dry rot incidence and scale infestation were observed during harvest. On comparing different treatments it was observed that dry rot due to *Macrophomina phaseolina* and scale infestation were found comparatively lesser in GAB48 and GAB107 applied plots. This has to be evaluated further.

Table 4: Growth promoting traits of apoplastic bacteria isolated from ginger

Isolate	Siderophore	HCN	Ammonia	Phosphate solubilization	IAA
IISR GAB 5	+	-	+	+	-
IISR GAB 24	+	-	+	+	+
IISR GAB 43	+	-	+	-	+
IISR GAB 48	+	-	+	-	+
IISR GAB 107	+	-	+	+	-
IISR GAB 146	+	-	+	+	-

Table 5: Enzyme activities of apoplastic bacteria isolated from ginger

Isolate	Amylase	Protease	Cellulase	Lipase
IISR GAB 5	+	+	+	-
IISR GAB 24	+	-	+	-
IISR GAB 43	-	-	-	-
IISR GAB 48	+	+	+	-
IISR GAB 107	+	+	+	-
IISR GAB 146	+	-	+	-

Table 6: Evaluation of ginger apoplastic bacteria under greenhouse conditions

Treatment	Total number of tillers produced	No. of tillers infected	% infection	Reduction of disease over control
Ab. control	261	27	10.34	89.66
Positive control	38	38	100	0
COC (0.25%)	71	71	100	0
IISR GAB 5	122	90	73.77	26.23
IISR GAB 24	131	104	79.39	20.61
IISR GAB 43	160	79	49.38	50.62
IISR GAB 48	145	108	74.48	25.52
IISR GAB 107	123	94	76.42	23.58
IISR GAB 148	127	103	81.10	18.9

Table 7: Field evaluation of apoplastic bacteria for control of pests and diseases of ginger

Treatment	Av. yield /bed (kg)	Av. dry rot (kg)	% dry rot	Av. scale infestation (kg)	% Scale infestation
Control	3.383	0.578	17.09	0.92	27.19
IISR GAB 5	3.350	0.532	15.88	0.05	1.49
IISR GAB 24	5.620	0.795	14.15	0.18	3.20
IISR GAB 43	5.550	0.647	11.66	0.05	0.90
IISR GAB 48	4.800	0.370	7.71	0.00	0.0
IISR GAB 107	4.320	0.290	6.71	0.00	0.0
IISR GAB 146	2.800	0.450	16.07	0.05	1.61
IISR COC 0.25%	3.13	0.355	11.34	0.000	0.00
LSD 0.05%	1.368	0.220	-	0.070	-

E. GENOMICS & BIOINFORMATICS

Whole genome sequencing of *Ralstonia solanacearum*

Two strains of *R. solanacearum* (GRs-SIK and GRs-MEP) were Illumina sequenced and the raw data has been assembled using A5-miseq. Both the strains have been annotated using Prokka (a software tool for the rapid annotation of prokaryotic genomes). In GRs-MEP there are 5120 CDS, 80 tRNA, and 1 tmRNA while GRs-SIK possesses 5080 CDS, 63 tRNA and 1 tmRNA. To better classify the predicted proteins from Prokka, a refined annotation has been done using Blast2GO with 1.0E-3 as e-value cut off

and 33 as HSP cut off length. The genomes were mined for various effector proteins and other virulence factors. Gene ontology (GO) distribution from the predicted CDS of GRs-SIK and GRs-MEP were classified into three major components *viz.* biological process (BP), molecular components (MF) and cellular components (CC)) (Fig. 6).

Sequence assembly and annotation

Sequence assembly of two strains of *R. solanacearum* (GRs-MEP and GRs-SIK) was performed using A5 pipeline and yielded 286 and 213 scaffolds, respectively. For both GRs-MEP and GRs-SIK genes were predicted using Prokka and revealed 5,201 and 5,114 genes with 5,120 genes were predicted as CDS in GRs-MEP and 5,080 in GRs-SIK. The annotation using BLAST2GO for GRs-MEP resulted in 5,039 annotated sequences and 81 were without any hits, whereas in GRs-SIK, 4,891 CDS could be annotated and 73 CDS were without any blast hits against NR.

Proteome comparison

Proteomes of both strains were compared against 10 other available strains of *R. solanacearum* using OrthoM-CL In total 6,510 orthologues protein clusters have been identified in the 12 strains of *R. solanacearum*. Singletons were also predicted for each strain, GRs-SIK had 20 singletons whereas GRs-MEP had 59 singletons (Table 8).
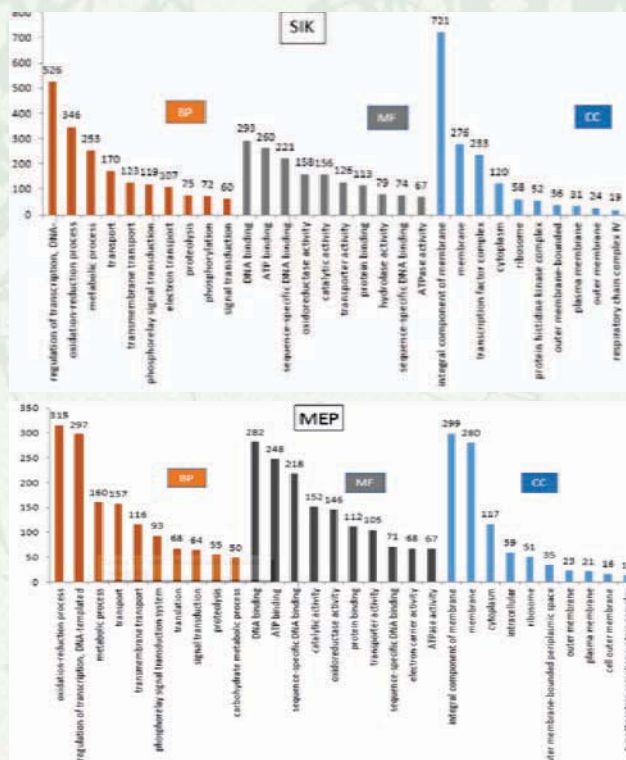

Fig. 6: Gene ontology (GO) distribution – from the predicted CDS of SIK and MEP, classified into three major components (Biological processes (BP), Molecular components (MF) and Cellular components (CC))

Table 8: The pair wise comparison of 12 strains of *R. solanacearum* using OrthoMCL

	CMR15	GM1000	IPO1609	MEP	Po82	RSFQY_4	RSK60	RSY45	SD54	SIK	UW551
CFBP2957	3808	3887	3757	3865	3949	3801	2865	3729	3827	3866	3665
CMR15	-	4012	3609	4006	3760	3940	2746	3950	3967	4006	3526
GM1000	-	-	3621	4318	3859	4332	2810	4237	4351	4319	3537
IPO1609	-	-	-	3632	3787	3588	2678	3562	3612	3631	3878
MEP	-	-	-	-	3836	4213	2851	4167	4247	4914	3550
Po82	-	-	-	-	-	3857	2814	3773	3881	3836	3694
RSFQY_4	-	-	-	-	-	-	2728	4203	4622	4213	3509
RSK60	-	-	-	-	-	-	-	2685	2750	2852	2570
RSY45	-	-	-	-	-	-	-	-	4204	4167	3482
SD54	-	-	-	-	-	-	-	-	-	4248	3527
SIK											3549

Prediction of pathogenic proteins

Collection of already characterized virulent genes from different strains of *R. solanacearum* was done and 41 virulence genes were found. Sequence similarity between these genes and CDS from GRs-MEP and GRs-SIK were analyzed. The predicted CDS of both the strains were further scanned for the presence of pathogenic proteins using MP3 and 316 CDS each were predicted in both (Fig. 7).
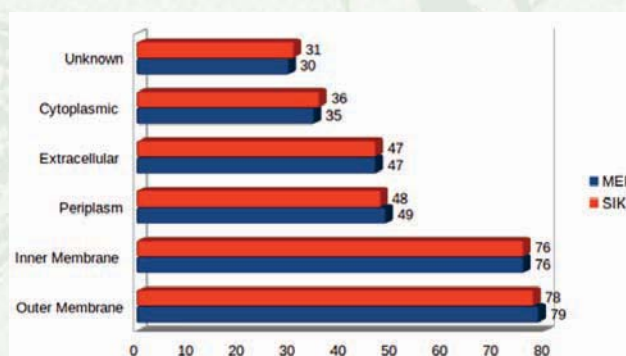
Fig. 7: The localization of predicted pathogenic proteins in two strains of *Ralstonia solanacearum* strains, GRs- MEP and GRs-SIK.

Reference based alignment and SNP calling

Reference based alignment with GMI1000 using BWA has shown that 83% of reads properly paired with the reference genome. SNP calling using GATK and Samtools indicated 4,368 SNPs in GRs-MEP and in GRs-SIK 4,648 SNPs were reported.

Type 3 effector prediction

All the Type 3 effectors (T3E) have been predicted in both the strains using T3E prediction tool. A total of 74 effectors were present in IISR strains in comparison to those of 11 other strains of *R. solanacearum*.

Classical secretory protein prediction

The classical secretory protein prediction for both GRs-MEP and GRs-SIK was done using the standalone tool SignalP4.1. GRs-MEP had 522 secretory proteins while GRs-SIK had a total of 517 secretory proteins. The localization of these classical secretory proteins was predicted using SOSUI Gram N to estimate the target regions for the secretory proteins (Fig. 8) .
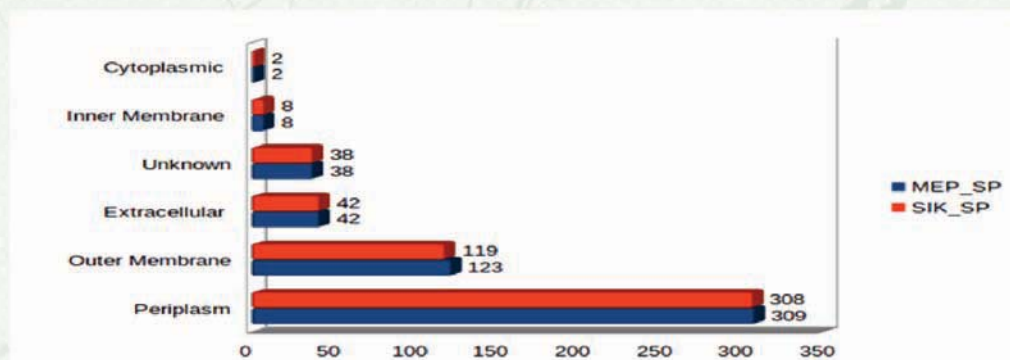

Fig. 8: The localization of predicted signal peptides of *Ralstonia solanacearum* strains, GRs-MEP and GRs-SIK

ICAR- ICAR RESEARCH COMPLEX FOR NEH REGION, UMIAM

Principal Investigator:
Dr. Amrita Banerjee

Co-investigator:
Dr. GT Behere

A. DIVERSITY

Collection of *Ralstonia solanacearum* isolates

A total of 20 *R. solanacearum* strains were collected from different solanaceous crops like tomato (6), potato (6), brinjal/eggplant (2), capsicum (1), as well as rhizospheric soil of solanaceous crops (5) from three different districts of Meghalaya *viz.*, West Jaintia Hills (6), East Khasi Hills (7) and and Ri-Bhoi (7) (Table 1). Isolation of the pathogen was carried out either from diseased plants (Fig. 1a and 1b) or from soil suspension on TZC medium. All strains yielded cream coloured, irregularly shaped, highly fluidal colonies with pink pigmentation in the centre (Fig. 1c). All were confirmed as *R. solanacearum*, as the PCR assay using specific primer pair 759/760 amplified an expected single fragment of 280 bp (Table 1 and Fig. 1d).

Biovar and phylotype determination

Results of biovar determination based on utilization of disaccharides and oxidation of hexose alcohols indicated that all the strains were bv 3 (Fig. 2a). Phylotype specific multiplex PCR showed amplicon of 144 bp in all strains (Fig. 2b). The amplification pattern grouped all the *R. solanacearum* strains isolated from solanaceous crops from Meghalaya under phylotype I, belonging to an Asian origin (Table 1).

Phylogenetic analysis

Amplification of *egl* gene of *R. solanacearum* using primer pair ENDO-F/ENDO-R generated a single band of 850 bp in 10 representative strains (Fig. 3). Further partial *egl* sequences (750 bp) from all representative strains (Table 1) along with 64 reference strains (Supplementary Table 1) were analysed to generate phylogenetic tree. The phylogenetic tree of *egl* sequences was constructed following neighbour joining (NJ) phylogency having 1000 bootstrap replicates. The phylogenetic position in *egl*-based tree was entirely consistent with their phylotype determination on the basis of multiplex PCR (Fig. 2b and Fig. 4). Thus, all our strains were under phylotype I. The major cluster of phylotype I was further divided into two sub groups of unknown (sub group-1) and known sequevars (sub group-2) (Fig. 4). None of the Meghalaya strains clustered with the unknown sequevars identified from plains of India (Fig. 4). Moreover, our strains within the major cluster (phylotype I) did not group based on the host or geographical location. All the strains were assigned sequevar numbers. Out of 10 strains from Meghalaya, eight were grouped along with sequevar 47, one each with sequevar 18 and sequevar 34 (Table 1 and Fig. 4).
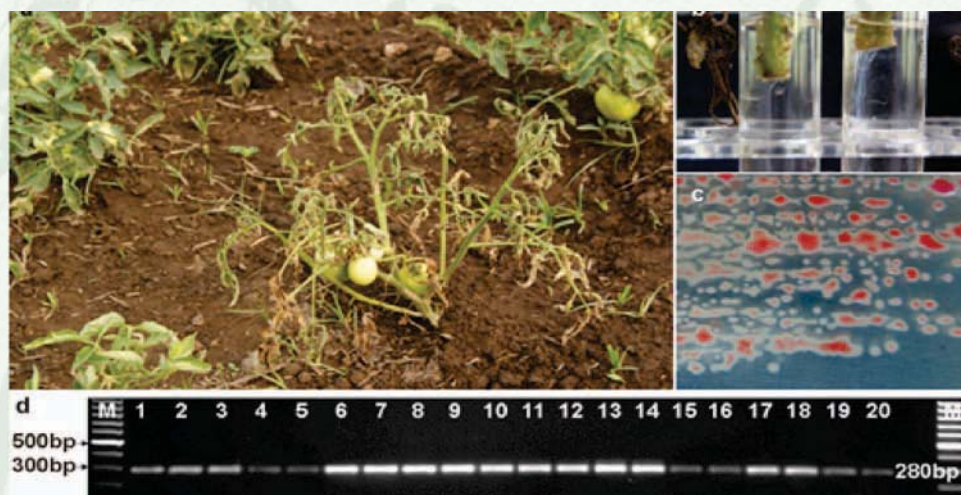
Fig. 1: Symptom and detection of bacterial wilt pathogen *Ralstonia solanacearum* (Clockwise from top left). Appearance of bacterial wilt infected tomato plants; Bacterial streaming in clear water from stem of infected plant; Typical *R. solanacearum* colonies on TZC agar medium; PCR-based confirmation of *R. solanacearum* strains using universal primer pair 759/760 (Lane M = 1 kb DNA ladder, lane 1-20 = strains of *R. solanacearum* collected from Meghalaya)
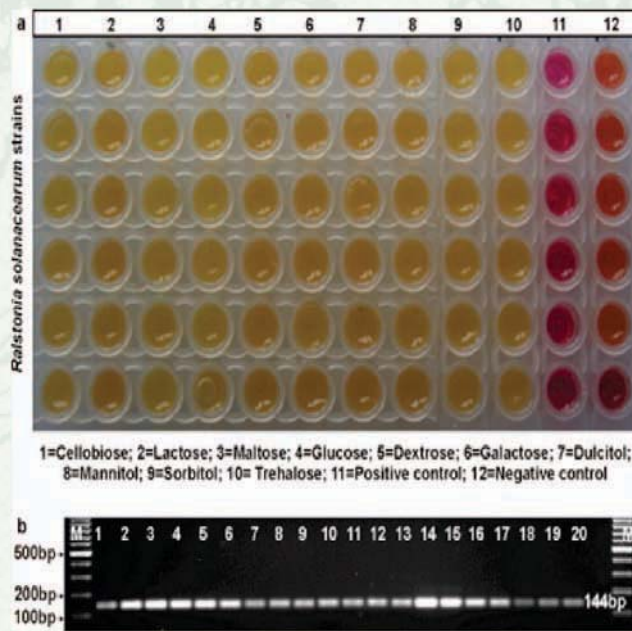
Fig. 2: Biovar and phylotype determination of solanaceous strains of *R. solanacearum* isolated from Meghalaya. (a) Results of biovar test showing the utilization pattern of sugar and sugar alcohols. (b) Phylotype specific multiplex PCR of 20 strains of *R. solanacearum* isolated from Meghalaya (Lane M = 1 kb DNA ladder, lane 1-20 = strains of *R. solanacearum* collected from Meghalaya)

Fig. 3: PCR amplification of endoglucanase (*egl*) gene from 10 representative strains of *R. solanacearum* isolated from Meghalaya using primer pair ENDO-F/ENDO-R (Lane M = 1 kb DNA ladder, lane 1-10 = strains of *R. solanacearum* collected from Meghalaya)

Fig. 4: Phylogenetic relationship based on partial nucleotide sequences of *egl* gene of *R. solanacearum* strains isolated from solanaceous crops in Meghalaya along with previously reported reference strains of *R. solanacearum*. The evolutionary history was inferred using the neighbour-joining method. 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%). The tree is 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 'Maximum Composite Likelihood' method and are in the units of the number of base substitutions per site. Each sequence is labelled with the GenBank accession number followed by strain/ isolate name. The strains under study are presented in bold form. The numerical values within the parenthesis indicate the sequevar number.

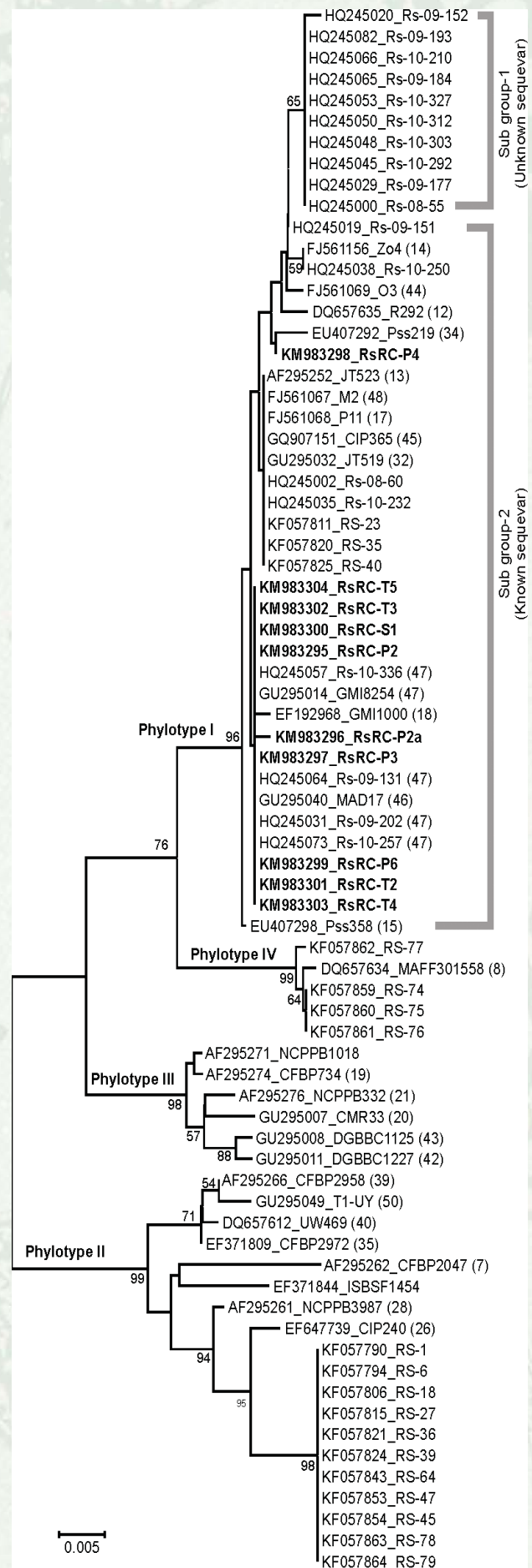Table 1: Details of *R. solanacearum* strains of solanaceous vegetables collected from Meghalaya

Strain	Location	District	Source for isolation	Biovar	PCR 759/760	Phylotype/Sequevar determined by PCR and egl-tree	Accession number NCBI/ PhytoFuRa repository(egl)
RsRc-T4	Umsning	RiBhoi	Stem/Tomato	3	+	I/47	KM983303/1261
RsRc-T3	Umsning	RiBhoi	Stem/Tomato	3	+	I/47	KM983302/1260
RsRc-C2	Umiam	RiBhoi	Stem/Capsicum	3	+	I	-
RsRc-T2	Umiam	RiBhoi	Stem/Tomato	3	+	I/47	KM983301/1259
RsRc-T2a	Umiam	RiBhoi	Stem/Tomato	3	+	I	-
RsRc-P2	Umiam	RiBhoi	Stem/ Potato	3	+	I/47	KM983295/1253
RsRc-P2a	Umiam	RiBhoi	Stem/Potato	3	+	I/18	KM983296/1254
RsRc-S4	Wahiajer	WestJaintia Hills	Rhizosphere soil/ Brinjal	3	+	I	-
RsRc-S2	Niriang	Westjaintia Hills	Rhizosphere soil/ Tomato	3	+	I	-
RsRc-E1	Niriang	Westjaintia Hills	Stem/Eggplant	3	+	I	-
RsRc-P4	Niriang	Westjaintia Hills	Stem/Potato	3	+	I/34	KM983298/1256
RsRc-B2	Niriang	Westjaintia Hills	Stem/Brinjal	3	+	I	-
RsRc-P3	Wahiajer	Westjaintia Hills	Stem/Potato	3	+	I/47	KM983297/1255
RsRc-S3	Mylliem	East Khasi Hills	Rhizosphere soil/ Potato	3	+	I	-
RsRc-S6	Mawjrong	East Khasi Hills	Rhizosphere soil/ Tomato	3	+	I	-
RsRc-T6	Lyngkien	East Khasi Hills	Stem/Tomato	3	+	I	-
RsRc-P5	Mawjrong	East Khasi Hills	Stem/Potato	3	+	I	-
RsRc-T5	Mawkdok	East Khasi Hills	Stem/Tomato	3	+	I/47	KM983304/1262
RsRc-P6	Umdiengpoh	East Khasi Hills	Stem/Potato	3	+	I/47	KM983299/1257
RsRc-S1	Shillong	East Khasi Hills	Rhizosphere soil/ Potato	3	+	I/47	KM983300/1258

B. DIAGNOSTICS

Evaluation of the new approach for rapid detection of bacterial wilt pathogen (*R. solanacearum*) from infected plants

A PCR-based rapid detection of *R. solanacearum* within 24 h was developed. In this method the bacterial ooze is used as a source for template DNA so that there is no necessity to isolate bacteria or extract DNA. The time required for the whole detection process (ooze test to gel electrophoresis) was less than 5 hours. The PCR based method developed in this report is very simple, robust and inexpensive. It has been successfully tested for more than fifty samples for *R. solanacearum* infection from various solanaceous vegetables.

C. DISEASE MANAGEMENT

Identification of potential tolerant/resistant source of brinjal against bacterial wilt

In case of brinjal, two local germplasm along with three released varieties were screened and 0-95% disease incidence was observed among the five tested varieties/germplasm. Among those five, one local germplasm (RL22) showed resistance against bacterial wilt same as Bholanath (resistant variety to bacterial wilt), while the other varieties/germplasm showed mean DSI of 66.67-95% (Table 2). We have collected infected plant samples from each plot and performed diagnosis assay upto molecular level. During ooze test and PCR detection both RL22 and Bholanath showed negative results. The resistant local germplasm will be tested under challenge inoculation for further confirmation of its resistance.

Table 2: Screening of brinjal varieties

Variety	Mean Disease Incidence (%)	Mean DSI	Ooze Test*	PCR detection*
V1 (Bholanath)	0.00a	0.00a	-	-
V2 (US28)	66.67b	64.00b	+	+
V3 (RL03)	95.00c	95.00c	+	+
V4 (US172)	78.33bc	86.00c	+	+
V5 (RL22)	0.00a	0.00a	-	-

Inhibition of *Ralstonia solanacearum* by volatile and non-volatile metabolites of *Pseudomonas putida*

The inhibitory effect of volatile and non-volatile metabolites of four *Pseudomonas putida* isolates were evaluated against *R. solanacearum*. Inhibition of *R. solanacearum* by volatile and non-volatile metabolites was studied. Initially, 1 ml of *P. putida* cultures (CFU 10^8ml^{-1}) was poured in 9 ml KB broth amended with 4.4 g l^{-1} glycine. Inoculated broth was incubated at 27ºC for 7 days. On the other hand, the inhibition by non-volatile metabolites was studied by pouring the *P. putida* cultures in KB broth amended with 1000 µM FeCl3+6H2O. For antagonistic assay, 1 ml of *R. solanacearum* (72 h) suspension was placed on CPG medium and kept for some time to settle down. The pre-soaked (volatile and non-volatile metabolites) filter paper discs (3 mm)were placed on the CPG medium inoculated with *R. solanacearum*. Finally, plates were incubated at 27ºC for 3 days to record the data. The filter paper discs soaked in rifampicin (25 ppm) and sterile distilled water were taken as positive and negative controls, respectively. Out of four, only one isolate (RC 105) showed relative inhibition of 72% in the form of volatile metabolites. Further experiment is in progress.

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Phytophthora

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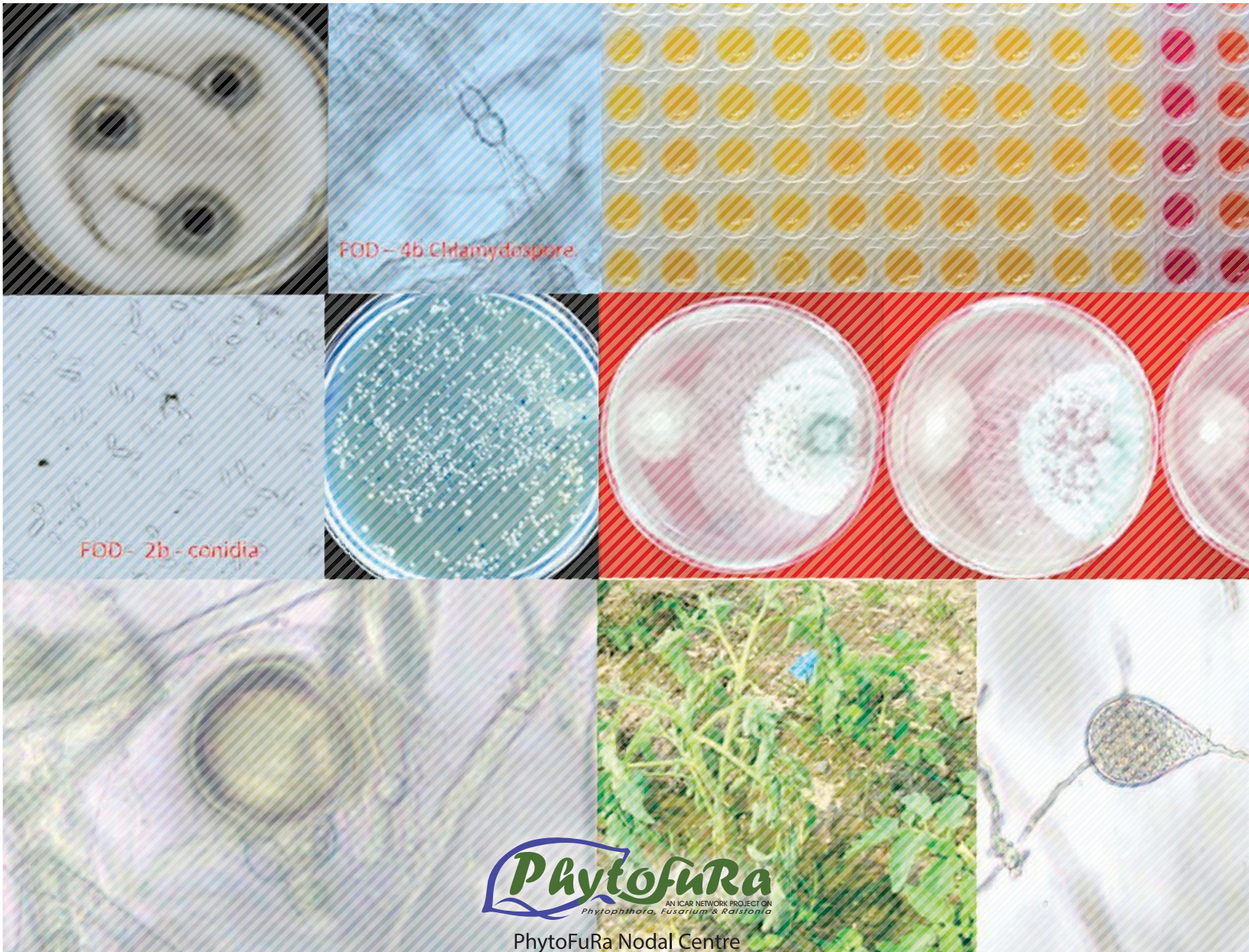
BUDGET EXPENDITURE
2014-15

Name of Institute		Opening Balance (₹ in lakhs)	Amount sanctioned for 2014-15 (₹ in lakhs)	Total amount (₹ in lakhs)	Expenditure of 2014 (₹ in lakhs)	Closing Balance (₹ in lakhs)
ICAR-IISR, Kozhikode		4.78	57.87	62.65	61.58	1.07
ICAR-RC NEH, Umiam		0.38	10.93	11.31	9.31	2.00
ICAR-IARI,	*Ralstonia*	0.49	9.87	10.36	7.76	2.60
New Delhi	*Fusarium*	2.95	9.37	12.32	10.72	1.60
ICAR-CPRI, Shimla		12.17	37.18	49.35	48.31	1.04
ICAR-IIHR, Bangalore		10.59	15.05	25.64	12.05	13.59
ICAR-CPCRI, Kasargod		14.20	9.00	23.20	9.02	14.18
ICAR-CTCRI, Trivandum		0	9.43	9.43	9.34	0.09
ICAR-CCRI, Nagpur		7.67	9.43	17.10	5.25	11.85
ICAR-IIPR, Kanpur		1.46	8.93	10.39	6.83	3.56
ICAR-DOR, Hyderabad		0.66	9.37	10.03	8.91	1.12
ICAR-IIVR, Varanasi		4.83	9.18	14.01	6.70	7.31
ICAR-NRC Banana, Trichy		2.84	7.86	10.70	7.84	2.86
ICAR-NBAIM, Mau		4.02	4.66	8.68	7.04	1.64
ICAR-CCARI, Goa		4.46	9.43	13.89	6.70	7.19
ICAR-SBI, Coimbatore		0	3.60	3.60	1.69	1.91
ICAR-NRCSS, Ajmer		0	2.30	2.30	1.71	0.59
RRI, Kottayam		0	3.60	3.60	0.43	3.17
YSPUHF, RC Kullu		4.28	6.44	10.72	8.34	2.38
Total		75.78	233.50	309.28	229.53	79.75

FOD – 4b Chlamydospore

FOD – 2b – conidia

PhytoFuRa

AN ICAR NETWORK PROJECT ON
Phytophthora, Fusarium & Ralstonia

PhytoFuRa Nodal Centre

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