PhytoFuRa
AN ICAR NETWORK PROJECT ON
Phytophthora, Fusarium & Ralstonia

Outreach Project on

PHYTOPHTHORA, *FUSARIUM* AND *RALSTONIA* DISEASES OF HORTICULTURAL AND FIELD CROPS

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PREFACE

With the rapidly growing international trade in plants and ongoing impacts of climate change, activities of plant pathogens like *Phytophthora*, *Fusarium* and *Ralstonia* are increasing, threatening the biodiversity and sustainability of our agro-ecosystems. On the other hand our understanding of these pathogens has tremendously improved with the advances in sequencing and genomic techniques. The Outreach Project on *Phytophthora*, *Fusarium* and *Ralstonia* Diseases of Horticultural and Field Crops (PhytoFuRa), operational in 17 centres distributed in nine states, was launched to harness the power of these new generation technologies and to bring in more synergy in our efforts to deal with these pathogens. PhytoFuRa has united our scientists and disease control experts with the overall aim of increasing our understanding of the biology and ecology of these serious plant pathogens. This knowledge, I am sure, will be used in the development of effective control and management protocols for the problems caused. This project has successfully completed one more year. During this period, the scientists made reasonable progress in studying the species spread of these pathogens, developing state-of-the art rapid molecular diagnostics, detecting host resistance and seeking sustainable techniques for management and control of the diseases. An isolate of *Phytophthora* infecting black pepper was completely sequenced, and transcriptomics of *Phytophthora-Piper* interaction was deciphered. Gene silencing approaches targeting the *Phytophthora infestans* Avr3a gene carrying RXLR motif are gaining momentum in potato. Several species specific primers were designed and proved as good candidates for *Fusarium* diagnostics. The predominance of Phylotype I in *Ralstonia solanacearum* collections from India was proved by employing an array of molecular tools. The knowledge emanated will be disseminated to end-users and authorities in the agriculture and horticulture sector, and to the general public. Another notable activity during this period was the organization of a ten days training programme on 'Application of Genomics and Bioinformatics in *Phytophthora*/*Ralstonia* Research' from February 8-17, 2011 at IISR, Kozhikode for the benefit of research fellows recruited under this project. The second review meeting was held at Kozhikode from 17-18 February 2011. This publication details the research progress achievements made from April 2010 to March 2011. Apart from the Executive Summary, the salient achievements are compiled centre-wise. I take this opportunity to thank all the investigators of the project for their commendable contribution. I would like to place on record my sincere gratitude to Dr. S. Ayyappan (Secretary, DARE and Director General, ICAR) and Dr. H.P. Singh (DDG, Horticulture) for their valuable guidance and keen interest in the project. The financial support for the project received from ICAR is gratefully acknowledged.

15-12-2011
Kozhikode

(M. Anandaraj)

EXECUTIVE SUMMARY

PHYTOPHTHORA

Biodiversity

Phytophthora was isolated from infected samples by direct plating in selective media and through baiting technique. Periodical survey of different apple orchards located in districts of Kullu, Mandi, Shimla and Kinnaur indicated that the incidence of collar rot disease (*Phytophthora cactorum*) varied between 3.1-72.5, 1.4-26.2, 0.9-12.4 and 0.3- 6.4 per cent, respectively. Maximum incidence of this disease was observed in apple orchard at Johal, Karjan of district Kullu, Ruhmini in Mandi, Tikkar - Pujarli in Shimla and Lippa in Kinnaur districts. A total of 397 new isolates from different hosts have been collected from different parts of India during the period.

Phytophthora repository

The National repository of Phytophthora have been revived and enriched with 305 isolates from 37 host species. This year 21 new isolates have been added to the repository.

Characterization

The *Phytophthora* isolates collected are being characterized by studying their colony morphology, sporangial morphology, pathogenic

Status of National Repository of Phytophthora at IISR, Calicut

Sl. No.	Host plant	No. of isolates collected (2010-11)	Total No. of isolates	Sl. No.	Host plant	No. of isolates collected (2010-11)	Total No. of isolates
1	Black pepper	7	165	20	*Piper chaba*	1	3
2	*Colocasia*	1	10	21	Tapioca	-	3
3	Tomato	-	09	22	*Bauhinia*	-	2
4	Vanilla	-	03	23	Potato	-	1
5	Coconut	1	09	24	Papaya	-	1
6	Strawberry	-	03	25	Clove	-	1
7	*Crossandra*	-	02	26	Carnation	-	1
8	Gerbera	-	02	27	*Vigna*	-	2
9	Periwinkle (*Vinca* sp.)	-	03	28	*Trichosanthes*	-	1
10	Betelvine	-	24	29	Brinjal	-	1
11	Cardamom	5	12	30	Sesamum	-	1
12	Cocoa	1	09	31	Clove	-	1
13	Rubber	-	08	32	Avocado	1	1
14	Capsicum	-	03	33	Yam	1	1
15	Nutmeg	-	03	34	*Diffenbachia*	-	1
16	Citrus	2	12	35	Pineapple	-	1
17	Arecanut	1	02	36	Apple	-	1
18	*Piper longum*	-	1	37	Geranium	-	1
19	*Plectranthus*	-	1			**Total**	**305**

variability, mating type determination, sensitivity to metalaxyl and also by employing various molecular tools. Studies on the impact of temperature, moisture and pH on disease development were also carried out. To study the genetic diversity, various tools like RAPD, SSR, AFLP, ITS rDNA etc. were used. Monitoring of *P. infestans* population for prevalence of physiological races in the states of Himachal Pradesh, West Bengal (Kalyani), Punjab (Jalandhar), Meghalaya (Shillong), Tamil Nadu (Ooty) and Karanatka (Hassan) revealed that the pathogen population in these states consisted of complex races ranging from 9-11 genes. Frequency of occurrence of 11 genes was 100% in HP hills, Meghalaya, Tamil Nadu and Karantaka while in West Bengal and Punjab it was 66.7% and 58.3%, respectively. The metalaxyl sensitivity of *P. capsici* ranged from an EC_{50} value of 0.0002 to 14.4 ppm and an EC_{90} value of 1.1-68.5 ppm. An isolate from Peruvannamuzhy (07-02) was found to be least sensitive with an EC_{90} value of 68.5 ppm. Metalaxyl sensitivity of 97 isolates was tested which revealed that 100.0% population of Ooty is tolerant to 50 ppm, 33.3% population of Shillong to 200 ppm, 66.7% population of Punjab, 33.3% of West Bengal, 17% of HP hills, 16% of UP and 14% of Karnataka are tolerant to 200 ppm.

SSR analysis of 126 *P. capsici* isolates clustered them into four clusters and 40 sub-clusters. Geographical origin did not form the basis for the clustering of isolates and isolates from different areas clustered together with the exception of a few isolates. *mt* haplotype analysis of *P. infestans* isolates revealed that only two haplotypes viz. *mt Ia* and *mt Ib* are prevalent in Indian population. However, their frequency varied from region to region. *mt Ia* type was dominant in all regions (63% in Shimla hills; 90% in Karnataka; 76% in U.P. and 100% in Uttarakhand). The frequency of *mt Ib* type ranged from 0 (Uttarakhand) to 37% in Shimla hills. ITS region of ribosomal RNA gene (rDNA) of 10 isolates were amplified and sequenced. Phylogenetic analysis revealed that ITS sequences of *P. nicotianae* and *P. palmivora* isolates derived from citrus orchards of central India and other parts of the world were highly conserved and variation was not geographically confined. Intra-species variation was observed more in *P. nicotianae* isolates than in *P. palmivora* isolates.

Diagnostics

Ten primers were synthesized for detection of *P. infestans* out of which seven showed specificity towards *P. infestans*. Others showed cross-reactivity with pathogens like *Fusarium oxysporum* or *Rhizoctonia solani*. A species-specific primer pair NIC 1 – NIC 2 was designed and tested for specific detection of *P. nicotianae* isolates. A PCR-RFLP technique was developed to detect *Phytophthora* spp. from citrus roots.

Epidemiology

A yield loss assessment model was developed for late blight on the basis of disease severity and age of the crop growth. JHULCAST model has been validated during December 2010-Janauary 2011. According to this model the probable date for appearance of late blight was 25- 28 December, 2010 and disease actually appeared on 27 December 2010.

Effect of temperature, pH, moisture and soil types on collar rot development was studied. A temperature of 25^{0}C was found to be optimum for the development of collar rot. Maximum incidence and severity of disease was observed at 90% soil moisture and minimum at 50% moisture level and it indicates that 50 per cent soil moisture is required for disease initiation. Soil pH at 5.0 was highly conducive for the development of disease whereas pH around neutral gave inhibitory effects to the development of collar rot disease. Clay type soil was highly conducive for the development of disease whereas sandy loam (gravelly) gave an inhibitory effect to the development of collar rot disease.

Studies on the effect of elevated temperature on host resistance, fungicides efficacy and aggression on *P. infestans* indicated that at 25^{0}C, ridomil (full dose) showed degradation after 14 days of spraying though the rate of degradation was very low compared to control while at 30^{0}C, degradation was more. At 25^{0}C curzate and mancozeb did not show any degradation even after 14 days while at 30^{0}C, degradation was found at 14 days after spraying. No degradation was recorded in acrobat even after 14 days of spraying at 20^{0}C, 25^{0}c and 30^{0}C (both half and full dose).

Phytophthora progapules were isolated from crown debris of dead coconut palms even after one year. Inoculum present in the crown of bud rot affected palms was found to be a potential source for disease spread. *Phytophthor*a was also isolated from coconut roots and soil at the base of affected palms. Rain water was also found to be a carrier for *Phytophthora* propagules. Dried cankers, mummified fruits (pods infected during the end of rainy season of previous year), soil, foliar infection, infected seedlings (self sown) and rain water were found to be the major sources of inoculum for *Phytophthora* diseases of cocoa.

Host-pathogen interaction

Studies on the effect of age of cocoa seedlings to *Phytophthora* infection during rainy season were initiated. Studies indicated that the disease decreases with the increasing age of cocoa seedlings.

In order to understand the mechanism of host tolerance/resistance in citrus against *P. nicotianae*, three rootstock genotypes, namely trifoliate orange (selected as tolerant), rough lemon and rangpur limewere screened. The total soluble phenolics of roots were found to increase in all rootstock genotypes, but their relative increase after infection were lowest in roots of trifoliate orange (tolerant) seedlings. Leaf phenolic levels were not changed upon infection and did not differ significantly between tolerant and susceptible plants.

Host resistance

Screening of ten rootstocks indicated that M26, M9, *Malus prunifolia* and *M. floribunda* are highly resistant and rootstocks MM106 (29.2 mm) and MM104 (27.5 mm) were highly susceptible. Under pot conditions M9, *M. prunifolia*, M26 and *M. floribundas* were recorded as highly resistant whereas M9 and *M. prunifolia* behaved as highly resistant in field conditions.

Fifty two black pepper lines, selected as association mapping population, were screened using leaf, stem and root inoculation methods. Most of the genotypes were grouped as either susceptible or moderately resistant. None of the genotypes were found to give resistant reaction. Accession Kumbachola (Acc. No. 1114), which was found to be tolerant to *Phytophthora* infection was also found to be resistant to 'Pollu' beetle and tolerant to drought.

To study the segregation of *Phytotphthora* resistance if any among the seedling progenies of *Piper colubrinum*, 178 seedlings were screened with *P. capsici*. Out of the progenies screened, 21 plants were resistant to *Phytophthora* whereas two plants were found to be susceptible. Others were found to show moderate resistance.

A set of three primers were designed for elicitin based upon information from the public databases. Out of the three primers, one set of primer ELICPHYF6 and ELICPHYR6 worked very well giving a product of 250 bp, which was subsequently cloned and sequenced. The sequence was perfectly matching with alpha elicitin of *P. capsici* from the database.

Transcriptome analysis was done in *Piper* to identify, characterize and catalogue all the transcripts expressed within leaf tissues challenged with *Phytophthora*. Preliminary analysis of the data revealed expression of many stress induced genes as well as genes related to secondary metabolism. The identified stress induced genes include catalase, chitinase class I and VII, glutathione-S-transferase, peroxidase, beta 1, 3-glucanase, Cu/Zn superoxide dismutase, manganese superoxide dismutase, MAP kinase, osmotin etc. Among the genes, those identical to genes involved in secondary metabolism were chalcone isomerase, chalcone synthase, cinnamate 4-hydroxylase, cinnamoyl-CoA reductase, geranyl pyrophosphate synthase, hmg-CoA reductase, lycopene beta cyclase, phenylalanine ammonia lyase, p-coumaroyl shikimate 3'- hydroxylase and transaldolase.

Genomics

Polymerase chain reaction (PCR) amplification of the ß-tubulin gene region of the template DNA from 9 isolates was performed using the primers BTUBF2 and BTUBR2 which amplifies ~ 750 bp product. PCR amplification of the elongation factor-1α gene region of the template DNA from eight isolates was performed using the primers EF1F and EF1R which amplifies ~950 bp product. Polymerase chain reaction (PCR) amplification of the Cytochrome Oxidase-1 gene region of the template DNA from seven isolates was performed using the primers COXF4N and COXR4N which amplifies ~950 bp product. Polymerase chain reaction (PCR) amplification of the cytochrome oxidase-II gene region of the template DNA from 10 isolates was performed using the primers FM35 and FMPhy which amplifies ~ 1000 bp product.

Gene silencing

The RNAi and amiRNA gene constructs were developed targeting the *P. infestans* Avr3a gene carrying RXLR motif. For RNAi gene construct, the strategy adopted was to develop inverted repeat cDNA fragment of *Phytophthora* Avr3a gene intervened by potato granule bound starch synthase (GBSS) intron (iIR-Avr3a).

Whole genome sequencing

De novo sequencing of an isolate (IISR_2) of *Phytophthora* from black pepper is in progress using Illumina's GAII technology.

FUSARIUM

Biodiversity

Seventy isolates of *F. oxysporum* f. sp. *ciceris* causing wilt of chickpea were characterized with the help of selected RAPD, ISSR, ITS, SSR and other gene specific markers. Forty six isolates of *F. udum* from 13 pigeon pea growing states have been studied for identification of variants. At CISH, Lucknow 154 isolates of *F. oxysporum* f. sp. *psidii* and *F. solani* were characterized by cultural, morphological and molecular methods. Diversity analysis using TEF-1-alpha gene specific primers were done for 54 isolates of *F. oxysporum* f. sp. *carthami*, collected from different geographical locations. A total of 102 *Fusarium* isolates including 62 isolates of *F. oxysporum* f. sp. *lycopersici* from IIVR, Varanasi, 20 isolates each of *F. udum* and *F. oxysporum* f. sp. *ciceri* from IIPR, Kanpur were maintained at NBAIM.

Digitization of all information related to geographical distribution occurrence, host specificity, pathogenicity, virulence etc. are under process to develop the database of Indian isolate of *Fusarium*.

Diagnostics

Molecular markers based on specific gene sequence/ DNA fragment were developed for detection of *F. oxysporum* f. sp. *ciceris*. DNA from *F. udum* representing distinct characters and of diverse ecological niches of the county was extracted and PCR conditions standardized with 10 random primers. Detection of *F. oxysporum* f. sp. *psidii* with species specific primer showing amplification of 183 bp PCR product was standardized at CISH Lucknow. Validated the SCAR marker developed for detection of Foc present in corm and pseudostem of infected banana and also from infested banana soil at National Research centre for Banana, Trichy. A 550 bp fragment was amplified from 30 isolates of *Fusarium* spp. using ITS region with universal primer ITS1 and ITS4 'and Cloning and sequencing of the PCR products is under progress. Out of 29 *Fusarium* specific primers used to characterize 50 isolates of *F. udum* representing distinct characters and diverse ecological niches, 4 primers (FDP 3, FDP4, FDP 25 and FD5 29) produced single amplicon of diagnostic value in all the isolates. Amplicons from fourteen isolates amplified with primer FDP 29 have been sequenced. The sequencing and homology search result showed maximum similarity with Translation Elongation Factor 1-alpha of different species of *Fusarium*. The similarity was as high as 99% for isolate Fu 88 with *F. udum* strain NRRL22949 translation elongation factor 1 alpha gene, partial cds. This high homology indicates the potential of the new sequences to be designed into *F. udum* specific markers. ITS region based markers were developed for detection of race/area specific (Rajasthan isolates-race 4) isolates of the *F. oxysporum* f. sp. *ciceris* causing wilt of chickpea. The markers were validated against 25 different Rajasthan isolates of the pathogen and it was able to amplify 322 bp size of amplicon. A SCAR marker was developed based on the ITS sequence which differentiates *F. oxysporum* f.sp. *carthami* from other species of *Fusarium*. For detection of *F. oxysporum* f. sp. *psidii* with species specific primer, 28S ribosomal RNA and 28S-18S ribosomal RNA intergenic spacer partial sequences were analysed using PRIMER3 programe.

Epidemiology

Four races of *F. oxysporum* f.sp. *carthami* found to be existing in safflower growing areas based on reaction to differential lines viz., 96-508-2-90, A-1, DSF-4 and DSF-6. *F. oxysporum* f. sp. *ciceris* isolates of chick pea were highly variable for causing wilt ranging from 0-100% on various varieties were included in a new set of differentials. Based on differential responses, the isolates were categorized in to eight groups and these groups may be considered as new races or pathotypes of the pathogen in India. Weather parameters viz., maximum and minimum temperature, humidity, and rainfall and diseases incidence were recorded in chilli and tomato during the growing season and correlation between weather and disease incidence is being done at end of the cropping season.

Host resistance

Virulence of 70 isolates against a new set of differential cultivars of chickpea will be analyzed. In safflower study was initiated to quantifying activities of defense enzymes viz., peroxidase, polyphenol oxidase, phenylalanine ammonia lyase (PAL) in safflower by biocontrol agents viz., *Trichoderma harzianum* Th4d, *T. viride* Tv5, *T. harzianum* T673, *T. viride* T7316, *T. viride* TvN13 and *T. harzianum* T693. The bioagents were selected based on their disease suppression against plant pathogens, saprophytic action and tolerance to high temperature.

Disease management

Pseudomonas fluorescens (Pf-80) and *Rhizobium ciceri* (Cp-66) were found to be insensitive against the fungicides evaluated including vitavax power. The efficacy of Pusa 5SD (*T. harzianum*), *P. fluorescens* (Pf-80), *R. ciceri* and fungicide vitavax powder alone and in combination was tested *in vitro* as seed treatment. Evaluated the potential two bioagents viz., *T. viride* and *T. harzianum* against safflower wilt, a field experiment was taken up during November 2010 in DOR wilt sick plot. Seven non-pathognic *Fusarium* isolates have been identified. Four of the non-pathognic isolates have been found to be not related to any of the known pathogenic isolates at molecular level. Field trial on the efficacy of these isolates is in progress.

Genomics

F. oxysporum phylogeny tree was constructed using bootstrap neighboring joining analysis and found that each cluster had the isolates from different states of India indicating the existence of high variability among the population. Successful amplification was obtained for uncultured *Fusarium* using *Fusarium* species specific primer. Genetic diversity analysis carried out for 180 isolates of *Foc* which includes *Foc* obtained from different parts of the banana growing regions of the country. The genome sequence and EST data were searched from different databases and compared for single sequence repeats. On the basis of

comparative analysis 40 SSR primers were designed for diversity analysis of different Indian isolates of *Fusarium*. Isolated genomic DNA of all the Indian isolates of *F. oxysporum* and variability within the population were analysed using SSR primers.

RALSTONIA

Biodiversity

In India, bacterial wilt is one of the important production constraints in crops such as tomato, brinjal, chilli, potato, and ginger in most of the states. During the period 2010-2011, a collection of 468 isolates of *Ralstonia solanacearum* representing diverse crops species such as tomato, chilli, eggplant, ginger and potato were made. The bacterial collection represented various Indian states such as Kerala, Goa, Karnataka, Tamil Nadu, Delhi, Orissa, Sikkim, Uttaranchal, Meghalaya, Mizoram, Manipur, Andaman and Nicobar Islands, Himachal Pradesh, Uttarakhand, Jammu and Kashmir, West Bengal and Jharkhand.

Characterization

The isolates collected from different areas of the country were characterized for various phenotypes such as pathogenicity on their respective hosts and biovar. The pathogen is identified based on 94 reactions in BIOLOG as *R. solanacearum*. The results indicated the dominance of biovar 3 of *R. solanacearum* in India. Some of the isolates from solanaceous crops are found to be biovar 4. Molecular methods for diversity analysis were standardized based on markers like 16s rDNA, *egl*, Rec N, BOX PCR, Rep PCR, phylotyping, PFGE and MLST. The results indicated the predominance of Phylotype I. The BOX PCR, RAPD and *hrp* gene sequence analysis could clearly differentiate the strains from different biovars. The methods adopted clearly showed the diversity of the pathogen from different geographical locations.

Diagnostics

Different primers were designed based on the gene sequences of hrp, 16-23 s regions which can specifically detect *R. solanacearum* from plant tissues and soil. Biovar specific PCR based rapid detection method is in progress.

Host resistance

Greenhouse and field experiments were conducted to search for resistance in crop plants. Ginger mutants irradiated with gamma rays were challenge inoculated with *R. solanacearum*. After three rounds of pathogen inoculation two mutants (HPO 5/15 and HPO 5/2) did not show any wilt symptoms. These mutants are being further evaluated. In brinjal, based on the screening of resistant varieties to bacterial wilt, Surya, Swetha and Utkal madhuri varieties could be used as donor parent in resistant breeding. Tomato cultivars, N- 5, H-24 and Feb-2 showed resistance against *R. solanacearum*.

Disease management

There are significant achievements in biological control of bacterial wilt. Planting antagonistic crops such as *Tagetus* spp. before planting of rhizomes is found to be reducing the bacterial wilt incidence in ginger. Microbial candidates found effective for biological control of *R. solanacearum* are *P. fluorescense*, *P. aeruginosa* and *Bacillus subtilis*. Some actinomycetes isolated from the rhizosphere soil of ginger showed antagonism against *R. solanacearum* under *in vitro* conditions. Further studies are in progress to exploit the activity of actinomycetes in the biocontrol of the pathogen. Bacteriophages which lyse the *Ralstonia* cells were isolated from soils collected from various solanaceous vegetables. Mass production of phages has been standardized and the potential phage isolates will be identified.

Training programme on Application of Genomics and Bioinformatics

Indian Institute of Spices Research (IISR) Calicut, hosted a ten days training programme on Application of Genomics and Bioinformatics in *Phytophthora*/*Ralstonia* Research from February 08-17 at the Institute campus. The programme was inaugurated by Dr. V. A. Parthasarathy, Director. The training was exclusively for investigators and research fellows working in PhytoFuRa project. It was conducted in two phases: Phase 1 – Basics of Molecular Biology and Bioinformatics (six days) and Phase 2 – Genomics and Proteomics (three days). Seventeen Research fellows and one scientist working in PhytoFuRa project have participated in the training programme. Apart from the institute faculty, Dr. R. Vishwanathan (SBI, Coimbatore), Dr. Vinod Scaria (IGIB, New Delhi), Dr. A. Kumar (IARI, New Delhi) & Dr. R. Ramesh (ICAR-RC, Goa) also delivered lectures.

Sub project - 1

PHYTOPHTHORA

Phytophthora species belong to a group of eukaryotic microorganisms classified as oomycetes that are phylogenetically distant from true fungi. Species of the oomycete genus *Phytophthora* are destructive pathogens, causing extensive losses in agricultural crops and natural ecosystems. Due to their distinct physiological and biochemical characteristics, it is difficult to efficiently control the diseases caused by these pathogens. Current disease control measures are largely dependent on application of chemicals, and novel approaches are urgently needed. It is difficult to control *Phytophthora* diseases in the tropics because of its wide host range and environmental conditions that are conducive to disease development. Generally the infection goes unnoticed until symptoms like foliar yellowing or wilting appears.

Under PhytoFuRa, studies are undertaken on seven species of *Phytophthora viz*. *P. cactorum* (apple), *P. capsici* (black pepper), *P. citrophthora* (citrus), *P. colocasiae* (taro), *P. infestans* (potato), *P. nicotianae* (citrus) and *P. palmivora* (coconut, cocoa and citrus).

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1 Biodiversity

a. Survey and collection

National Repository of Phytophthora is maintaining several species of *Phytophthora* from 35 host species. A total of 305 *Phytophthora* isolates have been retrieved and maintained in the repository (Table 1). Among these 21 are new collections made during 2010-2011.

In order to study the genetic diversity of nematodes, 53 soil and root samples were collected from different places in Kerala, Karnataka and Tamil Nadu. Out of these, 13 *R. similis* and 17 root knot nematode populations were collected and are being maintained under laboratory/greenhouse conditions.

Table 1. Details of *Phytophthora* isolates maintained in the repository

Host plant	No. of isolates collected during 2010-11	Total No. of isolates in the repository
Arecanut	1	2
Avocado	1	1
Betel vine	-	24
Black pepper	7	165
Cardamom	5	12
Citrus	2	12
Cocoa	1	9
Coconut	1	9
Colocasia	1	10
Piper chaba	1	3
Rubber	-	8
Tomato	-	9
Yam	1	1
Miscellaneous	-	40
Total	**21**	**305**

b. Morphological diversity

A total of 70 *Phytophthora* isolates were characterized for their colony morphology, while 42 were characterized for their sporangial morphology and 37 for their pathogenicity (Fig.1). In black pepper, out of the 137 isolates studied for virulence 110 were found highly virulent whereas 10 were found to be non-virulent.

c. Diversity studies using biological markers

Out of the 86 isolates characterized for their mating type, 57 are of A1 mating type while 29 are of A2 mating type. The sensitivity of *P.capsici* isolates towards Metalaxyl-mancozeb was studied for 81 isolates. The EC_{50} value of these isolates ranged from 0.0002 to 14.4 ppm while their EC_{90} value ranged from 1.1-68.5 ppm. Out of the 81 isolates studied, 07-02 (a stem isolate from Peruvannamuzhy, Calicut) was found to be the least sensitive with an EC_{90} value of 68.5. There were 19 highly sensitive isolates with an EC_{90} value less than 10 ppm.

d. Diversity studies using SSR markers

Diversity analysis of 126 black pepper isolates of *P. capsici* collected from different regions of India was attempted using SSR markers. The genomic DNA was ampli-

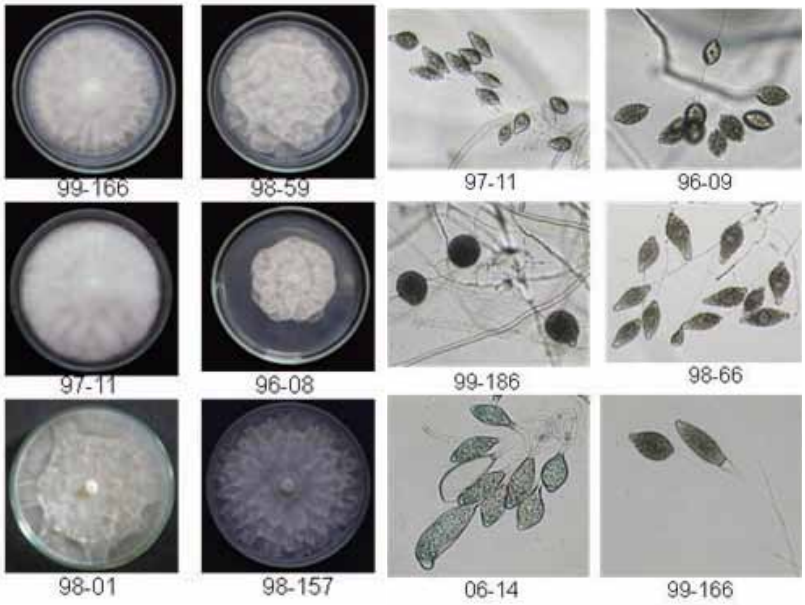


Fig.1. Colony morphology and sporangial morphology of a few *Phytophthora capsici* isolates

fied using 20 SSR primers, designed from entire genomes of *P. sojae*, *P. ramorum* and *P. infestans*. The amplified products were resolved on 3% agarose gel and a dendrogram of diversity was prepared (Fig. 2 & 3). The dendrogram indicated high diversity among isolates and there were five major groups further divided into 40 minor groups.

e. Molecular characterization of nematodes

Out of the 24 RAPD primers tested, eight primers resulted in differential amplification of genomic DNA of *R. similis*. The ITS region of nine isolates of *R. similis* from different places were amplified with 18-26S primer. A product of 920 bp was obtained using this primer set which was eluted and sequenced. The obtained sequences were assembled using CAP 3 programme and aligned (multiple sequence alignment) using Clustal W. Studies on genetic variations in these populations are in progress. Similarly the D2- D3 of 28S region of *R.similis* has been amplified with D2A-D3B primers. Sequence analysis is in progress. Fifty SSR primers were designed from EST sequences of *R.similis* and three primers from these were tested. Polymorphism was observed with one set of primer (EY193167, Forward primer: GCCCACGCTACTACTCGTTC; Reverse primer: CTGGAGACCCCAAAT GATTG). Species identity of root knot nematodes was confirmed with the use of species –specific SCAR primers. Out of the six populations tested, five (two each from Calicut and Kodagu, one from Coimbatore) belonged to *Meloidogyne incognita*.

2 Host resistance

a. Screening of association mapping population

Fifty two black pepper lines, selec ted as association mapping population, were screened using leaf, stem and root inoculation methods. In leaf and stem inoculations the size of lesion was scored as an index for disease resistance on a 0-4 lesion scale. Disease severity index (DSI) was calculated and the plants were rated as resistant, moderately resistant and susceptible in both leaf and stem inoculation methods separately and the average rating was taken as DSI. Plants with DSI < 30% were rated as resistant, 31 - 40% as moderately resistant and > 40% as susceptible. Most of the genotypes were grouped as either susceptible or moderately resistant. None of the genotypes were found to give resistant reaction (Table 2). The screened plants are under observation for 100 days. Accession Kumbachola (Acc. No. 1114), which was found to be tolerant to *Phytophthora* infection was also found to be resistant to 'Pollu' beetle and tolerant to drought.

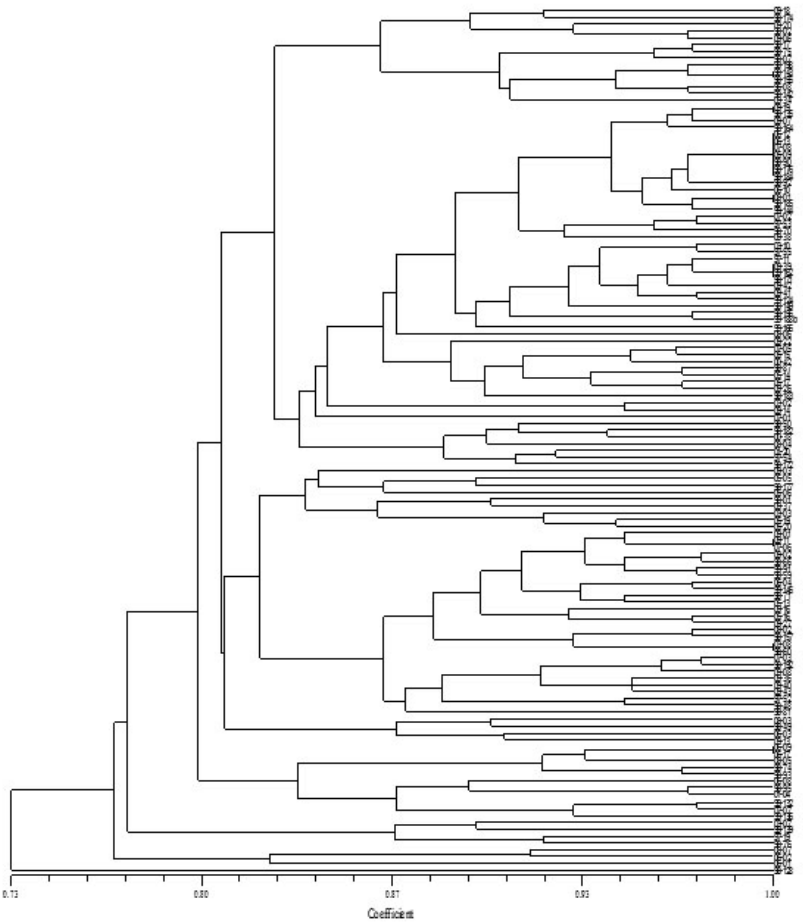


Fig. 2. A dendrogram depicting diversity of 126 *Phytophthora* isolates from black pepper

b. Segregation for *Phytophthora* resistance in *Piper colubrinum*

Seedling progenies (178 Nos.) of *Piper colubrinum*, reported as resistant to *Phytophthora*, nematodes and 'pollu' beetle, were screened with *P. capsici*. Out of the progenies screened, 21 plants were resistant to *Phytophthora* whereas two plants were found to be susceptible. Others were found to show moderate resistance.

c. Amplification and cloning of elicitin genes from *Phytophthora capsici*

Elicitin was isolated and cloned from Phytophthora capsici using a bioinformatics mediated approach. A set of three primers were designed based upon information from the public databases. Out of the three primers, one set of primer ELICPHYF6 and ELICPHYR6 worked very well giving a product of 250 bp, which was subsequently cloned and sequenced. The sequence was perfectly matching with alpha elicitin of *P. capsici* from the database (Fig. 4).

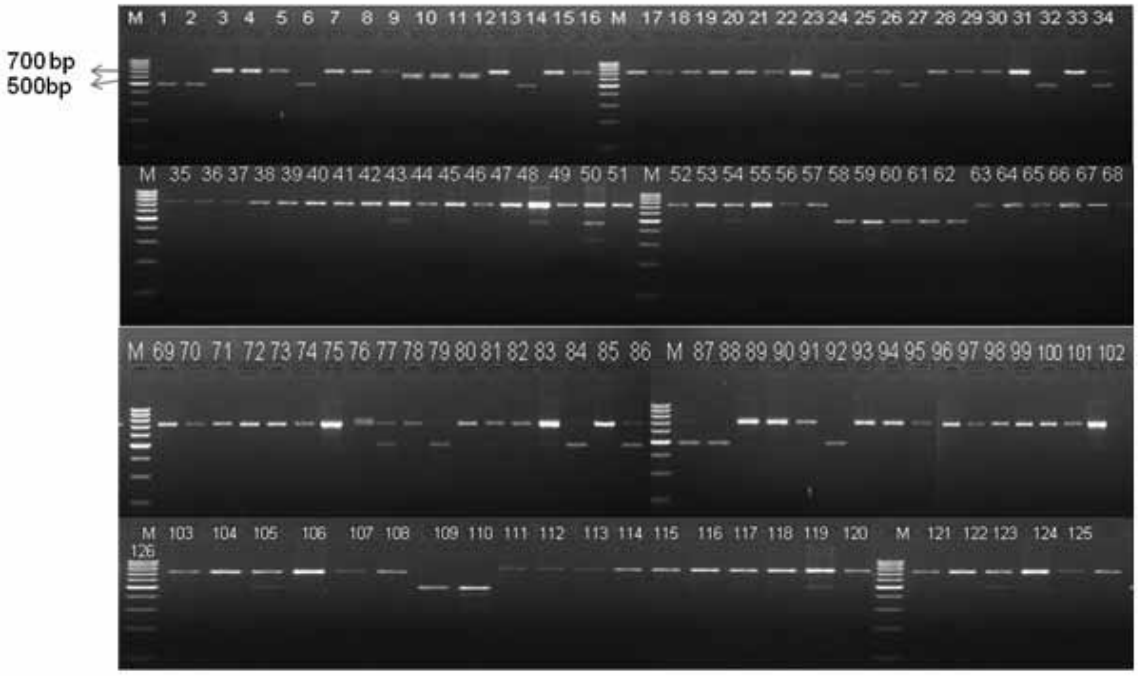
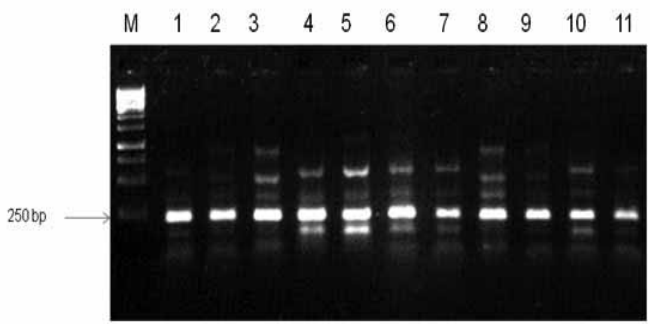


Fig. 3. Molecular profiling of 126 black pepper isolates of *P. capsici* using SSR marker, R4F-R5R.
Forward Primer: YATGGGWGGCATGATGAACC, Reverse Primer: AGGACCAGGAGATGGAGGAC; M: 100 bp ladder

Table 2. The list of moderately resistant genotypes selected after leaf and stem screening

Acc. No.	Genotype	Avg. leaf lesion (mm)	DSI(%)	Avg. stem lesion (mm)	DSI (%)	Avg. DSI (%)
984	Kalluvally	8	35	9	35	35
1069	-	9.33	50	6	25	37.5
1114	Kumbachola	8	35	5	35	35
1212	Karimalligesara	5.75	31.25	6	37.5	34.37
1230	Bilimalligesara	5	37.5	6.5	37.5	37.5
1231	Kalluvally	3.4	31.25	6	35	33.125
1324	Aimpiriyan	5	30	6	35	32.5
1386	Kattanadu Local	5	25	4	37.5	31.25
1389	Kattanadu Local	7	37.5	3	25	31.25
1605	Mullenkolly	8	35	6	35	35
HP 130	Neelamundi X Karimunda	4	25	10	43.75	34.37
HP 442	Perambramunda X P 1	3.3	25	11.33	50	37.5
HP 581	Panniyur X Balankotta	7.33	41.6	7.33	25	33.33
HP 750	Perambramunda X Karimunda	5	37.5	3	25	31.25
HP 780	Panniyur1 X Karimunda	7.6	33.3	6.25	31.25	32.29



Expected Size 220bp

GCGGACCATGATCTGGCTACTCGATGCTGACGGCTAC-
GCCGCTGCCCACGACGGCGCAGTACAAGCTCATGTGC-
GCGTCGACGGCGTGCAACACGATGATCTCCAAGATC-
GTGTCGCTGAACCCCCCTGACTGCGAGCTGACGGT-
GCCCACGAGCGGTCTGGTGCTCAACGTTAACTCGTAC-
GCGAACGGGTTCTCGACGAGTCCGAGAGGATCCTACT-
GTTCCGAGATGAGCTACATGGTTCGACGGCCCAAC-
GGGGCCCACATTTCCTTGATTCAGAGCAGATC-
GTGGGCAAGTTGAAAAAATGCCCAAAAGTTTTAAAA

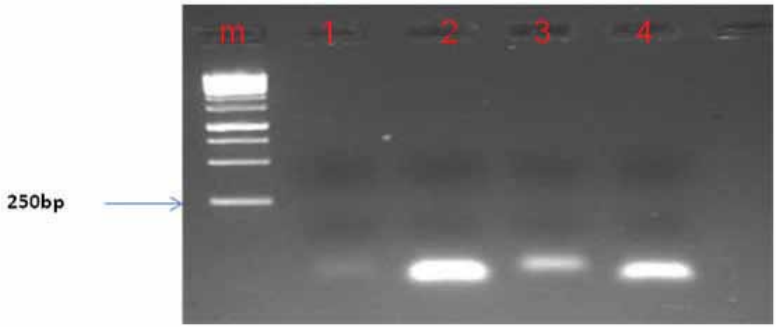
Fig. 4. Amplification and sequencing of elicitin gene from *P. capsici*

d. Amplification, cloning and sequencing of transcription factor gene from *P. colubrinum*

Targeted cloning of WRKY transcription factor genes from *P. colubrinum* was undertaken using RNA samples isolated from leaf samples of plants challenged with *P.capsici*. Amplification, cloning and sequencing of 143 bp gene fragment was found to be similar to WRKY sequences already identified in different plant species (Fig. 5).

e.Transcriptome analysis in *Piper*

With the purpose of generating a broad survey of genes associated plant – pathogen interactions, RNA was extracted from *P. colubrinum* and *P.nigrum* leaf tissues, challenged with *P. capsici*. RNA sequencing was done using Illumina (Genome Analyzer II) paired-end technology, each sequencing feature yielding 2 × 72 bp independent reads from either end of a DNA fragment. Preliminary analysis of the data revealed expression of many stress induced genes as well as genes related to secondary metabolism. The identified stress induced genes include catalase, chitinase class I and VII, glutathione-S-transferase, peroxidase, beta 1, 3-glucanase, Cu/Zn superoxide dismutase, manganese superoxide dismutase, MAP kinase, osmotin etc. Among the genes, those identical to genes involved in secondary metabolism were chalcone isomerase, chalcone synthase, cinnamate 4-hydroxylase, cinnamoyl-CoA reductase, geranyl pyrophosphate synthase, hmg-CoA reductase, lycopene beta cyclase, phenylalanine ammonia lyase, p-coumaroyl shikimate 3'- hydroxylase and transaldolase. A variety of transcription factors and genes involved in primary metabolism with significant similarity to those characterized in other plants were also identified.



Expected Size 143 bp

CGATTCGAGAGTTGATAGACTACGTTGCTTATTATCCTCCTGAGTATGACCCC-
GAGACAGTGACACCTTGCCAACATACGAGTACTGCTCCTCCTGGAGTAAAACT-
GAGCTTCCACGTCCTGCAGGTGCTGAACTTCCATCTTCGGTCATGAACCTC-
GCCGGATCGACTACCCCGCGAGTCACCCGCTGTCCAAAATAGGTTGTTA-
CAACAAAGAGCCTGTTGCTGGAGAAAAAATCCACATATTTGTAATGTAACG-
CATCCCTTCGACATTTTTAACGAAGGAACTGTTACTCACTTGTTAACTTCCTT-
GTGGGTAACGTATCCGGGTTCAAAGCTCTGTTGCTCCACGTTTGGAAGATTTGC-
GAATCCCTGTTGTTATGAAAAACTTCCCAGGACCGCCTCACGGTATCCATGTC-
GAGAGAGAAAATTTACACATGTATGCTCCCCTCTATTGAGAAGCATAAAAAACCTA-
AATTGGCGTTGTCTGCCAAAACTACCGACGAGCAGTTTATGATGTCTTCTGGT-
GATTTTCTTAAAAAC

Fig. 5. Amplification and sequencing of wrky gene (143 bp) from *Piper colubrinum*

3 Disease management

a. Isolation of endophytic fungi from black pepper and evaluation for their biocontrol potential against *Phytophthora capsici*

Forty five more isolates of endophytic fungi were isolated from black pepper making the total isolation to one hundred and twenty five. These endophytic fungi were isolated from four varieties of black pepper viz. Sreekara, Subhakara, Panniyur 1 and Panniyur 3. The isolations were made from roots, stem and leaves. Three mycological media viz. Rose Bengal Agar (RBA), Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA) were tested for the isolation of endophytes and MEA was found to be the best medium followed by PDA and RBA. The isolated endophytic fungi were evaluated against *P. capsici* by dual plate assay. Out of the 45 isolates tested during the period against *P. capsici*, nine isolates showed more than 70% inhibition under in vitro studies. Among the nine isolates, four of the isolates over grew *P.capsici* when incubated beyond 48 h. Morphological study of the short listed antagonistic endophytes revealed that seven of them were non-sporulating types and two were *Fusarium* species. The shortlisted isolates were further tested for their pathogenicity on black pepper using the variety Sreekara adopting the detached leaf assay. None of the isolates caused any lesion when incubated even up to seven days whereas the pathogen *P. capsici* could cause lesions with in 24 h. The shortlisted isolates are being evaluated for suppression of pathogens of black pepper.

b. Multi-location testing of *Trichoderma* isolates

Trichoderma isolates collected from 11 PhytoFuRa centers were sub-cultured, purified and were coded as PhytoFuRa 1-15 and sent to all the centers for evaluation against respective crops and target pathogens. They were tested against two pathogens viz. *P. capsici* (causing foot rot of black

pepper) and *Pythium aphanidermatum* (causing rhizome rot of turmeric) under *in vitro* conditions (Table 3). All the isolates except PhytoFuRa 7 showed >50% inhibition against both the pathogens. PhytoFuRa 7 showed only 46.23% inhibition against *P. aphanidermatum*.

Table 3. *In vitro* inhibition (%) of *Trichoderma* isolates against *Phytopthora capsici* and *Pythium aphanidermatum*

Isolate No.	Inhibition against *P.capsici* (%)	Inhibition against *P. aphanidermatum* (%)
PhytoFuRa 1	63.27 D	67.06 AB
PhytoFuRa 2	58.66 E	68.59 A
PhytoFuRa 3	59.07 E	66.49 AB
PhytoFuRa 4	64.38 CD	65.34 BCD
PhytoFuRa 5	50.92 G	63.33 CDE
PhytoFuRa 6	66.09 BC	62.45 DE
PhytoFuRa 7	52.66 FG	46.23 G
PhytoFuRa 8	59.90 E	62.26 E
PhytoFuRa 9	57.79 E	58.22 F
PhytoFuRa 10	68.67 B	66.30 AB
PhytoFuRa 11	54.63 F	66.00 ABC
PhytoFuRa 12	53.04 FG	64.45 BCDE
PhytoFuRa 13	67.07 BC	62.89 DE
PhytoFuRa 14	67.82 B	66.74 AB
PhytoFuRa 15	74.63 A	61.98 E

4 Developing bioinformatics support system

a. EST mining and secretome analysis to identify extracellular effector proteins from *Phytophthora capsici*

A genomics approach to identify extracellular effector proteins of *P.capsici* by secretome analysis was undertaken. All the 9814 ORFs of *P.capsici* available from NCBI were analyzed with SignalP, 652 ORFs were predicted to encode proteins with N-terminal signal peptides. In the set of 652 deduced proteins with N-terminal signal peptides, 354 were predicted to have no transmembrane domains. In the set of 354 ORFs, 34 ORFs were excluded due to presence of mitochondrial localization signal using TargetP. In the set of 320 ORFs, three ORFs were predicted to have ER site. Thus after the 652 ORFs were analyzed with TMHMM, TargetP and PS scan, 317 ORFs were predicted to be the secreted proteins. Further annotation of 317 ORFs identified 182 secreted proteins, 59 secreted effector proteins and 76 conserved hypothetical proteins. Signal method dependent EST mining predicted different kinds of effector genes, as candidates for testing in the laboratory and that may provide further understanding of plant–Oomycete pathosystems.

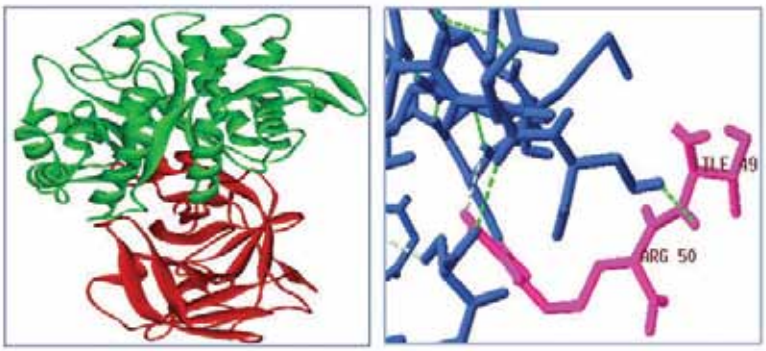


Fig. 6. The structure of EGase in complex with GIP, revealed a binding of the inhibitor to target enzyme using two residues: ILE49 and ARG 50.

b. Structural and docking studies of Glucanase Inhibitor Protein (GIP) from *P.capsici* with plant endo- β -1, 3-glucanases

Species of oomycete genus *Phytophthora* employ a counter defense by secreting Glucanase Inhibitor Protein (GIP) that specifically bind and inhibit plant endo glucanases. Functional annotation studies of *P.capsici* EST sequences have identified Glucanase Inhibitor Protein (GIP), a protein secreted by Oomycetes to counter defense action of endo- β -1, 3-glucanases secreted by plant hosts. The 3-D models of GIP from *P.capsici* and endo- β -1, 3-glucanases from *Capsicum annum*, (host of *P. capsici*) were constructed (Fig. 6). Furthermore, the docking studies were performed to understand the mode of interactions between endo-[beta]-1,3-glucanases and corresponding GIPs secreted by the oomycete pathogen *P. capsici*. This structural and docking information have potential implications to understand the mechanism of plant- pathogen recognition and interaction.

5 Developing PHYTOWEB portal for database creation and monitoring

The PhytoFuRa portal was maintained and updated with additional features. The scope of Phytolib, the literature database, was further enhanced by including literature on Fusarium and Ralstonia. Links to full texts of all the original articles published since 2000 are currently made available in this database. PhytoPD, a repository of Polymerase Chain Reaction primer sets, useful for the identification and detection of *Phytophthora* species, was developed and uploaded. It includes all the universal primers and species-specific primers for more than 30 species of *Phytophthora* published in literature.

CENTRAL PLANTATION CROPS RESEARCH INSTITUTE KASARAGOD

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1 Biodiversity

a. Cultural and morphological characterization

Cultural and morphological characters of *Phytophthora* isolates were studied to identify the species and to find out the extent of variability. Mycelial discs of 5 mm diameter were cut from the periphery of 3-day-old culture of each *Phytophthora* isolate grown on carrot agar medium. The plates were then incubated in dark at 23 ± 1^{0}C. The colony diameter was measured three days after inoculation. The colony diameter of *P. palmivora* isolates of coconut varied from 29.00 mm (KL-CO/86) to 61.83 mm (TN-CO-129). Studies on the diversity of the isolates indicated marked variability in five coconut *Phytophthora* isolates compared to *P. palmivora* isolates. Variations were also observed in shape and size of the sporangia and pedicel length.

Majority of the isolates out of the 364 cocoa isolates exhibited stellate/straite/combed pattern of growth. Colony diameter of the isolates on the 3rd day varied from 18.67 mm (KL/CA-153) to 56.67 mm (TN/CA-301). Sporangia of the isolates were mainly ovoid to ellipsoidal in shape with a round base and conspicuous papilla. Sporangial stalk was broad, short and occluded. Thus 364 out of the 369 cocoa *Phytophthora* isolates were identified as *P. palmivora*.

b. Studies on pathogenic variability

To study the pathogenic variability, 2-3 m old coconuts (WCT variety) were collected, washed thoroughly with tap water, and then surface sterilized with 0.1 % mercuric chloride solution. After washing thrice in sterile distilled water, a small cavity of 5 mm diameter and 2 mm depth was made on the surface at the soft perianth region by using sterile cork borer. Mycelial discs of similar size, taken from 7-day-old cultures of *P. palmivora* isolate, were placed in the cavity. The inoculated area was covered with thin layer of cotton dipped in sterile distilled water and the nuts were kept inside sterile polythene bags containing wet cotton to provide high humidity. The mouth of the bag was closed and incubated at 24±1^{0}C. Three replications were maintained for each isolate. The lesion size was measured six days after inoculation. Studies on the pathogenicity of 131 *P. palmivora* isolates tested on immature coconut revealed variability in their virulence. The lesion size varied from 7.92 (KL-CO/111) to 71.50 (KL-CO/16) cm^2. Thus the isolate KL-CO/16 isolated from Palakkad District was found to be the most virulent isolate. The pathogenecity tests on coconut seedlings also revealed the relative virulence of the isolates. When inoculated, the spindle leaf exhibited typical symptoms of bud rot such as wilting, brownish discoloration and drying within 15 days of inoculations.

To study the pathogenic variability of *P. palmivora* isolates from cocoa, nearly mature detached cocoa pods from a single accession were taken. Cocoa pods were surface sterilized by immersing in 0.1% mercuric chloride solution for 2 minutes and rinsing three times in sterile water. The method of inoculation (cork borer method) was same as explained earlier. Each pod was inoculated at the middle portion with a sporulating mycelial disc. The inoculated area was covered with wet cotton pad. Each inoculated pod was kept inside a polythene bag containing cotton boll dipped in sterile water to provide high humidity. The mouth of the bag was tied and incubated at 24±1^{0}C. Three replicate pods were maintained for each isolate. The length and breadth of resulting lesions were measured five days after inoculation and the area of the lesion was calculated. *P. palmivora* isolates KA-CA/260 from Dakshina Kannada District (Karnataka) causing black pod and AP-CA/334 from West Godavari District (Andhra Pradesh) causing canker disease were the most virulent isolates when inoculated on cocoa pods. The cocoa accession, less suscepti-

ble to black pod disease in Thrissur District with negligible lesion size on pod surface under natural conditions observed during 2009, did not show any natural infection during 2010. Unwounded pods did not take up infection when artificially inoculated. The lesion was very small and confined to a limited area when inoculated with wound. The accession was multiplied by soft wood grafting.

c. Studies on mating types

The compatibility types of *Phytophthora* isolates were determined by pairing each of them with *P. capsici* A1 (98-75) and P. capsici A2 (98-01) (received from IISR, Calicut). Disc of 5 mm dia. cut from the advancing margin of three – day old cultures were used for pairing the isolates. The mycelial disc of *P. capsici* (A1 or A2) and *Phytophthora* isolates to be tested were placed 50 mm apart on CA simultaneously. Inoculated plates were incubated at 20°C in dark for 15-20 days. The plates were then examined for the presence of sex organs and oospores. An isolates was considered A1 mating type when it produced oospores with A2 and not with A1. Similarly, a culture was considered as A2 mating type when it produced oospores with A1 type and not with A2. Out of the 131 *P. palmivora* isolates, 130 isolates were A2 mating types indicating the predominance of A2 mating type of *P. palmivora* causing coconut disease in India. Out of 364, *P. palmivora* isolates of cocoa, 350 isolates were A2 mating type indicating the predominance of A2 in *P. palmivora* population.

d. Molecular characterization of *Phytophthora* isolates

DNA isolation was standardized by CTAB method for molecular characterization of *Phytophthora* isolates.

2 Host-pathogen-environment interactions

a. Effect of environmental factors

Temperature and relative humidity (RH) inside a coconut garden and a cocoa – arecanut mixed garden in disease endemic areas were recorded. The data recorders were kept in Stevenson screen which was installed in the center of the garden. Rainfall data were also recorded from each garden. From this, i) monthly mean, minimum and maximum temperature; ii) monthly mean, minimum and maximum RH; and iii) number of rainy days and total rainfall per month are being compiled. Incidences of bud rot of coconut and black pod disease of cocoa were recorded at monthly intervals. Bud rot incidence started in the month of June, reached a peak during September and became the lowest during November. Black pod incidence initiated two weeks after the onset of monsoon reached a peak in August and the lowest incidence was recorded in the month of October (Table 4). Studies are in progress.

b. Effect of cultural practices

Effect of cultivation practices like organic or inorganic management on the soil population of *Phytophthora* was studied in coconut during premonsoon, monsoon and post-monsoon periods (Table 5). The same was studied in cocoa gardens with inorganic management and compared with that of neglected gardens (Table 6).

c. Studies on source of inocula

Phytophthora propagules were isolated from crown debris of dead coconut palms even after one year. Inoculum present in the crown of bud rot affected palms was found to be a potential source for disease spread. *Phytophthora* was also isolated from coconut roots and soil at the base of affected palms. Rain water was also found to be a carrier for *Phytophthora* propagules. Dried cankers, mummified fruits (pods infected during the end of rainy season of previous year), soil, foliar infection, infected seedlings (self sown) and rain water were found to be the major sources of inoculum for *Phytophthora* diseases of cocoa.

Table 4. Effect of environmental factors on *Phytophthora* disease incidence in coconut and cocoa

Crop	Disease incidence	Month	Temperature (°C)			Humidity (%)			No. of rainy days	Total rainfall (mm)
			Max	Min	Mean	Max	Min	Mean		
Coconut (Bud rot)	Starting	Jun	32.8	21.0	24.7	98.7	67.0	92.9	24	835.66
	Peak	Sep	29.7	21.6	24.5	98.1	65.7	90.1	24	598.16
	Lowest	Nov	29.7	20.8	24.0	99.6	74.5	93.6	17	363.55
Cocoa (BPD)	Starting	Jun	32.6	22.2	25.9	96.1	66.8	90.1	23	627.25
	Peak	Aug	28.9	22.4	24.9	100	84.9	97.7	30	821.75
	Lowest	Oct	29.5	22.5	25.0	100	83.1	94.9	17	277.00

Table 5. Effect of cultural practices on soil population of *Phytophthora* in cocoa gardens

Cultivation practice	Bait used	No. of baits used	No. of baits infected	Infection (%)
A. PRE-MONSOON				
ORGANIC PLOT	Male flower	90	8	8.9
	Small nuts	90	9	10.0
	Rachis	90	10	11.1
	Total	**270**	**27**	**10.0**
INORGANIC PLOT	Male flower	90	10	11.1
	Small nuts	90	6	6.7
	Rachis	90	14	15.5
	Total	**270**	**30**	**11.1**
NEGLECTED PLOT	Male flower	90	9	10.0
	Small nuts	90	6	6.7
	Rachis	90	14	15.5
	Total	**270**	**29**	**10.7**
	Total	**810**	**86**	**10.6**
B. MONSOON (July-Aug)				
ORGANIC PLOT	Male flower	90	16	17.8
	Small nuts	90	18	20.0
	Rachis	90	32	35.5
	Total	270	66	24.4
INORGANIC PLOT	Male flower	90	16	17.8
	Small nuts	90	24	26.7
	Rachis	90	32	35.5
	Total	270	72	26.7
NEGLECTED PLOT	Male flower	90	17	18.8
	Small nuts	90	33	36.7
	Rachis	90	24	26.7
	Total	270	74	27.4
	Total	**810**	**212**	**26.17**
C. POST MONSOON (Nov-Dec)				
ORGANIC PLOT	Male flower	90	13	14.4
	Small nuts	90	24	15.5
	Rachis	90	22	24.4
	Total	270	59	21.85
INORGANIC PLOT	Male flower	90	12	13.3
	Small nuts	90	9	10.0
	Rachis	90	12	13.3
	Total	270	33	12.2
NEGLECTED PLOT	Male flower	90	15	16.7
	Small nuts	90	18	20.0
	Rachis	90	16	17.8
	Total	270	49	18.14
	Total	**810**	**141**	**17.4**

Table 6. Effect of cultural practices on soil population of Phytophthora in cocoa gardens

Baiting method (pod tissue)	Cultivation practices					
	Inorganic garden			Neglected garden		
	Pre monsoon	Monsoon	Post monsoon	Pre monsoon	Monsoon	Post monsoon
Total no. of baits used	60	60	60	60	60	60
No. of baits infected	4	56	11	3	58	5
% of baits infected	6.7	93.3	18.3	5.0	96.7	8.3

3 Disease Management

a. Effect of age of the plant on *Phytophthora* infection

Studies on the effect of age of cocoa seedlings to *Phytophthora* infection during rainy season were initiated. The studies are in progress. Studies indicated that the disease decreases with the increasing age of cocoa seedlings. The disease incidence was very low or negligible in 4 month old seedlings. Seedling dieback was not observed in 5 to 6 months old seedlings (Table 7).

b. Field management of bud rot of coconut

A field trial against bud rot of coconut was laid out during the period under report. The treatments were given as prophylactic treatments at bimonthly interval during the rainy season. The first treatment was given just before the onset of south-west monsoon. Disease incidence was recorded at monthly intervals. Phosphorous acid and mancozeb treatments were effective in controlling bud rot disease of coconut (Table 8).

c. Integrated management of black pod disease of cocoa

Integrated field management trial was laid out to control black pod disease of cocoa. There were seven treatments with three replications including control. The treatments were given at monthly interval. Black pod disease incidence was recorded by counting the total no. of pods and no. of pods with disease incidence. The treatments, copper oxychloride (0.5%), akomin (0.5%) and cultural practices (alone) were found to be effective in the management of black pod disease (Table 9).

Table 7. Effect of age of cocoa seedlings to Phytophthora infection

Age of seedlings	Total No. of seedlings	Disease incidence			
		In open area		In shade	
		No. of plants	Percentage	No. of plants	Percentage
6 m	60	0	0.0	0	0.0
5 m	60	0	0.0	0	0.0
4 m	60	3	5.0	0	0.0
3 m	60	14	23.3	7	11.7
2 m	60	28	46.7	15	25.0
1 m	60	51	85.0	26	43.3
15 d	60	55	91.7	39	65.0
0 d (At sowing)	60	60	100.0	41	68.3

Table 8. Field trial for management of bud rot disease of coconut

Treatment	Bud rot affected palms (%)					
	Pre treatment	Post treatment				
		June -July	Aug -Sept	Oct-Nov	Dec-Jan	Total
Bordeaux mixture pouring to the crown	20.63	0	2.7	2.0	1.3	6.0
Pouring mancozeb solution to the crown and placing mancozeb sachets	38.87	0	0.7	0.7	0	1.3
Placing mancozeb sachets in the leaf axils	12.66	0	1.3	1.3	0	2.7
Bacillus soil drenching and placing *Bacillus* sachets	19.35	1.3	2.6	3.3	2.6	10.0
Placing *Trichoderma* sachets in the leaf axils	18.91	0	3.3	3.3	0.7	7.3
Phosphorus aid soil drenching	13.79	2.0	2.0	2.0	2.0	8.0
Pouring Phosphorus aid to the crown	16.66	0	0	0	0	0
Pouring Hexaconazole solution (0.5%) to the crown	10.7	0.7	1.3	1.3	0	3.3
Placing *T.harzianum* coir pith cake	22.5	0	0	0	0	0
Control	**29.82**	**1.3**	**2.6**	**4.6**	**2.6**	**11.3**

Table 9. Field management trial against black pod disease of Cocoa

Treatment	Disease incidence (%)			
	June	July	Aug	Sept
Copper oxichloride (COC) - 0.5 %	4.41	9.40	5.47	1.4
Ridomil -0.5 %	2.3	12.34	12.67	3.6
Ergon - 0.5 %	1.62	17.35	9.52	11.6
Samarath (Hexaconazole 2 % SC) - 0.5 %	10.30	11.16	18.99	18.3
Akomin (Phosphorous acid) - 0.5 %	12.17	9.96	9.64	2.3
Trichoderma viride	5.97	18.72	13.18	7.2
Cultural practices	4.41	19.24	25.79	3.0
Control	7.08	27.43	35.24	27.1

CENTRAL POTATO RESEARCH INSTITUTE, SHIMLA

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1 Biodiversity

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

One hundred ninety-six isolates of *Phytophtora infestans* were isolated from different geographic locations of India on Rye Agar Media and 256 isolates of *P. infestans* are being maintained. The new isolates were collected from H.P. hills (126), Karnataka (4), Tamil Nadu (3), Indo-Gangetic plains (20), Shillong (4), Kalyani (6), Jalandhar (24) and Tarai region (9). Extraction of DNA from 80 isolates has been done by using Qiagen kit and CTAB method.

b. Phenotypic differentiation of *P. infestans* isolates using biological marker

Monitoring of *P. infestans* population for prevalence of physiological races in the states of Himachal Pradesh, West Bengal (Kalyani), Punjab (Jalandhar), Meghalaya (Shillong), Tamil Nadu (Ooty) and Karanatka (Hassan) revealed that the pathogen population in these states consisted of complex races ranging from 9-11 genes. Frequency of occurrence of 11 genes was 100% in HP hills, Meghalaya, Tamil Nadu and Karantaka while in West Bengal and Punjab it was 66.7% and 58.3%, respectively (Table 10). Comparison of *P. infestans* population for metalaxyl sensitivity in different parts of the country revealed that there has been a marked increase in metalaxyl resistant population. Metalaxyl test of 97 isolates was done. In Indo-Gangetic Plains, 91% of the isolates were tolerant at 50 ppm while 58% of the isolates were tolerant at 100 ppm and 16% at 200 ppm. Eighty percent isolates of HP hills exhibited tolerance at 50 ppm while 39% isolates exhibited tolerance at 100 ppm; 17% at 200 ppm and 8% at 300 ppm. In Karnataka region 100% isolates were tolerant at 50 ppm whereas 71% isolates were tolerant at 100 ppm and 14% at 200 ppm. Population of Ooty region revealed that 100% tolerance at 50 ppm (Table 11). In West Bengal, 33.3% population and 66.7% of Punjab were tolerant at 200 ppm; 33.3% population of Shillong showed tolerance at 200 ppm. As per the status of mating types was concerned, the frequency of occurrence of A1 mating type was 100% in Karnataka, West Bengal and Punjab while A2 mating type w as recorded in Meghalaya (100%), H P hills (94%) and Tamil Nadu (66.7 %).

c. Genotype differentiation & fingerprinting of *P.infestans* using SSR markers

PCR conditions have been standardized with nine SSR primers. Further work is in progress.

Table 10. Racial spectrum of *Phytophthora infestans* in different parts of the country

Location	No. of isolates	Race identified	Frequency (%)	No. of genes
HP hills	47	1.2.3.4.5.6.7.8.9.10.11	100	11
West Bengal (Kalyani)	06	1.2.3.4.5.6.7.8.9.10.11	66.7	10-11
		1.2.3.4.5.6.7.8.10.11	16.7	
		1.2.3.4.5. 7.8.9.10.11	16.7	
Punjab (Jalandhar)	24	1.2.3.4.5.6.7.8.9.10.11	58.3	9-11
		1.2.3.4.5.6.7.8.9.11	58.3	
		1.2.3.4. 6.7.8.9.10.11	8.33	
		1.2.3.4.5.6.7.8.10.11	4.17	
		1.2.3.4.5.7.8.9.10.11	16.67	
		1.2.3.4.5.7.8.9.10.11	4.17	
		1.2.3.4.5.8.9.10.11	8.33	
Meghalaya (Shillong)	04	1.2.3.4.5.6.7.8.9.10.11	100.00	11
Tamil Nadu (Ooty)	03	1.2.3.4.5.6.7.8.9.10.11	100.00	11
Karnataka (Hassan)	04	1.2.3.4.5.6.7.8.9.10.11	100.00	11

Table 11. Metalaxyl sensitivity of *P. infestans*

Location	No.of isolates	Frequency of resistance (%)		
		50 ppm	100 ppm	200 ppm
HP hills	41	80	39	17
UP	43*	91	58	16
Karantaka	07**	100	71	14
Pantnagar	03	66	33	0
Ooty	03	100	0	0
West Bengal (Kalyani)	6	100	100	66.7
Punjab (Jalandhar)	24	100	100	100
Meghalaya (Shillong)	4	100	100	33.3

e. *mt* haplotype analysis for lineage determination

A sub set of population collected from Himachal Pradesh, Punjab, Rajasthan and West Bengal was analysed with primer F4-R4 (cut with EcoR1) for mitochondrial haplotyping which revealed that all the isolates belonged to the Ia haplotype. Another set of population from Shimla hills, Karnataka, UP, Tamil Nadu and Tarai, on analyzing with primers P3F1-P3R1 (digest with EcoR1) and P2F4 & P2R4 (digest with Msp1), revealed that only two haplotypes viz. *mt Ia* and *mt Ib* were prevalent in Indian population of *P. infestans* (Fig. 7). However, their frequency varied from region to region. *mt Ia* type was dominant in all regions - 76% in Shimla hills; 90% in Karnataka; 84 % in U.P., 100% in Tamil Nadu and 100% in Tarai. *mt Ib* type frequency ranged from 0 (Tarai) to 24 % in Shimla hills. It tends to suggest that the new population which was introduced during 2002 is on the rise and it is in the process of displacing the old population.

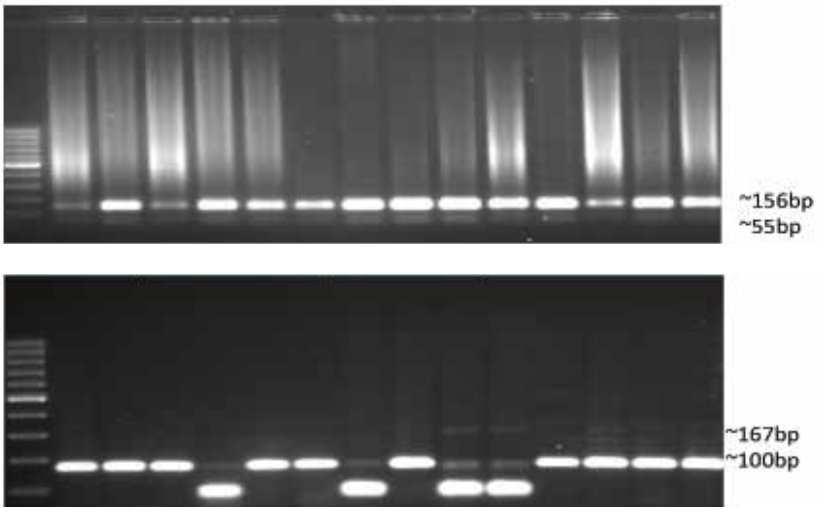

Fig. 7. Mt haplotyping with primer P3F1-P3R1 (cut with EcoR1) and primer P2F4 – P2R4 (digest with Msp1) Lane 1= Molecular marker; Lane 2-15 *P. infestans* isolates

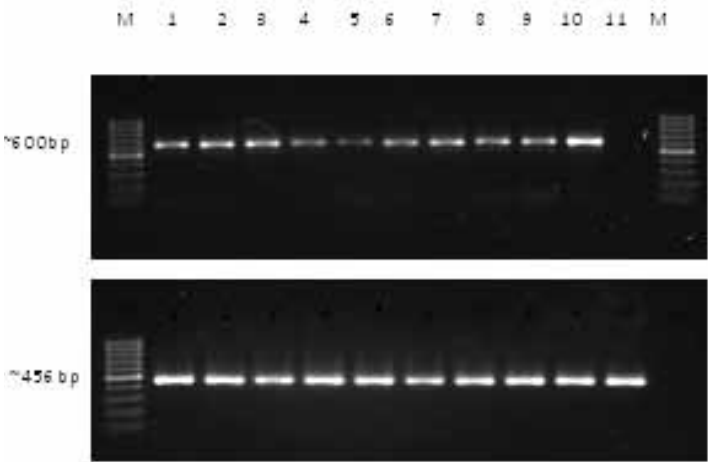

Fig. 8. PCR amplification of *P. infestans* isolates with (top) primer PINF/ITS5 and (bottom)

2 Diagnostics

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

Out of the 10 primers synthesized for detection of *P. infestans*, only seven primers showed specificity to *P. infestans*. PCR amplifications generated a DNA band of 600 bp with primer PINF/ITS-5 and 456 bp with primer PINF2/ITS3 (Fig. 8). The rapid NaOH tissue assay was used with primer set INF FW2 and INF REV for detection of *P. infestans* from artificially infected potato tubers, seed tubers (storage), infected potato leaf and pure culture of *P. infestans*. A single PCR product of approximately 600 bp size was detected. It was also demonstrated that PCR with INF FW2 & INF REV primer set could amplify DNA with a sensitivity limit of 10 fg.

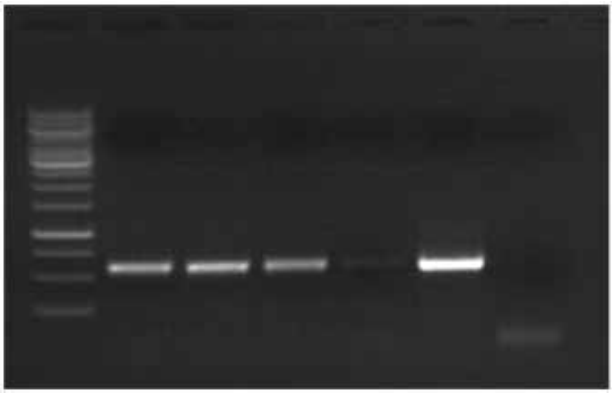

Fig. 9. PCR detection of *P. infestans* in artificially infected potato tubers. 1 = Ladder, 2 = symptomatic tissue, Healthy tissue (3= 5 mm away, 4= 10 mm away, 5= 20mm away), 6= DNA of *P. infestans*, 7= Control (un-inoculated tuber)

b. Detection of *P. infestans* in artificially infected tubers

P. infestans was detected in artificially infected tubers (Cv Kufri Jyoti) by the PCR assay and ethidium bromide staining of agarose gels at 4 & 6 days post-inoculation but not at 2 days post-inoculation, before the occurrence of visible symptoms. Moreover, amplifiedpr oducts of expected size were obtained from tuber lesions and sym ptomless areas at various distances from the tuber lesions (Fig. 9). No

amplification occurred with un-inoculated potato.

c. Detection of *P. infestans* in natural infected leaves

P. infestans was detected in naturally infected leaves (Cv Kufri Bahar) by the PCR assay using the primers PINF/ITS5. Successful amplification could be obtained with samples collected from leaf lesions and symptomless samples collected 5, 10 & 15 mm away from the lesion while no amplification occurred with healthy uninoculated leaves.

3 Epidemiology

a. Development of yield loss assessment models

A yield loss assessment model was developed based on three years' data. Y= -3.741446-0.046319xDS (%) +0.388629 X DAE where Y = yield loss (%), DS= Disease severity percentage and DAE = Days after emergence. Actual yield loss = P-Y where P= potential yield, Y= yield loss and R2= 0.5358261. The model will assess yield loss on the basis of disease severity and age of the crop growth.

b. Development of decision support system

JHULCAST model has been validated during December 2010-January 2011. According to this model the probable date for appearance of late blight was 25- 28 December 2010 and disease actually appeared on 27 December 2010.

c. Effect of elevated temperature on host resistance, fungicides efficacy and aggression on *P. infestans*

An experiment was conducted to study the effect of temperature on the efficacy of fungicides under in vitro conditions. Four fungicides *viz*. Acrobat (0.1%), Curzate (0.1%-cymoxanil + mancozeb), Mancozeb (0.125%) and Ridomil (0.1% -metalaxyl + mancozeb) were used in a detached leaves test at 20^{0}C and 25^{0}C. At 20^{0}C, Ridomil showed degradation after 14 days of spraying and at 25^{0}C, Curzate showed degradation after 7 days of spraying while mancozeb and Ridomil degraded after 14 days of spraying (Table 12). On testing the same treatments with double doses, Ridomil (full dose) showed degradation after 14 days of spraying at 25^{0}C, while at 30^{0}C the degradation was more. Curzate showed no degradation even after 14 days at 25^{0}C while it degraded 14 days after spraying at 30^{0}C (Table 13). At 25^{0}C mancozeb did not show any degradation while at 30^{0}C degradation was recorded after 14 days of spraying. No degradation was recorded for acrobat even after 14 days of spraying at any temperature or dosage.

d. Study of growth patterns of different *P. infestans* isolates at various temperatures

An experiment was also conducted for testing the adaptations of *P. infestans* isolates collected from different parts of the country. North western hills and Tarai region showed maximum growth diameters (88.75 mm) at 20^{0}C compared to western plains while at 25^{0}C maximum growth diameters were measured from Tarai and Plateau regions (85.87 mm).

Table 12. Effect of temperature on efficacy of fungicides (half dose)

Fungicide	Conc. (%)	LS (cm^2) 5 days after each inoculation					
		1 *DAS		7 *DAS		14 *DAS	
		20^{0}C	25^{0}C	20^{0}C	25^{0}C	20^{0}C	25^{0}C
Acrobat	0.1	0.0	0.0	0.0	0.0	0.0	0.0
Curzate	0.1	0.0	0.0	0.0	3.25	0.0	3.47
Mancozeb	0.12	0.0	0.0	0.0	0.0	0.0	3.35
Ridomil	0.1	0.0	0.0	0.0	0.0	1.28	1.33
Control		6.33	5.50	6.68	5.77	6.28	6.08

Table 13. Effect of temperature (25^{0}C & 30^{0}C) on efficacy of fungicides (full dose)

Fungicide	Conc. (%)	LS (cm2) 5 days after each inoculation					
		1 *DAS		7 *DAS		14 *DAS	
		25^{0}C	30^{0}C	25^{0}C	30^{0}C	25^{0}C	30^{0}C
Acrobat	0.2	0.0	0.0	0.0	0.0	0.0	0.0
Curzate	0.2	0.0	0.0	0.0	0.0	0.0	4.40
Mancozeb	0.25	0.0	0.0	0.0	0.0	0.0	4.16
Ridomil	0.2	0.0	0.0	0.0	0.0	1.64	4.23
Control		5.78	6.43	6.08	6.41	9.69	8.77

a. RNAi-mediated silencing of *Phytophthora* infestans Avr3a gene for imparting late blight resistance

The RNAi and amiRNA gene constructs were developed targeting the *P. infestans* Avr3a gene carrying RXLR motif. For RNAi gene construct, the strategy adopted was to develop inverted repeat cDNA fragment of *Phytophthora* Avr3a gene intervened by potato granule bound starch synthase (GBSS) intron (iIR-Avr3a). Genomic DNA of potato and *P. infestans* was isolated by CTAB procedure. Complete Avr3a cDNA was PCR amplified using primers (primer sequence is mentioned below) for sense and antisense fragments (Fig. 10).

Avr3a sense F:	AAAA CTGCAG (PstI) AA TCTAGA (XbaI) CGT CTG GCA ATT ATG CTG TCT GCT
Avr3a sense R:	CGCG GGATCC (BamHI) ATA TCC AGT GAG CCC CAG GTG CAT
Avr3a antisense F:	CGCG GAATTC (EcoRI) CT GAGCTC (SacI) CGT CTG GCA ATT ATG CTG TCT GCT
Avr3a antisense R:	CCGG GGTACC (KpnI) ATA TCC AGT GAG CCC CAG GTG CAT
Avr3a GBSSInt F:	CGCG GGATCC (BamHI) AAC TCT CCT GGA AGG TAG GTG TCA
Avr3a GBSSInt R:	CCCC GAATTC (EcoRI) ATCGAT (ClaI) ACTGGT (SpeI) GGTACC (KpnI) AAG TTC CTG CAT GAA CAC CAG TTG

Fig. 10. PCR amplification of *P. infestans* Avr3a cDNA. Lanes: M, DNA ladder; C, control without template DNA, S, Avr3a sense; A, Avr3a antisense.

GBSS intron was PCR amplified from potato genomic DNA using '*AVR3a* GBSS Int' primers. The amplified *Avr3a* sense cDNA was restricted with XbaI and BamHI and ligated onto XbaI and BamHI restricted pUC19 backbone to get pUC19::*Avr3a*-S. The amplified GBSS intron sequence was restricted with *Bam*HI and EcoRI and ligated onto pUC19::Avr3a-S backbone, generated by restricting with the same two enzymes, to get pUC19::Avr3a-S-I. KpnI and *Eco*RI restricted Avr3a antisense fragment was ligated onto pUC19::*Avr3a*-S-I backbone to get pUC19::iIR-*Avr3a* (Fig.11). Sequencing was done to confirm the presence of insert and nucleotide information. pUC19::iIR-*Avr3a* was restricted with XbaI and SacI, and iIR-Avr3a was gel eluted and ligated onto binary vector backbone pBI121 to get potato transformation vector cassette, pBI121::iIR-*Avr3a* in which the gene construct is under the control of constitutive CaMV 35S promoter. pBI121::iIR-*Avr3a* was introduced into *Agrobacterium tumefaciens* strain, EHA105, by freeze-thaw method. A total of 1200 internodal stem explants of Kufri Pukhraj and Kufri Khyati were transformed with iIR-*Avr3a* gene construct by *Agrobacterium-mediated* genetic transformation technique. About 300 adventitious shoot buds were obtained so far, which are being regenerated for differentiation into shoots.

b. Artificial microRNA (amiRNA) mediated silencing of *P. infestans* Avr3a gene for imparting late blight resistance

Five amiRNAs targeting five different regions of *Avr3a* mRNA and without any off-targets were selected for amiRNA construct development. The *Avr3a* amiRNA sequences and their target region in the *Avr3a* mRNA are mentioned in the next page.

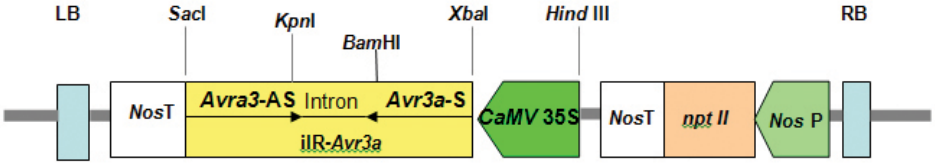

Fig.11. Map of T-DNA region of binary vector cassette, pBI121:iIR-Avr3a

1. Avr3a amiRNA-1 : 5’ TTT ACC CAT AAG CTG TTT CGC 3’
   Target region on Avr3a mRNA : 262-282
   Target gene 5’→3’/262-282 : GCG AAA CAG CTT ATG GGT AAT
   amiRNA reverse complement/1-21 : GCG AAA CAG CTT ATG GGT AAA
   One mismatch at amiRNA-1 5′-end position one.
2. Avr3a amiRNA-2 : 5’ TAT GTA GTG AGC TGG CGT CGC 3’
   Target region on Avr3a mRNA : 91-111
   Target gene 5’→3’/91-111 : GGG ACG CCA GCT CAC TAC ATA
   amiRNA reverse complement/1-21 : GCG ACG CCA GCT CAC TAC ATA
   One mismatch at amiRNA-2 position 20 from 5′-end.
3. Avr3a amiRNA-3 : 5’ TTG GTT TGG TCG ATT GCG CTG 3’
   Target region on Avr3a mRNA : 56-76
   Target gene 5’→3’/56-76 : CAG TGC AAT CGA CCA AAC CAA
   amiRNA reverse complement/1-21 : CAG CGC AAT CGA CCA AAC CAA
   One mismatch at amiRNA-3 position 18 from 5′-end.
4. Avr3a amiRNA-4 : 5’ TTC TGG TCT AGC GTA ACC CTA 3’
   Target region on Avr3a mRNA : 324-344
   Target gene 5’→3’/324-344 : CAG GGT TAC GCT AGA CCA GAT
   amiRNA reverse complement/1-21 : TAG GGT TAC GCT AGA CCA GAA
   Two mismatches at amiRNA-4 position 1 and 21 from 5′-end.
5. Avr3a amiRNA-5 : 5’ TTC TGA TTG TAC TTT GCG CGT 3’
   Target region on Avr3a mRNA : 381-401
   Target gene 5’→3’/381-401 : AGG CGC AAA GTA CAA TCA GAT
   amiRNA reverse complement/1-21 : ACG CGC AAA GTA CAA TCA GAA
   Two mismatches at amiRNA-4 position 1 and 20 from 5′-end.

Arabidopsis pre-miRNA 164b stem-loop backbone of 153 nucleotides (Fig. 12) was chosen for expression of Avr3a amiRNAs in transgenic potato. Appropriate primers were designed replacing *Arabidopsis* miRNA164b sequence and incorporating individual Avr3a amiRNA into the pre-miRNA backbone. Modified pre-miRNA 164b:Avr3a amiRNAs was PCR amplified by primer extension using error-proof Pfu Taq polymerase and appropriately designed primers. The amplified miR164b-amiRNAs were restricted with XbaI and SacI and ligated onto pUC19 backbone, generated by digesting with the same two enzymes to get pUC19::miR164b-Avr3a-amiRNA(s). Presence of insert and nucleotide sequence was confirmed by sequencing. Individual amiRNA construct was further religated onto the binary vector, pBI121, and placed under the control of constitutive CaMV 35S promoter to get pBI::miR164b-Avr3a-amiRNA(s) (Fig. 13). About 1000 internodal stem explants of two Indian potato cultivars, Kufri Pukhraj and Kufri Khyati were transformed with Avr3a-amiRNA-2 & -4 gene constructs by *Agrobacterium*-mediated genetic transformation technique. The co-cultivated explants were placed on regeneration media for initiation of adventitious shoot buds.

```
      a  ca      cauu      a ---- u  c  --a   g   a   a
gaugg gaag  gggcacgug  acuagcucau uaua  cac cuca caca  augcgu uauau ugcgg a
|||||  ||||  |||||||||  ||||||||||  ||||  |||  ||||  ||||  ||||||  |||||  |||||
cuacc cuuc  cccguguac  ugauugagua guau  gug gagu gugu  ugugua auaua guguu u
      a  ua      uucu      g  agua  u  u  gug   g   -   u
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Fig.12. Stem-loop structure and nucleotide sequence of *Arabidopsis* pre-miRNA164b. miRNA sequence is shown in red and miRNA* sequence is shown in bold black.

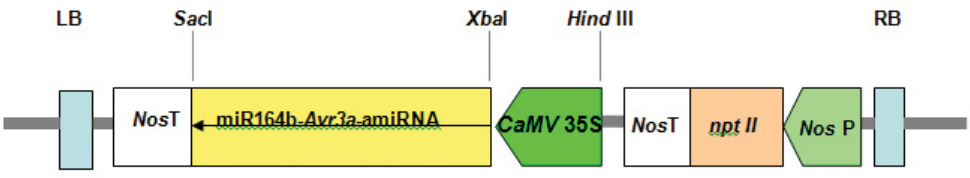

Fig. 13. Map of T-DNA region of binary vector cassette pBI121::miR164b-Avr3a-amiRNA.

5 Host resistance

a. Validation of molecular markers for R-genes (validation of PCR markers in late blight differentials and screening of parental lines/varieties)

Validation of R1 AS Markers (F 5'CAC TCG TGA CAT ATC CTC ACT A'.....3'; R 5'CAA CCC TGG CAT GCC ACG3') was done in a set of late blight differentials. Thereafter, the presence of R1 gene was checked by using this marker in 43 Indian potato varieties. The presence of R1 gene was confirmed in three varieties namely Kufri Sherpa, Kufri Kuber and Kufri Alankar only. Further, parental lines (39) currently being used in the late blight breeding programme were also analysed for the presence of R1 gene and only five lines namely SM/93-239, HB/82-372, HR 5-2 , HR 9-3, CP 3776 has been found to possess the gene. The R3a gene specific markers i.e. GP185 validated in R3 differential and its combination. Screening of parental lines was completed and 19 parental lines confirmed for presence R3a gene. Validation of R3a gene in potato varieties is in progress.

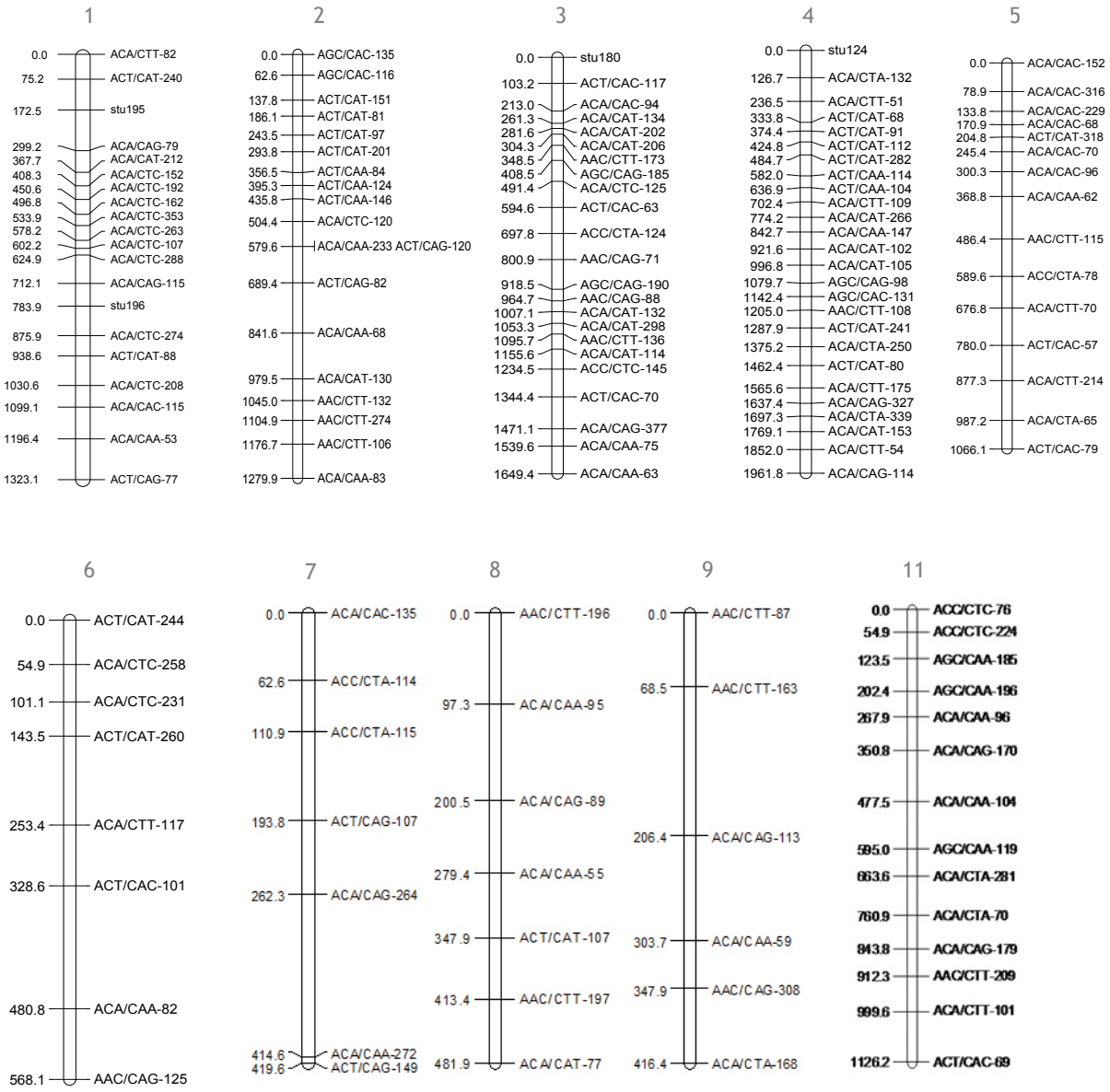

Fig. 14. Molecular linkage maps of *Solanum chacoense*

b. Maintenance of mapping population *in vitro*

A total of 128 genotypes including both the parents i.e. *Solanum spegazinni* and *Solanum chacoense* are being maintained under *in vitro* conditions. Mapping population was multiplied in tissue culture and further for tuber formation it was planted at Modipuram.

c. Molecular map of wild potato species *Solanum chacoense*

A mapping population for identification of horizontal resistance genes to late blight from the diploid wild potato species *S. chacoese* was developed by crossing with the susceptible species *S. spegazinni*. The population consisted of 126 segregating F1 progenies. A molecular map containing 132 markers had been prepared last year. To make the map denser, more AFLP markers were generated using two new sets of AFLP primer combinations. The data generated by these AFLP primers sets were analyzed and a total of 71 loci were recorded from the male parent *S. chacoense* that were absent in *S. spegazinni*. The expected segregation ratio of 1:1 was tested for all these loci in the population by Chi-square analysis using JoinMap 0.4 software and only 20 loci segregated as per the expected ratio. The presence/absence data of these 20 loci were added with the previously generated data of 132 markers, giving a total of 152 markers. A molecular linkage map of *S. chacoense* was prepared containing 152 markers from a total of 18 primer pair combinations (Fig.14) using the JoinMap programme (Version 0.4). The data generated so far could resolve only 10 linkage groups; the remaining two linkage groups contained only three markers each. More number of markers is needed for preparing a reasonable molecular map of those two linkage groups.

6 Disease management

a. Isolation of novel microorganisms with activity against *P. infestans*

Seventeen new bacteria have been isolated from rhizosphere and phyllosphere and 42 bacteria maintained in water at 4°C for further studies. Efficacy of these bacterial isolates against the *P. infestans* (Kasauli isolate) was also tested. Out of the 17 isolates, only three are good biosufactant producers and one of them showed biocontrol properties *in vitro* against *P. infestans*. For biosufactant assay two new methods have been standardized namely-haemolytic activity and tilted glass slide. Efficacy of *Pseudomonas* sp. I and *Pseudomonas* sp. II was tested by dual culture at different intervals through food poisoning, detached leaves and tuber slice methods. In food poisoning test, 100% inhibition of *P. infestans* was recorded at 0.25-5% concentration of culture filtrate of both bacteria. Biocontrol activities of both biosurfactant producing isolates of *Pseudomonas* sp. I and II were tested by using dual culture activity in. Forty eight hours old spore suspension and culture filtrate both exhibited better results than control under all the three methods tested in. Both isolates of *Pseudomonas* spp. also revealed positive result with *Rhizoctonia solani* and *Fusarium* spp in dual culture test.

b. Extraction, purification and characterization of microbial metabolites with activity against *P. infestans*

Two methods i.e. chloroform: methanol extraction and chilled acetone extraction were standardized for extraction and purification of microbial metabolites. Chloroform –methanol extraction is better than chilled acetone extraction method in terms of recovery percentage. Further extraction and purification of metabolites is under progress.

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1 Biodiversity

a. Isolation, purification and maintenance of *Phytophthora* cultures

Taro leaves infected by leaf blight were collected from Kerala and Andhra Pradesh. *P. colocasiae* was isolated from mature leaves of taro showing typical symptoms of taro blight. For isolation leaf tissue segments of 2-3 cm from infected area were excised from lesion margins. The leaf segments were sterilized in 1% sodium hypochlorite for 2 min, rinsed twice with sterile distilled water and placed onto *Phytophthora* selective media (rye agar amended with 20 mg l^{-1} rifamycin, 200 mg l^{-1} vancomycin, 200 mg l^{-1} ampicillin, 68 mg l^{-1} pentachloronitrobenzene and 50 mg L-1 50% benlate) in Petri dishes and incubated in Petri dishes for 4 to 5 days at 20^{0}C and mycelia were then transferred and maintained on potato dextrose agar medium (PDA; 250 g l^{-1} potato, 20 g l^{-1} dextrose and 20 g l^{-1} agar). A total of 23 pure cultures were added to the culture repository along with the eight cultures obtained from IISR, Calicut. Two isolates of *P. palmivora* were maintained in laboratory and a new isolate was isolated from Palode, Trivandrum.

b. Mating type assay

The mating type of an isolate was determined by pairing of unknown isolates with standard A2 isolates on potato dextrose agar. The isolate to be tested was placed on one side of the Petri dish and the A2 standard isolate was placed on the other side. The plates were incubated at 28°C in the dark for about 10 days or until oospores were formed. On the basis of the appearance of oospores a tested isolate was classified as mating type A1 or A2. If it produced oospores with the A2 standard isolate it was classified as A1 mating type; an isolate that did not create oospores was determined as A2 mating type. Out of the 30 isolates of *P. colocasia* tested, nine were A1 and the rest A2 mating type.

c. Screening of growth media

Seven different media were tested to find out the best medium suited for the optimum growth of *P. palmivora*. Potato Dextrose Agar (PDA), Carrot Agar, fungal agar, oat meal agar, corn meal agar and V8 Juice Agar were used as a source for solid media. Small blocks (4 mm) of actively growing cultures taken from the periphery were used to inoculate on above said agar plates. The plates were incubated at optimum temperature 28°C for 4-6 days. Among the solid media tested, carrot agar and corn meal supported the maximum mycelial growth followed by fungal agar, PDA, oat meal and V8 agar.

d. Effect of temperature on mycelial growth

Effect of various temperatures on the mycelial growth of *P.palmivora* was monitored on carrot agar. For studying the effect of temperature on the growth of *P.palmivora*, small agar blocks (4mm) of actively growing cultures taken from the periphery of the actively growing culture were used to inoculate onto the center of fresh carrot agar Petri plates. The cultures were incubated on a wide range of temperature viz. 20^{0}C, 25^{0}C, 30^{0}C, 35^{0}C and 40^{0}C. Among the various temperatures used, 30^{0}C favored the maximum growth rate of P.palmivora followed by 25^{0}C and 20^{0}C. No growth was observed on plates at 40^{0}C.

e. Virulence assay

For pathogenecity assay, young taro leaves (two months old plant)

were detached from the plant of similar age and size. Mycelial plug of (newly collected isolates) size 5 mm was placed on the abaxial side of the leaf and 1.5% agar was added over the plug. Wet cotton was placed over the plug. Then, the leaf was placed up on a moistened filter paper in plastic tray. Trays were covered with transparent plastic sheets. Leaves were incubated at 28±2°C and the disease development was recorded daily for 7 days. The isolates were grouped based on the severity of the disease developed on the leaves (Table 14). For pathogenecity assay in tuber, the variety Sree Kiran was used. A piece of mycelial plug (P7) of 5 mm was placed in to the incision made on the surface of the tuber. Moistened cotton was placed over the plug to facilitate the development of pathogen growth. The corms were then incubated in plastic tray covered with moistened filter paper and checked after six days.

f. Metalaxyl sensitivity

Responses to the fungicide metalaxyl were determined by growing the isolates on potato dextrose agar amended with Ridomil. The stock solution of the Ridomil (250 mg metalaxyl ml^{-1}) was prepared in sterile distilled water and added to PDA at 0.1, 1, 5, 10, and 100 µg ml^{-1} concentration. Mycelial plugs of size 5 mm were placed on plates of different concentrations and incubated in the dark at 28±2°C. The radius of the mycelial growth was recorded after seven days. An isolate was scored as sensitive (S) if colony growth on media amended with 5 µg ml^{-1} metalaxyl was less than 40% of the isolate growth on non amended media. Moderate isolates (M) exhibited growth on media amended with 5 µg ml^{-1} greater than 40% of that on nonamended media, but growth on media amended with 100 µg ml^{-1} metalaxyl less than 40% of that on nonamended media. Resistant isolates (R) exhibited growth on media amended with 100 µg ml^{-1} greater than 40% of that on nonamended media. The mating type, metalaxyl sensitivity and grouping of isolates based on virulence is presented in table 14.

Table. 14. Characterization of *Phytophthora* isolates collected from tuber crops

Isolate	Mating type	Virulence	Metalaxyl sensitivity
P1	A2	+	S
P2	A2	+	S
P3	A2	+++	S
P4	A2	+++	M
P5	A1	+	S
P6	A1	+++	S
P7	A2	+++	M
P8	A2	--	S
P9	A2	+++	M
P10	Culture lost due to contamination		
P11	A2	--	S
P12	A2	--	S
P13	Culture removed from collection.		
P14	A2	--	S
P15	A2	--	S
P16	A2	+++	S
P17	A2	++	S
P18	A2	++	S
P19	A2	--	M
P20	A2	+	M
P21	A2	+++	S
P22	A2	+++	S
P23	A2	+++	M
P24	A2	+++	S
98-35b	A2	--	R
98-111	A1	--	R
02-04	A1	--	M
98-35a	A1	--	R
02-08	A1	--	S
02-07	A1	--	S
02-03	A1	--	M
02-05	A1	--	M

For virulence, +++ most virulent, ++ moderate, + least virulent and - avirulent. For metalaxyl sensitivity, R - Resistant, M - moderately resistant and S - sensitive

g. Protein profile of *P. colocasiae* at different temperatures

Proteins are produced due to various metabolic activities. Pathogen changes its protein profile when it is exposed to adverse conditions or stress and the proteins are termed as heat shock proteins. Keeping in this mind, an attempt was made to study the protein profile of P. colocasiae at different temperatures. Isolate of *P. colocasiae* (P7) was used in this study. The isolate was grown in defined liquid medium under continuous stirring (50 rpm) at various temperatures viz., 25°C, 30°C and 35°C for seven days. The colourless culture filtrates (100 mL) were filtered through 0.45 µm Millipore filters. The filter sterilized culture filtrate was concentrated by acetone precipitation and dialyzed for 24 h against cold distilled water (Sigma, dialysis bag, molecular cut-off 8000). Electrophoresis under denaturing condition (SDS-PAGE) was performed with 4% acrylamide in the stacking gel and 12% acrylamide in the separating gel. Gels were stained with silver nitrate. Low molecular mark standard proteins (Fermentas) were used for molecular mass determination.

The growth medium used support-

ed the profuse growth of mycelia. By the 7th day mycelial clumps covered the entire base of Erlenmeyer flask. Total protein was isolated from the culture filtrate of isolate P7 grown in defined liquid medium. Acetone precipitation was employed for the isolation of protein from culture filtrate. SDS PAGE fractioned the proteins in to different bands based on their molecular weight. Presence of a unique band was observed in the culture filtrate which was incubated at 35^{0}C (Fig. 15).

h. ITS characterization of *P. colocasiae* isolates

In order to find out the diversity among the isolates and also confirm them at molecular level ITS characterization was performed. The genomic DNA of *P.colocasiae* isolates was obtained by using standardized extraction protocol. The nucleic acid dissolved in TE buffer were treated with 3 µl of RNase A (20 mg ml^{-1}), incubated at 37°C, and stored at –20°C until use. ITS primers 1 and 4, was used in this reaction. The PCR reaction mixtures were heated at an initial step of 94°C for 2 minutes and then subjected to 35 cycles of following programme: 94°C for 30 s, 60°C for 1 minute, 72°C for 1 minute 45 s. After the last cycle temperature was maintained at 72°C for eight minutes. Amplified products were resolved on a 1.5% agarose gel containing 0.5 mg ml^{-1} ethidium bromide and visualized under UV light. The PCR reaction yielded a product of 750 bp. ITS sequencing of the PCR was performed in randomly selected six isolates to confirm them at molecular level (Fig. 16).

i. Isolation of elicitor from *P. colocasiae*

Elicitor from the culture filtrate of *P. colocasiae* (P 7) was isolated and its biological effects on taro plant to induce typical hypersensitive response (HR) were studied. The isolate was grown in defined liquid medium under continuous stirring (50 rpm) at 27^{0}C for seven days.

Fig. 15. Protein profile of *P. colocasiae* grown at different temperatures 1-25^{0}C, 2-30^{0}C 3-35^{0}C and M-Marker

The synthetic media under shaking condition at 50 rpm gave rapid and uniform growth of the mycelia. It was observed that by 8th day mycelia covered the entire surface of the medium.

The colourless culture filtrates (100 mL) were filtered through Whatman No. 1 qualitative filter paper, 1.2-mm (RA) and 0.45-mm (HA) Millipore filters. The filter sterilized culture filtrate was concentrated by ammonium sulphate precipitation and dialyzed for 24 h against cold distilled water (Sigma, dialysis bag, molecular cut-off 8000). Gradient precipitation (0 to 100%) was employed for the purification. Visible pellets were obtained from 60% saturation onwards which became intense reaching 70%. Each saturated fraction was recovered by centrifugation and the pellets were dissolved in 1 ml of sterile water following which they were dialyzed for 24 h against cold distilled water. The dialyzed sample was viscous in nature. The dialyzed sample was loaded on a gel filtration column (Sephadex G-50, Sigma), and equilibrated with sterile distilled water at 4^{0}C.

Electrophoresis under denaturing condition (SDS-PAGE) was performed with 4% acrylamide in the stacking gel and 12% acrylamide in the separating gel. Gels were stained with silver nitrate. Low molecular mark standard proteins (Fermentas) were used for molecular mass determination. Out of the various percentages tried 12% was best suited for crude elicitor protein. The protein profile of the crude protein is depicted in figure 17. Gel filtration chromatography is being standardized in order to isolate the elicitor molecule.

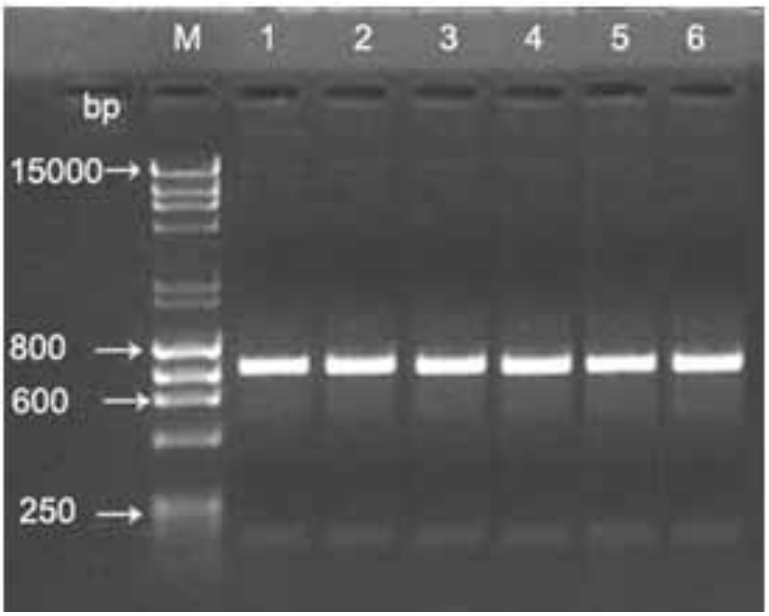

Fig. 16. ITS characterization of *P. colocasiae*
1-P 3, 2 - P 7, 3 - P 8, 4 - P10, 5 - P14, 6 - P16

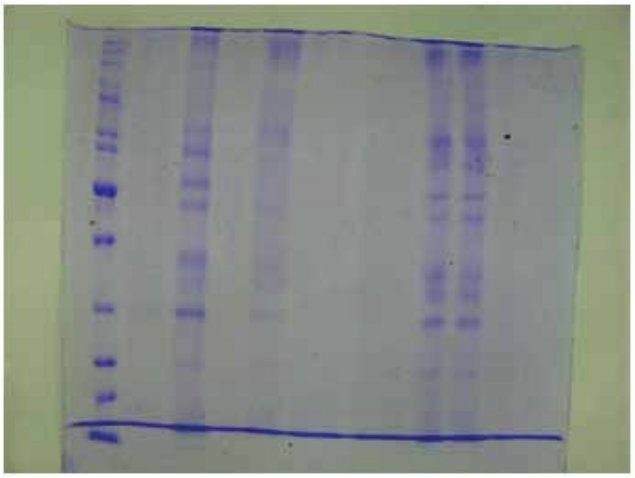

Fig. 17. SDS PAGE profile of crude elicitor fraction

The crude dialyzed fraction was injected into the primary vein of taro leaf for checking the eliciting activity. After 1-2 days the infiltrated areas appeared shiny. After 2-3 days, these zones became necrotic and fluoresced under UV light, suggesting the accumulation of phenolic compounds. The detached leaf assay produced (variety Sree Rashmi and Sree Kiran) produced HR. There was formation of a yellow halo around the inoculated region after 2-3 days. It was observed that the HR did not widen after reaching a particular dimension in the resistant variety. This may be attributed to the natural resistance of the cultivar against the pathogen.

Of the crude filtrate analyzed, the filtrate which was precipitated with 75% of ammonium sulphate gave eliciting activity. Of the types of leaves analyzed, younger leaves of both varieties gave faster eliciting activity followed by mid-aged leaves. However, after 96 h, there was no increase in the eliciting activity. No reaction was observed in mature leaves.

Among the 14 accessions checked, eight of them gave faster response (i.e. yellow halo around the inoculated region) after 24 h itself and the response became stable after 96 h. Three of the accessions gave delayed visible response after 48 h. No response was observed on three accessions.

j. Molecular analysis of *P. colocasiae* isolates

Molecular markers like RAPD and AFLP were employed for studying the diversity in *P. colocasia* isolates. AFLP technique was used for the genetic differentiation of 14 isolates of *P. colocasiae* collected from different region of India by cluster analysis based on allele frequencies of gene loci. A set of eight screened random decamer oligonucleotides primers of RAPD kit T were used for RAPD analysis. Amplified products were resolved on a 2% agarose gel containing 0.5 mg ml^{-1} ethidium bromide and visualized under UV light for scoring for the presence or absence of bands. Analysis of the scored data is in progress.

AFLP analysis of the selectively amplified products is under progress.

2 Disease management

a. Dual culture assay

Ten isolates of *Trichoderma* spp. obtained from culture repository of CTCRI were evaluated *in vitro* as antagonists against isolate of *P. colocasiae*. Dual cultures were carried out by using one-week-old cultures of *P. colocasiae* and *Trichoderma* spp. on PDA. *Trichoderma* isolates demonstrated high antagonistic action to *P. colocasiae*. All isolates of *Trichoderma* grew considerably faster on PDA than the pathogen. Out of the ten isolates evaluated six of them were superior in suppressing the growth of both *P.colocasiae* and *P. palmivora*.

b. Effect of volatile metabolites

Six effective *Trichoderma* selected from dual culture assay was used for the study. The effect of volatile metabolites from *Trichoderma* against *P. colocasiae* was tested. There was no significant inhibition in all tested isolates when compared to control. This suggests that isolates lack or stringently produce effective volatile compounds.

c. Effect of diffusible metabolites

The six effective *Trichoderma* spp. selected from dual culture assay was used for the study. The effect of non-volatile metabolites from *Trichoderma* species against *P. colocasiae* was studied. All of the tested isolates effectively inhibited the growth of the pathogen. Among the isolates tested, T7 showed maximum activity in which no growth was observed.

d. Spore count of the bioformulated biocnotrol agent

The effective biocontrol agent was formulated on talc, vermicompost, talc with wheat bran (5:1) and vermicompost with wheat bran (5:1). The spore count was continued and checked every two months. Among the substrates used, vermicompost with wheat bran had more spore count followed by talc formulation (Table 15).

e. *In vivo* study of *Trichoderma* against *P.colocasiae*

The effective *Trichoderma* strain (T7) obtained from *in vitro* screening was used for the study. The taro corms (variety Sree Rashmi) were raised in the pots enriched with vermicompost. The experiment was carried out in two ways: i) Leaf was inoculated with the pathogen for the disease symptom development and then the effective *Trichoderma* strain (T7) was sprayed over the leaf. Disease severity was monitored and recorded; ii) initially the leaves were sprayed with isolate T7 and left overnight. Then the leaves were inoculated with pathogen and the disease development was censored. For infection, taro leaves of same age were in-

oculated on their abaxial surfaces with 50 µl of spore suspension of *P. colocasiae*. The performance of effective strain of *Trichoderma* obtained by *in vitro* assay showed no correlation with *in vivo* evaluation as it was less effective in controlling the pathogen.

f. Antagonistic activity of *Trichoderma* spp. isolates received from all the centers

The 15 *Trichoderma* isolates (PF1 to PF 15) received were tested for their antagonistic activity against P.colocasiae by dual culture assay as mentioned above. Of the isolates tested, the isolates PF 2, 5 and 12 were more aggressive which entirely overlaid the pathogen. The isolates PF 8 and 12 were least in controlling the pathogen growth.

g. Inhibitory effect of growth regulators on *P. colocasiae*

To study the effect of growth regulators, the mycelial discs (5 mm dia) from 6-day old cultures of *P. colocasiae*/*P. palmivora* were inoculated onto carrot agar plates amended with the various concentrations of growth regulators NAA and IAA (from 0 to 2000 ppm). The plates were incubated at 28^{0}C and the mycelial growth rate was recorded. NAA has completely inhibited the growth of *P. colocasiae* at 1900 ppm, followed by IAA at 2000 ppm. In the case of *P. palmivora*, BAP has completely inhibited the growth of the pathogen at 50 ppm followed by NAA and IAA. There was change in morphology on plates with 1500 ppm of IAA. The present *in vitro* study shows that the growth regulator effectively suppresses the pathogen's growth. The results of the study may be exploited for controlling the pathogen.

h. Screening of fungicides against *P. colocasiae*

A preliminary study was taken up to investigate the effect of various fungicides on growth of *P. colocasiae*, to explore its potential in controlling taro leaf blight disease. The fungicides employed in this study include: Ridomil, Mancozeb (Dithane-45) which are routinely employed and widely accepted by farmers, newly marketed Akoton (Sodium bicarbonate formulation) and Biofight (a synthetic formulation). Suitable dilutions of each chemical were prepared separately with sterile distilled water and incorporated into standardized amount of sterile molten Potato Dextrose Agar (PDA) kept at 45°C grading different concentration range of active ingredients based on preliminary screening of the fungicides. Each plate was inoculated with *P. colocasiae* and was incubated in dark at 28 ±2°C. Mycelial growth on the fungicide-amended media was measured as a percentage against control.

Table 15. Sporulation of *Trichoderma sp* (T7) on different substrates

Substrate	Spore count/ g of substrate			
	6 m	8 m	10 m	12 m
Talc	4 x 10^6	2 x 10^6	2 x 10^6	x 10^5
Vermicompost	9 x 10^7	3 x 10^6	8 X 10^5	x 10^5
Vermicompost + Wheat bran	8 x 10^8	6 x 10^7	7 x 10^7	6 x 10^6

All the fungicides employed significantly inhibited the growth of *P. colocasiae* when compared to control. The inhibitory effect of all fungicides generally increased with increase in concentration. Among the fungicides evaluated, Biofight followed by Dithane were highly efficient in inhibiting the growth of the pathogen. The growth of the pathogen was completely arrested at 1% in the case of Biofight and 1.5% in the case of Dithane. There was moderate rate of inhibition in the lower concentrations used.

The effective inhibitory concentration determined by the *in vitro* study was used for the *in vivo* glasshouse study. The taro corms (variety Sree Reshmi) were raised in pots enriched with vermicompost. The experiment was carried out in two ways: i) Inoculating leaves and spraying the fungicides the effective concentrations once the disease symptoms are expressed; ii) Spraying first with the fungicides and then inoculating with the pathogen. For infection, taro leaves of same age were inoculated on their abaxial surfaces with 50 µl of spore suspension of P. *colocasiae*. In the first experiment, Biofight restricted the spread of the disease. But other fungicides failed to control disease development and the leaves became fully destroyed. While in the second experiment, the fungicide Biofight and Ridomil controlled the development of the disease. Akoton showed less activity in controlling the disease development and the use of raw (without dilution) Akoton caused some blights on the leaves of taro. In summary, the effective concentrations of fungicides determined *in vitro* failed to suppress the growth of the pathogen *in vivo*. Hence, for *in vivo* study, various concentrations of the fungicides have to be further screened in order to determine an effective concentration.

i. Micro propagation of taro for production of healthy planting materials

A simple, economical and rapid multiplication protocol was developed for mass propagation of taro. Four varieties of taro viz. *Colocasia esculenta var*. Muktakeshi, Telia, Sree Resmi, and Sree Kiran were used for the study. The excised sprouting tips were used as the explants. A two-step protocol was executed for the rapid micro propagation of taro. The explants received two subsequent transfers of media, an initial shoot development media followed by root induction media whereby they developed into actively growing plantlets. The basic culture medium used for shoot initiation consisted of MS medium, 30 g l^{-1} sucrose, 7 g l^{-1} agar supplemented with 5 m g^{-1} BA and 1 m g^{-1} NAA. The pH of the medium was adjusted to 5.8 prior to autoclaving. Multiple shoots were obtained after 2 – 3 weeks of culture on the medium. Subculture of these multiple shoots was repeated every 4 weeks interval on the same medium. Following the incubation period the regenerated shoots were aseptically transferred and cultured onto MS basal medium with 30 g l^{-1} sucrose for the induction of roots. They initiated root development after 5-7 days of incubation. After sufficient growth and development of explants into healthy plantlets, they were transferred to greenhouse under 70% shade and planted in disposable cups with potting media (Vermiculite + sand, 1:1) for hardening. Majority of the plantlets survived on the pot mixture.

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1 Biodiversity

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

Periodical survey of different apple orchards located in districts of Kullu, Mandi, Shimla and Kinnaur indicated that the incidence of collar rot disease (*Phytophthora cactorum*) varied between 3.1-72.5, 1.4-26.2, 0.9-12.4 and 0.3-6.4 per cent, respectively (Table 16). Maximum incidence of this disease was observed in apple orchard at Johal in Anni block and Karjan in Naggar block of district Kullu, Ruhmini in Mandi, Tikkar in Shimla and Lippa in Kinnaur districts of Himachal Pradesh. In total 250 soil sample were collected from the different apple orchards infested soil.

Table 16. Status of *Phytophthora* collar rot in major apple growing districts of Himachal Pradesh during 2010

Mandi District		Kullu District		Kinnaur District		Shimla District	
Place	Incidence (%)	Place	Incidence (%)	Place	Incidence (%)	Place	Incidence (%)
Chiuni	9.6	Jan	3.2	Nichar	3.5	Garog	8.7
Jankhari	2.4	Shat	3.4	Garade	4.7	Chathla	8.6
Tehta	4.8	Jallugram	11.8	Chagaon	2.0	Khneti	4.2
Thatchi	7.5	Tepribain	4.8	Urni	4.0	Kyrai	3.8
Bhrayan	6.2	Jibhi	5.1	Kalpa	2.8	Chaman	4.6
Panjain	2.0	Khanag	49.1	Batseri	2.0	Ratnari	5.2
Manjhali Dhar	8.6	Johal	72.5	Kuppa	3.4	Rawalakiar	3.2
Seri	5.9	Chohani	64.0	Kilba-I	3.9	Bhagi	4.4
Bakhrot	2.2	Kungus	14.1	Sangla	4.1	Pujari	8.2
Churag	3.6	Karana	5.5	Shudarag Dakhon	2.6	Tikkar	12.4
Manjholi	5.3	Chowai	8.9.	Pangi	4.0	Surantha	4.4
Kandhi	3.9	Amarbagh	5.2	Kalpa	3.4	Jubbal	4.3
Rohanda	6.5	Dalash	3.3	Duni	2.8	Nihari	3.8
Bagsiad	4.8	Bradha	12.5	Chaunglig	1.7	Kalemo	8.6
Kelodhar	3.1	Raison	3.2	Talangi	0.8	Khanog	5.3
Pendho	3.1	Naggar	10.8	Pooh	0.4	Barthata	8.4
Baddu	7.5	Haripur	4.4	Ribba	1.5	Batargalu	5.5
Ratocha	3.9	Karjan	6.5	Rispa	0.3	Narkanda	4.4
Ruhmini	26.2	Dobhi	3.6	Rarang	1.4	Matiana	5.8
Chhatri	15.5	Banjar	4.8	Jangi	3.5	Kandyali	3.2
Pangana	4.0	Telangi	6.8	Lippa	6.4	Silaru	3.5
Danda Pani	4.2	Kamand	4.5	Giabong	1.3	Laphu gati	6.0
Mahunag	3.6	Dallighrat	5.6	Roggi	3.9	Hulli	5.2
Karsog	7.6	Bhaliayani	8.3	Kilba II	4.9	Kuddu	4.5
Barokhari	4.8	Seobagh	4.0	Bhabanagar	3.6	Aunti	2.6
Kandhi Faliar	8.8	Jari	5.2	Shrabo	1.3	Annu	0.9
Bijhai	4.2	Puud	3.1	Telangi	3.1	Thanedhar	4.5
Panarsa	1.5	Chansari	3.2			Kotgarh	5.7
Takoli	1.4	Summa	8.8			Nankhari	6.4
Kotala	6.8	Badai	3.6			Kotkhai	5.2
Janjelli	7.5	Sainj	5.2			Kufri Baghi	6.4
		Gadsa	5.8			Rohru	2.4
		Barsaini	7.2			Jawanda	4.7

Table 17. Morphological characters of *Phytophthora cactorum* isolates of apple

Isolate	Mycelial character	Size of sporangium (µm)	Size of oospore (µm)	Size of papilla (µm)
PC1	Mycelium hyaline coenocytic, colony whitish, fluffy radiate	35.4-41.5 x19.6-29.4 (37.9-25.2)	23-25	3.2-9.6 x3.2-6.4 (6.8-5.9)
PC2	Mycelium hyaline coenocytic, colony creamish white slightly fluffy, radiate	26.5 -45.1 x19.6-36.2 (33.4x23.8)	22-24	3.2-6.4 x 3.2-6.4 (6.1 x5.9)
PC3	Mycelium hyaline coenocytic, colony whitish, slightly fluffy radiate	28.5 -43.3 x18.3 -31.4 (31.4x22.7)	22-25	3.2-6.4 x3.2x6.4 (5.2x 3.6)
PC4	Mycelium hyaline coenocytic, colony whitish, fluffy radiate	25.5-39.2 x17.6-27.4 (28.6 x21.1)	22-24	3.2-9.6 x3.2-6.4 (6.6 x 4.9)
PC5	Mycelium hyaline coenocytic, colony creamish white, slightly fluffy radiate	24.5-39.6 x 17.6 -29.4 (29.7 x 20.8)	23-25	3.2-6.4 x 3.2-6.4 (6.1 x5.9)
PC6	Mycelium hyaline coenocytic, colony whitish, fluffy radiate	33.1-42.0 x 19.6-27.2 (39.5-22.3)	22-24	3.2-6.4 x 3.3-6.4 (5.8-6.0)
PC7	Mycelium hyaline coenocytic, colony creamish white, slightly fluffy radiate	35.2-41.3 x 19.6-25.2 (38.3 -21.7)	22-24	3.2-6.4 x 3.3- 6.4 (4.5 x4.2)

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

In all, 18 isolates of *Phytophthora* and six isolates of *Pythium* have been isolated from the soil samples collected from Kullu and Mandi apple growing areas. Ten and three isolates have been isolated from apple soil of Shimla and Kinnaur districts, respectively. Seven virulent isolates of collar rot pathogen *P. cactorum*, collected from areas of their maximum incidence, and two *Pythium sp*. (*ultimum*) were identified based on pathogenicity tests. Morphological studies revealed that maximum size of sporangium and width of mycelium were recorded in PC1 followed by PC2 and PC6 (Table 17).

c. Virulence studies

Virulence of the above isolates was tested on a susceptible rootstock MM106 by excised twig method. Isolate PC1 exhibited maximum lesion size (64.6mm) followed by isolate 2 and 6 (Table 18). Virulent strains produced dark brown lesions whereas less virulent ones produced light brown lesions.

Table 18. Virulence of *P. cactorum* isolates to apple root stock MM106 (most susceptible) by excised twig method

Isolate	Average lesion size (mm)			Lesion colour
	3 days	6 days	9 days	
PC1	12.2	29.4	64.6	Dark brown
PC2	10.4	20.6	50.8	Dark brown
PC3	5.3	12.7	32.4	Light brown
PC4	6.8	16.8	38.0	Slightly dark brown
PC5	5.2	11.8	33.4	Light brown
PC6	9.2	27.2	46.8	Slightly dark brown
PC7	5.8	12.4	32.0	Light brown
CD 5%	2.17	4.21	5.72	-

Sporangium of PC6 PC1 Isolate PC2 Isolate

Fig. 18. Sporangial characters of *P. Cactorum* isolates of apple

d. Effect of solid media on growth of *P. cactorum* isolates

Six solid media were evaluated for

their effect on diametric growth of different isolates of target pathogen and also to find out the best basal media for their vegetative growth. The results obtained indicated that oat and cornmeal media supported maximum growth of the isolates of *P. cactorum* (Table 19). Further it was observed that irrespective of medium PC1 resulted in maximum growth and was followed by PC2 and PC6. Apple bark extract was found the least preferred medium by all the isolates.

e. Effect of temperature on the growth of *P. cactorum* isolates

Studies on the effect of different temperature on the vegetative growth of *P. cactorum* isolates indicated that all the isolates of the target pathogen could grow from a temperature ranging between 5^{0}C -35^{0}C. However, maximum mean vegetative growth (66.33 mm) was observed at 25^{0}C. Amongst the isolates tested PC1 gave the maximum diametric growth followed by PC2 and PC6 (Table 20).

f. Effect of pH on growth of *P. cactorum* isolates

Studies on the effect of different pH levels on the vegetative growth of *P. cactorum* isolates indicated that all the isolates could grow from pH ranging between 4.0-8.5, however maximum mean vegetative growth (68.05 mm) was observed at 4.5 (Table 21). Amongst the isolates tested PC1 gave the maximum diametric growth followed by PC2 and PC6.

Table 19. Effect of different solid media on the growth of *Phytophthora cactorum* isolates of apple

pH	Average colony diameter (mm)							Mean
	PC1	PC2	PC3	PC4	PC5	PC6	PC7	
Potato dextrose agar	75.67	64.67	52.33	50.67	51.67	64.00	52.00	58.72
Oat meal agar	77.33	69.00	64.00	62.67	59.67	71.67	60.67	66.43
Corn meal agar	78.67	67.33	52.67	51.33	56.00	66.67	58.00	61.52
Pea meal agar	60.00	63.33	47.33	54.33	53.33	64.33	57.33	57.12
Apple bark extract agar	22.33	32.67	26.67	24.67	31.67	34.33	28.33	28.67
Czapeck-Dox agar	58.67	48.67	40.67	43.67	39.00	46.00	42.67	45.61

CD at 5% Medium = 5.67 Isolates = 4.21 Temp. x Isolates = 9.64

Table 20. Effect of temperature on the growth of *Phytophthora cactorum* isolates of apple

Temp. (°C)	Average colony diameter (mm)							Mean
	PC1	PC2	PC3	PC4	PC5	PC6	PC7	
5	7.00	7.00	7.00	6.67	7.00	7.00	7.00	6.95
10	21.33	17.67	15.67	18.33	14.67	20.67	14.33	17.52
15	35.67	31.67	31.33	32.33	25.33	33.67	26.67	30.95
20	63.33	56.33	48.67	49.00	42.67	54.67	45.33	51.42
25	88.33	71.67	67.00	61.33	51.67	70.33	54.00	66.33
30	36.67	32.00	28.67	32.00	26.00	34.67	27.33	31.05
35	6.67	6.67	7.00	7.00	6.67	6.67	6.67	6.76

CD at 5% Temperature = 0.54 Isolates = 0.45 Temp. x Isolates = 1.32

Table 21. Effect of pH on the growth of *Phytophthora cactorum* isolates of apple

pH	Average Colony diameter (mm)							Mean
	PC1	PC2	PC3	PC4	PC5	PC6	PC7	
4.0	52.00	47.67	41.67	41.33	41.67	46.00	42.33	44.66
4.5	76.33	72.00	67.33	63.33	62.67	70.33	64.67	68.05
5.0	70.67	66.67	60.33	56.67	55.33	66.00	59.00	62.08
5.5	68.33	61.00	51.67	48.33	47.67	60.33	50.67	55.40
6.0	57.00	48.33	42.00	39.67	38.00	47.67	40.33	44.71
6.5	47.67	42.67	37.67	35.33	32.00	40.33	36.67	38.91
7.0	42.00	37.00	29.33	25.00	25.33	35.00	27.33	31.56
7.5	38.33	31.33	20.67	18.67	16.33	29.67	17.67	24.66
8.0	23.67	17.33	12.00	11.33	10.00	15.67	11.33	14.47
8.5	9.33	8.33	7.67	7.33	6.67	8.00	7.00	7.75

CD at 5% Temperature = 0.96 Isolates = 0.69 Temp. x Isolates = 2.14

2 Epidemiology

a. Effect of temperature

Inoculations with *P. cactorum* was made on a highly susceptible rootstock MM106 by excised twig method and the Petri-plates containing excised twigs inoculated were incubated at 5, 10, 15, 20, 25 and 30^{0}C. Each treatment was replicated thrice and observations regarding incubation period and lesion colour were recorded periodically. The study showed that temperature has a significant role to play in the disease development as incubation period and the lesion colour varied with temperature. A temperature of 25^{0}C was found to be optimum for the development of collar rot as the pathogen produced the disease symptoms within three days after inoculation, while at 5^{0}C, the disease appeared after 18 days.

b. Effect of soil moisture

In order to study the effect of soil moisture on the development of collar rot disease, one year old plant of susceptible apple rootstock, MM106 planted in earthen pots were inoculated with the virulent isolate (PC1) of *P. cactorum* at 50, 60, 70, 80 and 90% soil moisture levels and data were recorded after 90 days of inoculation. The study indicated that maximum incidence and severity of disease was observed at 90% soil moisture and minimum at 50% moisture level.

c. Effect of pH

The effect of pH regimes (5.0-7.0) was studied on the development of collar rot disease on the susceptible apple rootstocks MM106. The plants were inoculated with the virulent isolate PC1. The data were recorded after 80 days of inoculation which indicated that soil pH at 5.0 was highly conducive for the development of disease whereas pH around neutral gave inhibitory effects to the development of collar rot disease.

d. Effect of soil type

The effect of soil type was studied on the incidence and severity of collar rot disease in pot culture on susceptible rootstocks (MM106). The plants were inoculated with a virulent strain by adding the inoculum @ 2% (w/w). The data was recorded periodically for 80 days. The experiment proved that clay type soil was highly conducive for the development of disease whereas sandy loam (gravelly) gave inhibitory effects to the development of collar rot disease.

e. Studies on rhizosphere microflora

Fungal and bacterial microflora from the soil samples collected from healthy and diseased areas were estimated by using Rose Bengal supplemented PDA/malt extract and nutrient agar medium, respectively. Results obtained indicated that *Phytophthora* containing soil sample had low population of *Trichoderma species*, *Penicillium* species and bacterial species whereas, *Aspergillus* species and *Pythium* spp. were present in higher level and reverse was true for non *Phytophthora* soils.

3 Host resistance

a. Screening of rootstocks

Ten root stocks were screened by the excised twig method and the results obtained after six days of inoculation indicated that M26, M9, *Malus prunifolia*, and *Malus floribunda* as highly resistant with 2.42, 2.20, 2.74, and 3.78 mm, respectively. Rootstocks MM106 (29.2 mm) and MM104 (27.50 mm) were highly susceptible. The same rootstocks were screened under pot conditions and the data were recorded periodically up to three months following a 0-5 disease rating scale. Based on percent disease index (PDI) calculated, M9, *Malus prunifolia*, M26 and *Malus floribundas* were recorded as highly resistant with PDI of 1.42, 1.06, 2.63 and 2.34 per cent, respectively. Rootstocks MM106 (PDI= 71.6) and MM104 (PDI=62.6) were highly susceptible after three months.

In a separate experiment, these rootstocks were also planted in the sick plots during Feb. 2010 for their evaluation. Additional inoculum of target pathogen was added @ 60 g/ bed (1 m^2) in the month of April 2010. Observations on disease severity were recorded periodically up to Nov. 2010 and PDI was calculated for each rootstock. Data obtained indicated that only M9 and *Malus prunifolia* behaved as highly resistant with PDI of 1.31 and 1.65%, respectively whereas MM106 and MM104 were highly susceptible (Table 22).

b. Screening of apple cultivars

Twenty different cultivars were

Table 22. Reaction of apple rootstocks against *Phytophthora cactorum* under pot and field (nursery) conditions

Apple rootstock	Disease index (%)		Reaction
	Pot conditions	Field conditions	
Malus floribunda	2.34	5.26	R
M. prunifolia	1.06	1.31	HR
M4	7.22	7.75	R
M7	21.36	14.55	MR
M9	1.42	1.65	HR
M26	2.63	6.71	R
MM104	62.61	56.32	HS
MM106	71.68	65.58	HS
MM111	26.36	14.34	MR
MM115	14.37	8.24	R
CD (P=0.05)	2.17	3.52	-

HR= Highly resistant, R= Resistant, M=Moderately resistant, S= susceptible, HS= Highly susceptible

screened against *P. cactorum* by following the excised twig inoculation method at pink bud stage during 2010. The study indicated that cv. Vance Delicious was highly resistant (lesion size 5.0 mm), Royal Delicious gave moderately resistant reaction (8.66 mm) whereas Wellspur (32.33 mm) and Starking Delicious (29.67 mm) were highly susceptible.

Seeds of ten pollinizer cultivars viz., Golden Delicious, Red Gold, Red Fuji, Scarlet Gala, Tydeman,s Early Worcester, Gold spur, Mollies Delicious, Granny Smith, Commercial and winter Delicious were collected, stratified and sown in the pot containing sterile soil. The inoculum of the target pathogen was added @2% (w/w) at four leaf stage. The results obtained indicated that winter delicious saplings showed maximum tolerance as minimum mortality (20.6%) was observed. It was followed by Golden Delicious and Mollies Delicious.

4 Disease management

a Evaluation of fungal biocontrol agents

A pot study to evaluate fungal antagonists effective under *in vitro* conditions indicated that addition of *T harzianum-15* (cfu 5.2x10^4 /ml; 50 ml/pot and 50 g of *Trichoderma* culture grown on wheat bran) provided 82.1, 71.5 and 62.6% control when applied 20, 10 and 0 days prior to inoculation of pathogen @1% (w/w) (Table 23). The seedlings were also dipped in a suspension of biocontrol agents (cfu 5.2x10^4/ml) for 15 minutes prior to planting in pots. Further eight effective (moderate to high) fungal BCAs were evaluated in sick plots in a nursery by adding their bran culture @ 150 g/ m2. One-year-old apple seedlings/grafted plants (15 Nos.) were planted in each plot in the third week of March after dipping (upto collar portion) in talc based spore suspension (2.4x10^6 cfu/ml) for 15 minutes. The talc based formulation (2.4x10^6 cfu/ml) of respective fungal antagonists was also added in soil @ 0.2% /m2 (10 g in 5 liters of water) in the last week of April. Additional inoculum of the collar rot pathogen was also incorporated @ 60 g/m^2 in the first week of April to maintain the desirable inoculum threshold level. Data on disease severity were recorded periodically using a 0-5 disease rating scale. Final observations were recorded in the month of December 2010 by uprooting the plants. *T. harzianum-15*, *T. longibrachiatum*; *T. viride-23* and *Penicillium funiculosum* were found individually highly effective and gave disease control of 76.1, 75.6, 74.8 and 70.2 per cent under sick plots. In naturally infested soil, similar trend with slightly higher control was obtained, which was to the tune of 80.2, 82.4, 79.3 and 74.1, respectively.

b. Evaluation of bacterial biocontrol agents

In vitro evaluation of bacterial antagonists against the target pathogen indicated that *Bacillus sp*. 3, 11, 21, *Enterobacter aerogenes* (obtained from IMTECH- Chandigarh) and *Pseudomonas sp*. 6 and 14 were effective (Table 24). Further, pot culture evaluation indicated that *Bacillus sp*. 11 followed by *E. aerogenes*, *Bacillus sp*. 3 and *Pseudomonas sp*. were highly effective when added (cfu 108 /ml @ 25ml/ pot) seven days prior to addition of the pathogen @1% (w/w). Similar experiments were carried out in nursery for evaluation of *in vitro* effective bacterial isolates. These were multiplied on coconut coir under laboratory conditions and then separately added @ 150 g/m^2 in sick plots (1x1m size) during first week of March. One-year-old apple seedlings/ grafted plants on seedlings (20 No.) were planted in each plot during third week of March after dipping (upto collar portion) in cell suspension (9x10^9 cfu/ml) of each bacterial antagonist for 15 minutes. The talc based formulation (1.6 x 10^{12} cfu/ml) of

Table 23. Evaluation of fungal antagonists against collar rot pathogens of apple in sick pot conditions

Fungal antagonist	Disease control (%)				
	In pots			In field	
	20	10	0*	Artificiallys-ickened soil	Naturally sick soil
T. harzianum - 6	74.1	60.2	51.4	64.2	69.1
T. harzianum -15	82.1	71.5	62.6	76.1	80.2
T. virens -2	73.6	65.2	50.1	65.2	70.2
T. longibrachiatum	80.8	65.3	58.5	75.6	82.4
T. hematum	73.1	64.8	50.5	61.2	68.2
T. viride -5	72.1	61.2	52.7	60.5	67.9
T. viride - 23	80.3	67.1	55.2	74.8	79.3
Penicillium funiculosum	76.2	65.1	52.3	70.2	74.1

* Days prior to pathogen inoculation
(0 days means that both biocontrol agent and pathogen were added simultaneously).

Table 24. Evaluation of bacterial antagonists against collar rot pathogen of apple under *in vitro* and pot conditions

Fungal antagonist	Growth inhibition (%) of pathogen		Mean	Disease control (%)		
	Dual culture method	Culture filtrate method		Pot condi-tions	Artificially sickened soil	Naturally sick soil
Bacillus sp -3	65.1	66.5	65.8	69.2	62.4	65.8
Bacillus sp -11	74.5	75.7	75.1	78.2	70.6	77.6
Bacillus sp-21	62.3	63.1	62.7	60.1	60.1	64.9
Enterobacter aerogenes	70.2	71.4	70.8	74.2	69.8	75.3
Pseudomonas sp -6	69.2	67.2	68.2	66.5	56.7	60.2
Pseudomonas sp-14	61.8	62.4	62.1	52.2	55.2	63.5
CD at 5 % level	0.93	0.78	0.86	2.96	3.05	2.67

Table 25. Effect of combined application of biocontrol agents and effective fungicides against collar rot (*P. cactorum*) disease in apple under pot culture conditions.

Treatment	Conc. (%)	Disease control (%) after 45 days of inoculation			
		7 days before	0 day**	7 days after	12 days after
Trichoderma viride-5 (TV 5)	2% (bran* culture) + 0.2% (talc based culture)=A	79.6	68.2	58.5	41.2
Bacillus sp.-11 (BS-11)	2% (coconut coir culture) 0.2% (talc based culture)=B	86.5	78.8	70.2	58.5
Mancozeb	0.3	75.6	67.2	59.1	42.3
Mancozeb	0.4	89.5	79.9	66.2	50.8
TV5+ mancozeb	A+0.3	88.4	78.5	64.3	49.8
TV5+ mancozeb	A+0.4	94.6	82.6	69.6	56.0
BS11+ mancozeb	B+0.3	92.5	84.1	75.0	62.5
BS11+ mancozeb	B+0.4	98.4	89.6	79.8	69.8
TV5 + BS 11 + mancozeb	A+B+0.3	96.9	88.1	80.4	68.1
TV 5 + BS11 + mancozeb	A+B+0.4	100.0	91.2	85.5	74.3
TV 5 + BS 11	A+B	88.9	82.1	74.3	61.2
Control	-	1.6	0.6	1.2	1.4
CD at 5% level	-	2.75	2.96	2.67	3.19

*TV 5 cfu/g = 2.5 x 106 in talc based culture; TV 5 cfu/g = 2.0 x 103 in bran based culture; BS11 cfu/g = 9.1 x 109 in coconut coir based culture; BS11 cfu/g = 9.1 x 109 in talc based culture; ** = target pathogen and treatments were added simultaneously

each bacterial antagonist was also added @ 10 g/m^2 in the last week of April. Additional inocula of collar rot pathogen were also put @ 60 g/m^2 in the first week of April to maintain the desired inoculum threshold level. Data on disease severity were recorded periodically using a 0-5 disease rating scale. Final data were recorded in the month of December 2010 by uprooting the plants. Perusal of data (Table 24) indicated that addition of *B. subtilis-11* was recorded as highly effective in controlling collar rot disease under nursery conditions and provided 70.6% disease control. It was followed by *E. aerogenes* (69.8%), *Pseudomonas sp*. 6 (66.7%) and *B. subtilis -3* (62.4%) in order. In naturally infested soil, similar trend with slightly higher disease control was obtained, which was to the tune of 77.6, 75.3, 70.2 and 65.8%, respectively (Table 24). *Pseudomonas sp*. 6 and 14 were less effective under filed conditions.

c. Studies on fungicide compatibility with effective BCAs

Twelve and six biocontrol agents (BCAs) of fungal and bacterial origin, respectively were screened for their compatibility with two more commonly used fungicides viz., mancozeb and metalaxyl MZ under laboratory conditions by adopting food poisoning technique. Results obtained indicated that most of the BCAs of fungal origin showed complete incompatibility with these two fungicides except *T. viride 5*, and *T. harzianum 15* and T. *longibrachiatum*. The above three BCAs were grown repeatedly for six times on the potato-dextrose agar medium poisoned with target fungicides (mancozeb, metalaxyl MZ) at an higher concentrations (2500-3000 ppm and 500-1000 ppm), which resulted in further growth inhibition.

Two biocontrol agents (BCAs) viz., *Trichoderma viride-5* (TV5) and *Bacillus sp.-11* (BS-11) were added individually @ 2% bran/coconut coir culture + 0.2% talc based formulation as well as in combination with mancozeb (0.3, 0.4%) seven days prior to inoculation, simultaneously at the time of inoculation, and also 7 and 12 days of inoculation with target pathogen. 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). The data on per cent disease control was recorded after 45 days of inoculation (Table 25). Perusal of data indicated that pre-inoculation treatments with either BCAs or mancozeb exhibited more disease control in comparison to their post inoculation applications. Addition of mancozeb at higher concentration (0.4%) gave enhanced disease control (> 18-28%). Further the combined application of TV5 and mancozeb at both the concentration provided less disease control in comparison to combined addition of BS-11 and mancozeb at all the duration as mentioned above (Table 25).

A similar trial was conducted under field conditions in the nursery (sick plots). Fifteen apple seedlings were planted in each plot of 1m^2 and each treatment was replicated three times. Data on disease mortality was recorded periodically and final data on disease severity by following 0-5 disease rating scale was recorded in the month of Dec. 2010 by uprooting the plants. Under field conditions a slightly low disease control was exhibited compared to results obtained under pot conditions (Table 26). Pre-plant application of a combination of TV5, BS11 and mancozeb (0.4%) was highly effective to control collar rot infection. '

d. Evaluation of soil amendments, fertilizers, non-host crops, and bio-fumigation against targe pathogens under pot and field conditions

Soil amendments: Fifteen different amendments (plant leaves/ seeds, cakes) were evaluated

against the target disease under pot conditions. The amendments were added in the sick soil @1.5 per cent (w/w) seven days before planting the apple seedlings during the month of March 2010. Disease incidence was recorded periodically upto four months. The results obtained indicated that addition of dried leaves of *Vitex negundo*, *Murraya koningii*, tirmira, castor, *Lantana*, both dried leaves and seeds of *Melia azedarach*, mustard leaves cakes of mustard and neem were found individually effective in controlling the disease (Table 27). Above soil amendments comprising dried leaves of plants (5 No.), seeds of *M.azedarach*, cakes of mustard, neem and cotton were added separately @200 g/m^2 in the already made sick plots seven days before planting of one year old seedlings in the first week of March 2010. Disease incidence was recorded periodically and final data was taken in the month of Dec. 2010 by uprooting the plants. Amendment with cakes of mustard, leaves of *V. negundo* and seeds of *M. azedarach* were found quite effective.

Fertilizers: Evaluation of fourteen different chemicals/ fertilizers after their addition in sick soil under pot conditions indicated that phosphate (super phosphate) and sulphate (calcium sulphate, potassium sulphate, magnesium sulphate) and carbonate (copper carbonate and calcium carbonate) containing chemicals applied/ added @3 g/Kg soil and borax @2 g/Kg soil were effective to some extent and provided 62.5, 52.1, 48.2, 42.6, 58.2, 47.3, 45.2 per cent disease control, respectively. Nitrate fertilizers viz. calcium ammonium nitrate, potassium nitrate added @ 4 g/Kg soil remained ineffective.

Non-host crops: Plants of six non-host crops viz., maize, beans, cabbage, chillies, tegetes and squash were grown separately in the pots containing sick soil during Kharif season 2009 whereas, onion, garlic, mustard, cauliflower, peas and wheat were grown during Rabi season 2009-10. Stratified seeds of apple were sown in these pots during Sept. 2009 and March 2010. The data on disease severity were recorded periodically upto 45 days. The pots where mustard, tagetes and onion were planted yielded disease control upto an extent of 61.4, 57.2, 53.2 per cent, respectively (Table 28). Plants of twelve non-host crops as above were grown separately in sick plots during 2009-10. One-year-old apple seedlings were planted in these plots during the month of March 2010. In plots where mustard and tagetes were grown, disease control up to 60.3 and 54.2 per cent, respectively, was recorded.

Bio-fumigation (mulching whole plant): Bio-fumigation of sick soil in pots was done by mulching whole plant of six crops viz., mustard, tagetes, taramira (*Brassica juncea*), peas, cabbage and turnip @20 per cent (w/w) during January 2010 and one-year old plants of susceptible rootstock MM 106 (2No.) were planted in such treated pots during March 2010. Data on disease severity was recorded periodically and final data was recorded after 90 days. Bio-fumigation with mustard; taramira and tagetes provided 80.2, 75.4 and 70.2 PDC, respectively (Table 29). Bio-fumigation of sick soil was done by mulching it with whole plant of six crops viz., mustard, tagetes, taramira, peas, cabbage, and turnip during 2009-10. Planting of one year old apple seedlings in the treated plots during March 2010 revealed that bio-fumigation with mustard, taramira, tagetes and turnip was effective.

e. Effect of soil solarization on disease control

To study the effect of soil solarization, transparent polyethylene sheets of different thickness i.e. 25, 50 and 75 µm was separately spread over the naturally infested soil and already sickened plots at HRS-Seobagh during the month of June - August for 45, 60 and 75 days during 2009. The plots were well irrigated before spreading polyethylene sheet to conserve the moisture as well as to get higher temperature under the sheet. The soil from the already sickened and solarized plots was also used for pot experiments. For this one-year old apple plants were planted in pots (15 cm dia). These were then kept in a polyhouse for infection to occur. Data on collar rot incidence were recorded after every 15 days upto 60 days. Stratified seeds of apple were also sown in pots (25 cm dia) having solarized sick soil

Table 26. Effect of combined application of biocontrol agents and effective fungicides against collar rot (P. cactorum) disease in apple under field conditions in the nursery

Treatment	Conc. (%)	Disease control (%) after 45 days of inoculation			
		7 days before	0 day**	7 days after	12 days after
Trichoderma viride-5 (TV 5)	150g bran* culture + 50 g talc based culture=A	74.6	64.2	55.5	39.2
Bacillus sp.-11 (BS-11)	150 g coconut coir culture + 50g talc based culture= B	76.5	70.8	65.2	51.5
Mancozeb	0.3	69.6	60.2	52.1	40.3
Mancozeb	0.4	72.5	69.9	58.2	45.8
TV5+ mancozeb	A+0.3	80.4	70.5	61.3	43.8
TV5+ mancozeb	A+0.4	88.6	75.6	64.6	55.0
BS11+ mancozeb	B+0.3	78.5	71.1	62.0	53.5
BS11+ mancozeb	B+0.4	87.4	81.6	72.8	60.8
TV5 + BS 11 + mancozeb	A+B+0.3	84.9	78.1	72.4	64.1
TV 5 + BS11 + mancozeb	A+B+0.4	95.2	87.2	79.5	74.3
TV 5 + BS 11	A+B	80.9	71.1	60.3	57.2
Control	-	1.2	0.4	1.8	0.5
CD at 5% level	-	3.72	3.08	2.71	3.27

during the month of September and data on seedling mortality were also recorded. In another experiment, one year old seedlings of apple were also planted during Feb. 2010 in sterilized soil to study the impact of this non-chemical method of disease control. Data on disease severity of target disease were recorded periodically and disease control was calculated for each treatment. Data on the increase in soil temperature at different soil depths and prevention of loss of soil moisture (%) both with and without polyethylene sheets were recorded and correlated with disease development. Bits of actively growing culture of individual target pathogens were kept at different soil depths (5-30 cm) to study the effect of solarization on eradication of pathogen.

Planting of apple seedlings in the pots containing solarized (60 days below 25 µm thick sheet) sick soil of target pathogens provided 88.3% control of collar rot disease. Similarly raising of apple seedlings from stratified seeds in the pots containing solarized sick soil gave 89.6% control of collar rot. Results of the study indicated that spreading of polythene sheet of 25 µm thickness for two months duration (June 1 – July 30) was significantly superior over 50 µm and 75 µm thickness sheets. It was further observed that soil solarization for 60 days (June 1-July 30) was superior to 45 days and at par with 75 days; and provided 93.6 and 90.6% control of collar rot pathogen in naturally and artificially infested solarized soil under 25 µm sheet thickness.

Recording of data on the increase in soil temperature at different soil depths and prevention of loss of soil moisture (%) both with and without polyethylene sheet revealed an increase in temperature ranging between 9.1-13.9^{0}C and prevented the soil moisture loss up to an extent of 51.8-63.8%. The propagules/ mycelium of the target pathogen kept upto 25 cm soil depth (5-30 cm) got killed on solarization.

Table 27. Effect of soil amendments in controlling the collar rot disease under pot conditions

Crop	Disease control (%)	
	Pot conditions	Field conditions
Vitex negundo	78.4	72.2
Murraya koningii	71.2	61.2
Lantana camara	64.1	60.9
Melia azedarach (leaves)	68.6	62.1
Melia azedarach (seeds))	75.1	70.1
Castor	69.4	62.4
Tirmira (*Xanthoxyllum esculantum*)	68.2	61.2
Artemisia vulgaris	48.2	38.3
Soap nut	62.1	58.2
Mustard leaf	66.2	60.2
Mustard cakes	75.5	72.4
Cotton cakes	60.1	56.1
Sunflower cakes	56.4	52.5
Neem cakes	69.0	62.2
Soybean cakes	54. 3	48.4
CD (P=0.05)	2.48	3.76

Table 28. Effect of different non host crops against collar rot pathogen of apple under pot and field (sick plot) conditions

Crop	Disease control (%)	
	Pot conditions	Field conditions
Maize	41.2	38.6
Beans	12.5	12.9
Cabbage	50.7	44.6
Chillies	34.0	21.8
Tegetes	57.2	54.2
Squash	18.4	8.6
Onion	53.2	48.6
Mustard	61.4	60.3
Cauliflower	48.5	41.0
Peas	34.2	22.8
Wheat	40.5	36.2
Garlic	46.1	40.1
CD (P=0.05)	2.48	2.96

Table 29. Effect of different non host crops against collar rot pathogen of apple under pot and field (sick plot) conditions

Crop	Disease control (%)	
	Pot conditions	Field conditions
Mustard	80.2	73.1
Taramira	75.4	64.1
Peas	44.1	39.0
Cabbage	62.3	56.4
Tegetes	70.2	60.2
Turnip	68.4	57.6
CD (P=0.05)	1.84	3.72

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Dr. RAM DUTTA

1 Biodiversity

A survey was taken up in government orchards and progressive farmers' gardens of Meghalaya, Assam, Arunachal Pradesh and Sikkim. Information related to crop loss was collected based on farmers' opinion as well as onsite observations of the affected orchards. Disease samples were collected and used for isolation of pathogen and bio-agents. Thirtyfive (cumulative) isolates of *Phytophthora* were isolated and preserved from citrus (Mandarin, Rough lemon, Pummelo) so far. Based on morphological studies of sporangia, branching habit of sporangiophores and colony characters, most of the isolates resembled characters of *P. citrophthora* and *P. palmivora*. In the PCR based identification using (5'-GTCGACGTCCTGCTTGGCACTCTG-3'), (5'-CGGTGCTCCGCGACTGTTGTCCAC-3') primers, seven isolates from Arunachal Pradesh were confirmed as *P. citrophthora*. Identity of the remaining isolates is being confirmed with different species specific primers.

2 Diagnostics

Pathogenicity of the above isolates is being tested using potted Mandarin seedlings. Root rot infection ratings based on a 10 point scale are being recorded. The pathogenicity of 16 isolates was confirmed, which produced the specific symptoms of disease. The rest of the isolates have been inoculated on fresh seedlings to check their pathogenicity.

3 Disease management

Fungal BCAs were isolated on *Trichoderma* semi-selective medium from healthy citrus soils. So far seven *Trichoderma* spp. could be collected and identified as *T. virens*, *T. viride*, *T. harzianum*, *T. longibrachiatum*, *T. crassum*, *T. ressei* and *T. pseudokoningii*. Bio-efficacy test under protected conditions is yet to be taken up.

NATIONAL BUREAU OF AGRICULTURALLY IMPORTANT INSECTS, BANGALORE

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1 Disease management

a. Studies on elicitors from *Trichoderma* spp.

Isolation, purification and characterization: After initial screening of 65 isolates of *Trichoderma* that elicit ISR in chilli plants, 10 isolates were selected for further studies. Selected isolates are Th-9, Th-10, Th-16, Th-19, Tv-10, Tv-3o, Tv-115, Tvs-5, Tvs-7 and Tvs-8 (Th - *T. harzianum*, Tv - *T. viride*, Tvs - *T. virens*). *Trichoderma* cultures were grown on PDB at ambient temperature for 10 days. The mycelial mat was harvested and rinsed with sterile water for several times. The mycelia were resuspended in sterile water (five ml g^{-1} of mycelia) and blended with homogenizer at full speed for two minutes. The mycellial slurry was filtered through two layers of muslin cloth and the residue obtained on muslin cloth was homogenized three more times in water followed by once in a mixture of chloroform and methanol (1:1) and finally in acetone. This preparation was air dried and the fraction was condensed as mycellial walls. Elicitor was extracted from mycellial walls by suspending one gram walls in 100 ml of distilled water and autoclaving 20 min. The autoclaved suspension was filtered, clarified by centrifugation and concentrated to 10 ml under reduced pressure.

Evaluation of elicitors: A greenhouse bioassay was carried out using 30 days old chilli (Byadagi variety) seedlings to study the efficacy of purified elicitors. For pathogen inoculation sporangia ($5x10^5$) of *P. capsici* 06-16 (IISR, Calicut) was used. Pathogen was inoculated after three days of elicitor treatment. Treatment with elicitors of Th 9, Th 10, Th 16 and Th 19 provided 60-70% reduction in *Phytophthora* incidence in chilli plants. High concentration of elicitor in Th 9 and Th 10 caused yellowing of leaves.

b. Estimation of carbohydrate content in the elicitors

The cell wall preparations obtained from *Trichoderma* spp. were analysed for the carbohydrate content by DNS method. The carbohydrate content in terms of glucose equivalents is given in Fig. 18. The isolate Th 16 had high glucose content (60 mg/ml) compared to other isolates. Glucose content in other isolates varied between 20-40 mg/ml (Fig.19) Standardization of quantum of elicitor application to the plants: For standardization of treatment of plants with cell wall elicitor preparation one ml of elicitor with above mentioned glucose equivalent content was used for seedling dip method of application. Chilli se edlings were treated for 30 s with the elicitor preparations. No hypersensitive reaction or stunting of growth was observed in plants treated with elicitors of

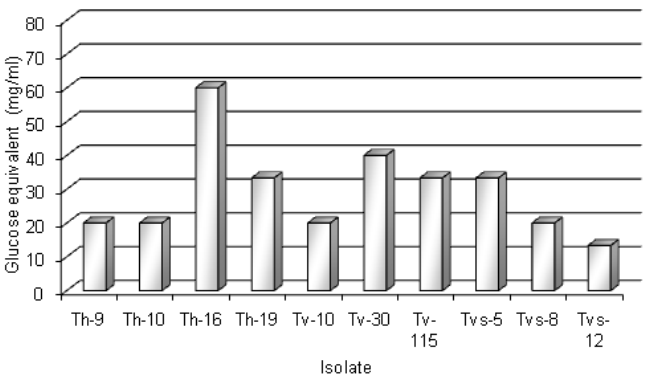

Fig. 19. The carbohydrate content in elicitors of different isolates of *Trichoderma* spp.

Th-9, Th-10, Th-16, Th-19, Tv-10, Tvs-5 and Tvs-8. But in plants treated with Tvs-7 and Tv-30, yellowing was observed.

PCR amplification of elicitors: Sm1 (Small protein 1), a novel proteinaceous nonenzymatic elicitor secreted by *T. virens*, efficiently elicit plant defense response and systemic resistance against a foliar pathogen of dicot cotton (*Gossypium hirsutum*). The protective activity of Sm1 was associated with the accumulation of reactive oxygen species and phenolic compounds, and increased levels of transcription of the defense genes regulated by SA and JA/ET as well as genes involved in the biosynthesis of sesquinterpenoid phytoalexins. For selected isolates of *Trichoderma* (Th-9, Th-10, Th-16, Th-19, Tv-10, Tv-30, Th-115, Tvs-5, Tvs-8 and Tvs-12) that elicited ISR in chilli plants, amplification of Sm1 gene was carried out using specific primers SmF-(5'-GTCTCCTACGACACCGGCTA-3') and SmR (5'-GTCGAG CGCAATG TTGAA-3'). The optimum conditions for PCR included, initial lid heating or hot start at 94°C for 5 min followed by 28 cycles each consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 20 seconds, extension at 72°C for 20 seconds and then a final extension of 72°C for 5 min. DNA amplification was done with 11 isolates. The amplified products were checked on 1.4% agarose gel out of which three isolates showed amplification at 264 bp (Fig. 20). The PCR product was sequenced (Ocimum Pvt. Ltd) and were compared using NCBI blast programme. Nucleic acid sequences of Sm1 gene was matching with Sm1 protein of *T. virens* (DQ121133.1). Sm1 amplification was observed in *T. virens* isolates TVS-5, TVS-8 and TVS-12.

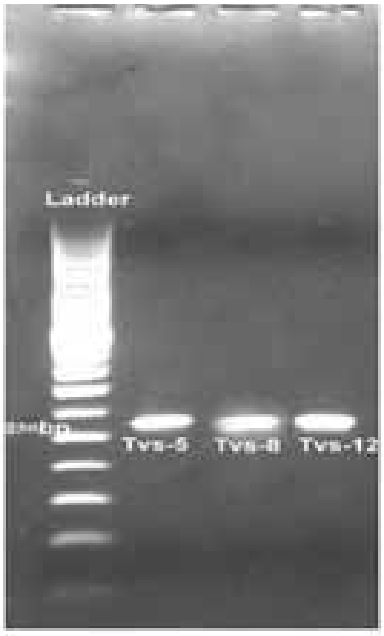

Fig. 20. Sm1 gene amplification in *Trichoderma virens* isolates

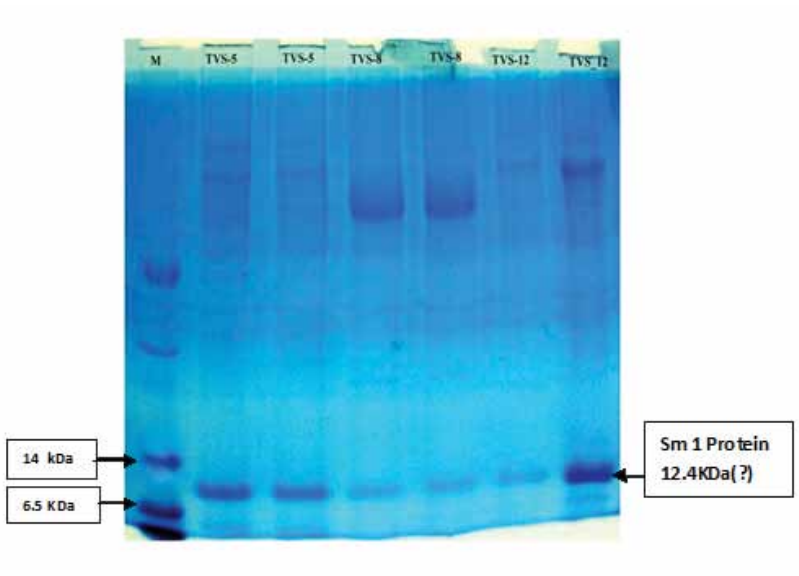

Fig. 21: SDS-PAGE Coomassie Brilliant Blue R-250 staining for Sm1 protein detection. Lane1-Marker, Lane2-Tvs5 (1stelute), Lane3-Tvs5 (2ndelute), Lane4-Tvs8 (1stelute), Lane5-Tvs8 (2ndelute), Lane6-Tvs12 (2ndelute), Lane7-Tvs12 (1stelute).

Isolation, purification and identification of Sm1 protein: Sm1 protein was isolated from three T. virens isolates –NBAII TVS 5, NBAII TVS 8 and NABII TVS 12 were used in this study. The broth cultures of these isolates were prepared by inoculating a spore suspension of $2x10^6$ spores / ml in 300 ml of Vogels minimal media and incubating on a rotary shaker at 150 rpm for 5 days at 27°C. After incubation mycelial biomass was filtered through muslin cloth and again filtered through 1 µm filter paper. Proteins were precipitated by 80% ammomium sulphate at 4°C and centrifuged at 10,000 rpm for 30 min at 4°C. Pellets were resuspended in small amounts of 10 mM Tris, pH 7.8 and dialysed against same buffer using 8KDa cutoff dialysis membrane for two days. Protein concentration was determined by Lowry's method using BSA as a standard. For SDS-PAGE 15% gel was used and 50 µg protein was loaded in each well and electrophoresis was done at 100V. On staining with Coomassie brilliant blue R-250, low molecular weight protein bands were observed between 6.5 kDa and 14 kDa in TVS-5 and TVS-12 and TVS-8 (Fig. 21).

b. Biochemical analysis of plants treated with elicitors from *Trichoderma*

Samples from the pot culture experiments were used for the biochemical assays. For ISR studies 1, 7, 14, 21, and 28 day-old plant

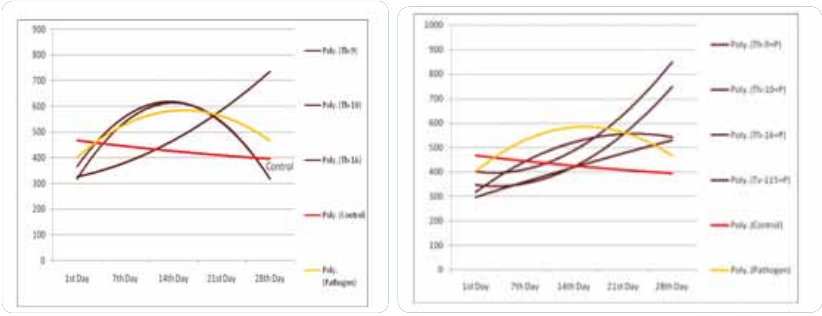

Fig. 22. Phenol content in plants treated with elicitor (µg/g of tissue)

material was used. Leaf and stem samples were collected for enzyme assays of peroxidase, polyphenol oxidase, PAL, total proteins and total phenols. Colorimetric assays were carried out to assay these defense related enzymes and compounds. Activity gel electrophoresis with native gels was carried to study the isoforms of peroxidase and polyphenol oxidase induced by the elicitor treatment. All gels were analyzed for presence or absence of bands and photographed immediately after incubation and staining.

Phenylalanine ammonia lyase: PAL induction in chilli plants increased till 7th day. Among the 10 isolates tested, Th-10 and Th-16 (1.07 and 0.64 change in OD/min/g) showed more PAL activity compared to control (0.23). On 7th day PAL activity increased gradually in all treatments and maximum activity was observed in Th-19 and Tvs-7 treated plants (0.88 and 0.97, respectively). In elicitor + pathogen treated plants PAL activity was high after 24 h in Th-10 (0.68) treated plants compared to control and pathogen alone treated plants. On 7th day maximum PAL activity was observed in Th-9 treated plants (0.93).

Peroxidase: In elicitor treated plants, the peroxidase activity was more compared to control. In Th-19 and Tv-115 treated plants maximum peroxidase activity was observed on 7th day and after that the activity gradually decreased. In plants treated with elicitors from Th-19, Th-16 and Tvs-7, there was gradual increase in peroxidase activity upto 28th day. In elicitor + pathogen treated plants, peroxidase activity was on increase compared to control and pathogen alone. Among the 10 isolates tested, Th-9 treated plants showed less peroxidase activity compared to increasing activity in the remaining isolates. Peroxidase activity could be located in the elicitor treated plants upto 28 days through gel elctrophoresis. In plants treated with elicitors of the *Trichoderma* isolates Th16, Th19 Th10 and TVS5 very thick bands of peroxidase could be observed while in untreated plants only three faint or thin peroxidase bands could be seen on 28th day indicating the role of elicitor in inducing high peroxidase activity in chilli and their role in ISR.

Polyphenol oxidase: In elicitor treated plants, among 10 isolates Th-9 and Tv-115 showed gradual increase in PPO activity and maximum activity was observed on 28th day (0.35, 0.28) compared to control. Tvs-5 showed maximum activity on 14th day (0.31) and decreased by 28th day. In elicitor + pathogen treated plants, among the 10 isolates tested, Tvs-5 showed maximum PPO activity on 14th day (0.56) and in the remaining isolates PPO activity was more on 14th day that gradually decreased by 28th day. Electrophoresis studies using 9% resolving and 5% stacking gels four isoforms on 7th day and only 3 isoforms on 28th day in untreated plants while in plants treated with elicitors of Th10, Th16, TV10, Tv30, Tvs7, Tvs8 there were five isoforms on 7th day.

Phenols: In elicitor treated plants, among 10 isolates Th-9 showed increasing phenol content and maximum activity was observed on 28th day (840µg/g).Th-16 showed maximum activity on 21st day (720 µg/g) and decreased on 28 th day compared to control (Fig. 22). In elicitor + pathogen treated plants gradual increase in phenol content was observed in plants treated with Th-9, Th-10, Th-16 and Tv-115 and maximum phenol content was observed on 28th day (580, 940, 600, 780 µg/g).

Total proteins: Among 10 isolates tested, maximum protein induction was observed in plants treated with Th-9 and Th-10 on 14th day (5.75 and 5.25 mg/g). In elicitor + pathogen treated plants protein induction was increasing gradually and maximum induction was observed on 28th day. Th-10, Th-16 and Tv-10 showed maximum protein content (6.95, 6 and 6 mg/g of tissue) on 28th day compared to control.

c. Validation of bioefficacy of *Trichoderma* isolates provided under PhytoFuRa scheme

Dual plate assay: Dual plating of the 15 *Trichoderma* isolates was done against the tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici* and per cent inhibition was calculated compared to control (pathogen alone). Most of the isolates showed overgrowth on pathogen (hyper parasitism) and isolates PFR-1, PFR-4, PFR-12 and PFR-15 showed pigmentation and isolates PFR-2 and PFR-14 inhibition zones (Table 30).

Table 30. Percentage inhibition of *Fusarium oxysporum* f. sp. *lycopersici* by *Trichoderma* isolates in dual plate assay

Isolate No.	Inhibition (%)	Remarks
PFR-1	29.19	Inhibition zone at contact point. Yellow coloration (pigment production)
PFR-2	26.37	Inhibition zone was observed. Pathogen growth inhibited. Overgrowth by *Trichoderma*.
PFR-3	26.85	Pathogen growth inhibited. Overgrowth by *Trichoderma*.
PFR-4	27.30	Yellow coloration (pigment production), Pathogen growth inhibited. Inhibition zone at contact point
PFR-5	28.22	Pathogen growth inhibited. Overgrowth by *Trichoderma*.
PFR-6	25.44	Pathogen growth inhibited. Overgrowth by *Trichoderma*.
PFR-7	27.26	Overgrowth by *Trichoderma*.
PFR-8	26.11	Pathogen growth inhibited. Inhibition zone at contact point
PFR-9	25.89	Pathogen growth inhibited. Overgrowth by *Trichoderma*
PFR-10	24.52	Pathogen growth inhibited. Overgrowth by *Trichoderma*
PFR-11	29.63	Pathogen growth inhibited. Overgrowth by *Trichoderma*
PFR-12	24.52	Yellow coloration (pigment production). Overgrowth by *Trichoderma*
PFR-13	30.52	Pathogen growth inhibited. Initially inhibition zone observed. Later overgrowth by *Trichoderma*.
PFR-14	28.19	Pathogen growth inhibited. Inhibition zone at contact point
PFR-15	30.56	Yellow coloration (pigment production), Inhibition zone at contact point

Greenhouse evaluation: Evaluation of talc formulations of the above 15 isolates of *Trichoderma* was done in a greenhouse using tomato plants (Lakshmi variety) and cocopeat as the substrate. *Trichoderma* treatment was done with seedling dip method and later pathogen (*Fusarium oxysporum* f. sp. *lycopersici*) was inoculated @2x106 spores/ml by drenching method. The experiment is in progress.

NATIONAL RESEARCH CENTRE FOR CITRUS NAGPUR

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1 Biodiversity

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

Soil, root, leaf, bark and fruit samples were collected from citrus orchards situated in Vidarbha region of Maharashtra, Nalgonda Dist. of A. P., Hosiarpur region of Punjab and parts of Sikkim. Soil samples were collected from the rhizosphere of each plant at 5- 30 cm depth alongwith feeder roots. Samples were then assayed on a selective medium containing pimaracin-ampicilin-rifampicin-PCNB-hymexazol (PARPH) and the propagules per cm^3 soil were determined by dilution plating. In total, 32 orchards and 8 nurseries were surveyed in different citrus growing areas. *P. nicotianae* and *P. palmivora* could be isolated in the range of 4 - 116 propagules / cc soil. Baiting technique (using rough lemon leaf bits) was followed to isolate *Phytophthora* spp. from the soil samples which failed to produce any colony in selective medium. Altogether, 17 isolates of *P. nicotianae* and five isolates of *P. palmivora* were isolated and purified. These isolates have been included in our existing ac cessions of *Phytophthora* spp. making the total collection as 101 isolates. The same sets of cultures were also maintained in CMA plates by periodical subculturing in a BOD incubator at ~25^0 C. Two cultures of *Phytophthora* spp. viz. NRCPh-21 (*P. palmivora*) and NRCPh-66 (*P. nicotianae*) were submitted in the IISR, Calicut *Phytophthora* repository.

b. Survey for nematode infestation

A survey was carried out in mandarin orchards and nurseries at various locations of Kalmeshwar and Katol talukas of Nagpur District of Vidarbha region during the Ambia season of 2010 for assessing nematode infection and infestation. As many as 14 Nagpur mandarin orchards were surveyed. Ten to fifteen sub samples of soil and roots were drawn from each orchard representing area of the orchard to make one composite sample. Two representative samples (250 cc soil each) from each composite sample were processed for nematode extraction. Roots were stained in acid fuchsin lactophenol for observing the root infection. Of the orchards surveyed, 52% were having high (more than 1000 juveniles/ 250 cc soil), 22% medium (501-1000 juveniles/ 250 cc soil) and 14% low (1-500 juveniles/ 250 cc soil) populations of *Tylenchulus semipenetrans*. Twelve per cent of the orchards were free from *T. semipenetrans* infestation. Maximum nematode population recorded was 1864/100 cc in soil and 135.9/ g in roots.

c. Colony characteristics

Colony morphology was recorded as pattern, nature of margin and growth rate of isolates on V8 agar media after four days of growth at 25+10C in the dark. The isolates NRCPh 21 – NRCPh 86 were studied during the period. In general, colony of *P. nicotianae* isolates showed dense cottony mycelium to cottony aerial mycelium with no specific pattern growth whereas *P. palmivora* isolates produced a stellate striated pattern colony. However, on PDA, the colony of *P. nicotianae* isolates showed dense cottony mycelium to cottony mycelium to slightly stellate pattern growth whereas *P. palmivora* isolates produced a stellate striated pattern colony. In P. nicotianae isolates, 11 different types of colony patterns on V8 agar medium and seven different types of colony patterns on PDA were observed, whereas in P. palmivora isolates, three different colony patterns on V8 agar medium and five patterns on PDA were observed. The growth rate varied from 6.06 – 16.00 on V8 and 4.25 - 8.62 mm/ day on PDA for above isolates.

d. Sporangial morphology

Sporangium morphology for 86 isolates was checked through agar-disk-in-water technique. *P. nicotianae* isolates produced more rounded pear shaped sporangia that were noncaducous, whereas *P. palmivora* isolates produced ovoid sporangia (mostly on sympodial sporangiophores) that were caducous but with short pedicel. Ovoid,

spherical, globose, limoniform, obturbinate, ellipsoid were the most common sporangial shapes observed in all *Phytophthora* isolates investigated. Occasionally, bipapillate sporangium of *P. nicotianae* was also observed.Mating types

The mating types of the collected isolates (NRCPh 1-86) were detected in crosses between the known mating types with unknown isolated samples on carrot agar medium incubated at 20^{0}C at dark for 3-4 days to develop oospores. The known A1 mating type of *PhytoPhthora nicotianae* (ATCC-MYA-4036) was procured from American Type Culture collection (ATCC), Manassa, VA, USA through their Indian distributor, LGC Promochem India Pvt. Ltd., Bangalore. Till date, out of 86, nine isolates of *P. nicotianae* (NRCPh 18a, 19, 24, 35, 37, 43, 45, 60 and 62) were observed to form oogonia and antheridia with MYA-4036. Hence these are A2 mating type. The oospore formed was round and 23-25 mm in diameter (Fig. 23). Rest 77 isolates were found to be A1 mating type. In both *P. nicotianae* and *P. palmivora* isolates oogonium was observed with an amphigynous anthredium.

Fig. 23: Oospore of Phytophthora nicotianae (NRCPh 60) produced by mating with a known A1 isolate, ATCC-MYA-4036.

e. Molecular diversity analysis

Culture preparation and DNA extraction methods for already collected *Phytophthora* isolates were standardized. *Phytophthora* isolates NRCPh 47-86 were used in the study. Five 5 mm cut bits of actively expanding mycelium from single hyphal tip cultures of *Phytophthora* isolates were transferred to 75 ml of carrot broth (prepared by grinding 200 g of carrot, and boiled in 500 ml of distilled water. The extract was brought to one liter and autoclaved for 30 min) or V8 broth in 150 ml conical flask and incubated in dark for 3-4 days at 25+ 1^{0}C. Mycelial mats were washed with sterile distilled water and dried briefly under vacuum before being frozen at -20^{0}C. Frozen mats were ground with a sterile mortar and pestle. Total genomic DNA from approx. 100 mg of ground mycelium was extracted using standard method and Qiagen DNeasy Plant mini kit according to manufacturer's directions. DNA pellets were resuspended in TE buffer, and DNA concentrations were estimated using a Eppendorf Biophotometer.

Polymerase chain reaction (PCR) amplification of the ITS region of the template DNA was performed using the primers ITS 4 (5'-GAAGGTGAAGTCGTAACAAGG-3') and ITS 6 (5'- TCCTCCGCTTATTGATATGC-3'). ITS 6 is a universal primer designed to amplify a part of rDNA of Oomycota; it amplifies ~ 900 bp product in combination with ITS 4 primer. PCR was conducted in 25 µl reaction volume using 0.2 ml PCR tubes and a PCR machine with a hot lid. Each reaction mixture contained: dNTPs (0.2 mM), $MgCl^2$ (2 mM), Forward primer (ITS 6) to a final conc. of 1 µm, reverse primer (ITS 4) to a final conc. of 1 µm, 10X PCR buffer, Taq polymerase 1 Unit and 1 µl Template DNA. For 25µl thin walled PCR tube reactions, a single step at 94°C for 3 min followed by 35 cycles of 94°C for 30 s , 50°C for 30 s, 72°C for 60 s and and a final single step at 72°C for 10 min. PCR was conducted in a Mastercycler personal PCR machine (Eppendorf, Germany). The amplified DNAs (PCR products) were analyzed on 1% agarose gel stained with ethidium bromide. The PCR amplicons obtained from all the isolates were of expected size (~900 bp). A 10 µl sample of the PCR product was digested with the restriction enzymes MspI and AluI, according to the enzymes manufacturer's instructions and the restricted products were electrophoresed in 2.5% agarose gel and documented. The results of RFLP analysis of the PCR products of all the isolates under study revealed a difference in their restriction pattern. MspI digestions revealed three bands of 404, 390 (seen as one broad band of 400 bp) and 120 bp in isolate nos. 47-86 (except isolate no. 79) (Fig 24). Correspondingly, AluI digestion revealed three bands of 745, 117 and 52 bp in isolate nos. 47-86 (except isolate no. 79) (Fig. 24). Comparative data of RFLP analysis available at www.phytid.org/list.asp suggest isolate nos. 47-86 (except 79) as P. nicotianae. The RFLP data for NRCPh 79 indicates as *P. palmivora*. This also confirms our non-molecular morphological assessment.

The RFLP patterns obtained on digestion of the PCR product with RsaI, for isolate nos. NRCPh 47-60 are typical of *P. nicotianae* and the profile obtained for isolate nos. NRCPh 20, 25, 26, 27 and 30 are characteristic of *P. palmivora*.

f. Detection of *Phytophthora nicotianae* specific primers

P. nicotianae – specific primer pair NIC 1 – NIC2 was designed. PCR amplification of genomic DNAs using the primer pair NIC 1 – NIC2 generated a ~750 bp sequence for isolate nos. NRCPh 1-12, 16-19, 22 and 24, confirming that the pathogens were *P. nicotianae*. In case of *P. palmivora* isolates (nos. NRCPh 13, 14, 20, 21 and 25-30) no products were observed.

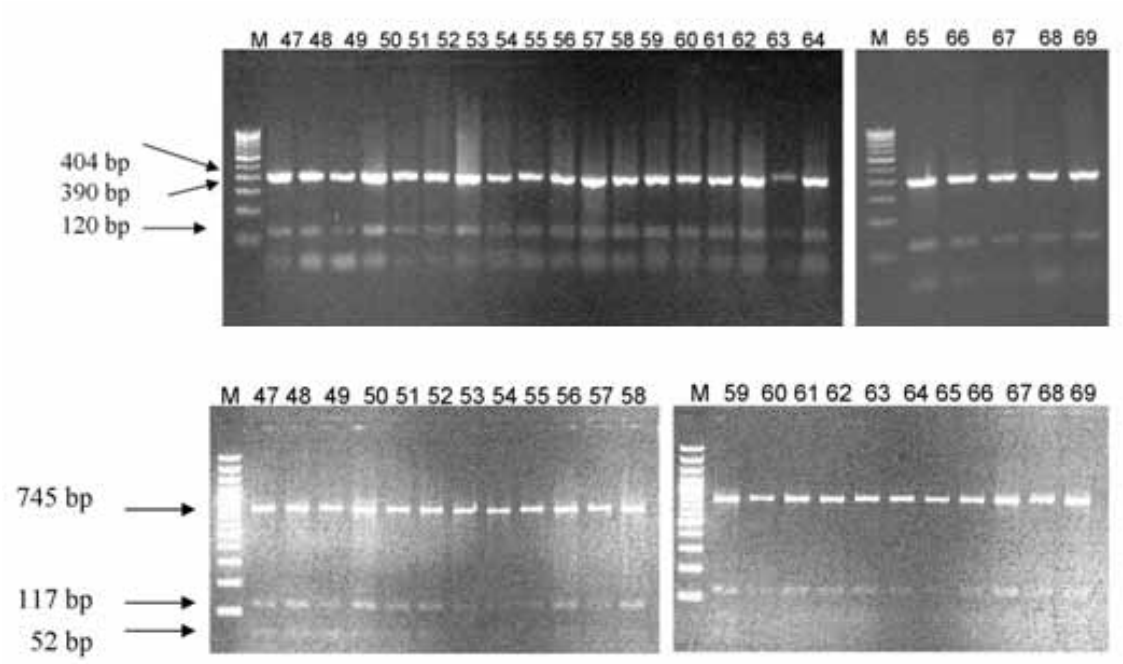

Fig. 24: RFLP profiles of *Phytophthora* isolates after digestion of ITS4/ITS6 amplification products with restriction enzymes. (Top) MspI and (bottom) Alu 1

g. ITS sequencing

Ten amplicons (for isolate Acc nos. NRCPh 27, 29, 31, 37, 41, 52, 56, 58, 61 and 66) obtained with primers ITS 4 and ITS 6 were purified using QiaQuick PCR purification kit (Qiagen), according to the manufacturer's protocol. The purified PCR products were sequenced bidirectionally at the commercially available automated DNA sequencing facility (Chromus Biotech, Bangalore). Search for homologies in the GenBank databases (http://www.ncbi.nlm.nih.gov/blast) was carried out using the BLAST program. When these sequences were compared with the known *Phytophthora* species available in the GenBank, ~ 99% homology (at the ITS 1, 5.8S and ITS 2 regions of the nuclear ribosomal DNA) was observed with *P. nicotianae* and *P. palmivora*. ITS sequences of four isolates of *P. nicotianae* (NRCPh- 1, -4, -6 and -7) were submitted in the GenBank databases under the accession numbers HM807369, HM807370, HM807371 and HM807372, respectively. A total of 29 rDNA ITS region sequences of *Phytophthora* were compared for diversity using MEGA version 4. Sequences were aligned with Clustal W followed by construction of phylogenetic tree by means of maximum parsimony (500 Bootstrap) method in MEGA 4. Multiple Sequence Alignment (MSA) depicted addition, deletion, substitution (transition & transversion) of nucleotide/s in the ITS region. The sequences of *P. nicotianae* showed scattered variation in ITS 1 as compared to ITS2 region, 5.8 S region was almost conserved.

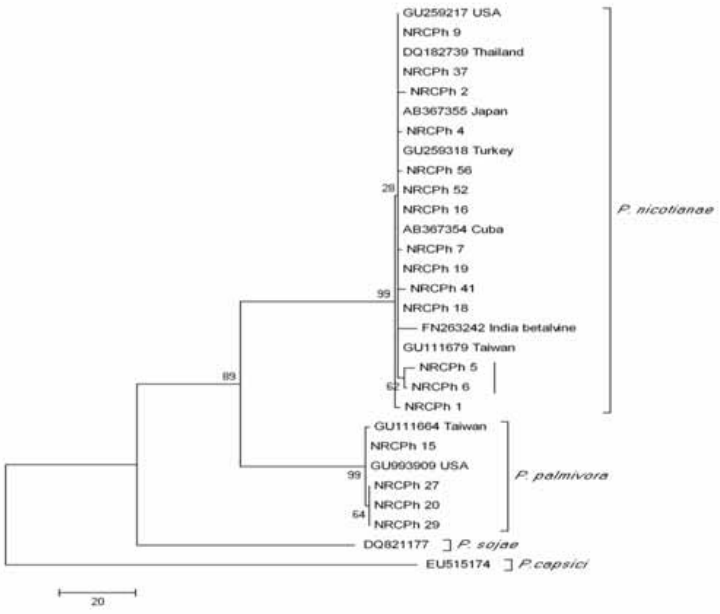

Fig. 25. Phylogenetic tree depicting relationships among *Phytophthora nicotianae* and *P. palmivora* isolates

Sequences of *P. palmivora* showed minor variation in ITS2 only, ITS1 and 5.8S was 100% conserved. Tree generated revealed diversity of sequences, it formed two major clusters of *P. nicotianae* and *P. palmivora*, respectively. *P. capsici* and *P. sojae* sequences were outliners and were included in the analysis to aggravate the diversity (Fig. 25). ITS sequences of *P. nicotianae* and *P. palmivora* isolates derived from citrus orchards of central India and other parts of the world were found highly conserved, thus variation was not geographically confined. Intra-species variation was observed more in *P. nicotianae* isolates than in *P. palmivora* isolates.

h. PCR amplification and sequencing of ß-tubulin, EF-1α, COX-1 and COX-II gene regions

PCR amplification of the ß-tubulin, elongation factor-1α (EF-1α), Cytochrome Oxidase-I (COX-1) and II (COX-II) gene regions of the tem-Plate DNA from different isolates of *Phytophthora* was performed using the respective primers and the standard protocol described earlier (Table 31; Fig. 26). Fourteen amplified PCR products with respect to above genes were given for sequencing to Chromus Biotech, Bangalore.

Table 31. Amplification of ß-tubulin, EF-1α, COX-1 and COX-II genes in *Phytophthora* isolates

Gene	Primers used	No. of isolates	used Size of Amplicon (bp)
ß-tubulin	BTUBF2 5'-CGG TAA CAA CTG GGC CAA GG-3' BTUBR2 5'-GAT CCA CTC AAC GAA GTA CG-3'	9 (NRCPh 15, 16, 17, 18, 19, 20, 21, 22 and 23)	750
Elongation factor-1α	EF1F: 5'-TCA CGA TCG ACA TTG CCC TG-3' EF1R: 5'-ACG GCT CGA GGA TGA CCA TG-3'	8 (NRCPh14, 15, 16, 17, 18, 19, 20, 21 and 22)	950
Cytochrome Oxidase-II	FM35: 5'-CAG AAC CTT GGC AAT TAG G-3' FMPhy: 5'-GCA AAA GCA CTA AAA ATT AAA TAT AA-3'	10 (NRCPh 70, 71, 72, 73, 74, 75, 76, 77, 78, 79)	1000

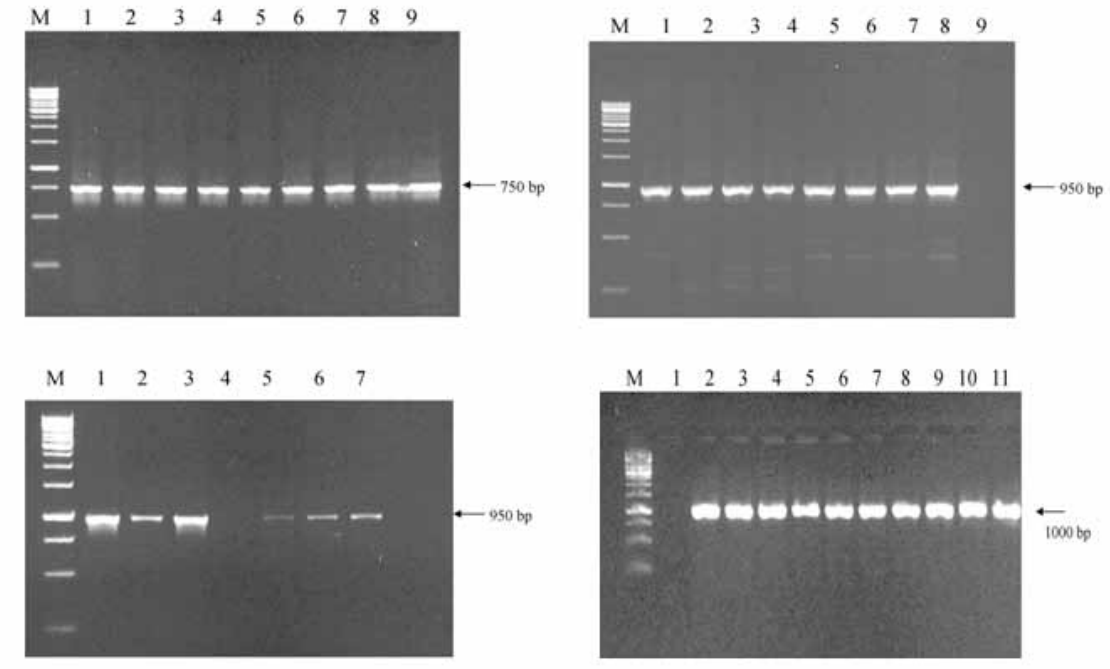

Fig. 26. PCR amplification of different markers in *Phytophthora* spp. (Top left) ß-tubulin, Isolates NRCPh 15 - 23, (Top right) EF-1α, Isolates NRCPh 15 - 22, (Bottom left) COX-I, Isolates NRCPh 15, 18, 27, 56, 58, 66 and 71, and (Bottom right) COX-II, Isolates NRCPh 70-79.

2 Diagnostics

a. Detection of *Phytophthora* spp. in citrus roots and soil using PCR-RFLP

Tests were conducted on roots collected from Nagpur mandarin plants showing symptoms of mild to moderate decline. All rootlet samples were washed with tap water, dried with blotting paper, cut into segments and ground in liquid nitrogen with a mortar and pestle to produce a powder. DNA was extracted from 100 mg of ground tissue using the Qiagen DNeasy Plant mini kit according to manufacturer's instructions. DNA was quantified using an Eppendorf Biophotometer.

PCR amplification of ribosomal ITS regions was performed with the primers ITS4 (5'-TCCTC-CGCTTATTGATATGC- 3') and DC6 (5'-GAGGGACTTTTGGGT AATCA-3'), which specifically amplify ribosomal DNA from the major pathogenic oomycete groups *Pythium*, *Phytophthora* and the downy mildews. PCR products were then amplified in a second, semi-nested round, using universal primers ITS4 and ITS6. PCR reactions were performed in a total volume of 25 µl using the reaction mixture as mentioned earlier. Ten µl of the amplification products generated with primer pair ITS4 and ITS6 were digested with restriction enzyme MspI in a total volume of 20 µl according to the manufacturer's instructions. Reaction products were analyzed by electrophoresis in 2.5% agarose gels. Restriction fragment patterns were compared with data provided at www.phytid.org/list.asp.

The primer pair DC6/ITS4 produces 1.2 kb fragment and an amplification product of ~ 900 bp, typical for the genus *Phytophthora*. MspI digestions revealed two clear bands of 508 and 389 bp indicating the presence of *P. palmivora* in eighteen samples (Fig 27). In six of those samples presence of *P. nicotianae* was also observed. The species-specific primer pair NIC1/ NIC 2 is also being investigated for diagnosis of *P. nicotianae* infection in roots.

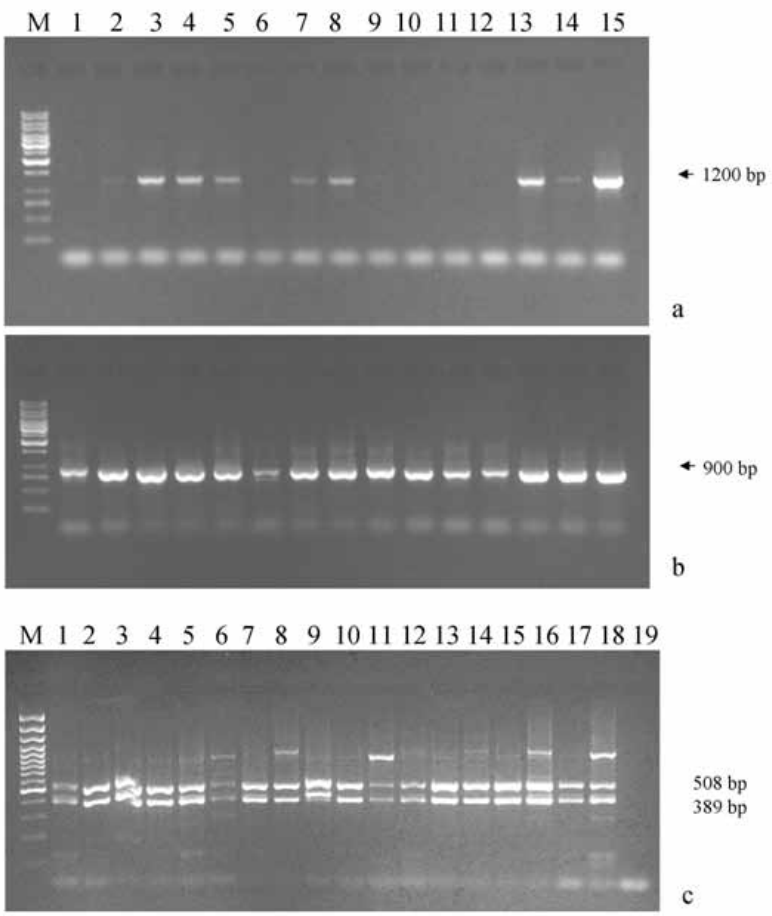

Fig. 27. Molecular identification of *Phytophthora* spp. in citrus roots through nested PCR. (a) Simple PCR with primers DC6 and ITS4, (b) Nested PCR with primers ITS4 and ITS6, and (c) Restriction profile with MspI. Lane 1-18: Root samples collected from infected citrus trees, M – 1 kb marker.

Work has also been initiated to detect *Phytophthora* spp. in soils. Soil samples were collected from rhizosphere of several citrus plants (Nagpur mandarin, pummelo, Sour orange, trifoliate and Mosambi sweet orange. A total of 14 soil samples were collected from NRC Citrus experimental farm. Soil DNA was extracted using Ultraclean Soil DNA isolation Kit (Mo Bio Laboratories, USA) and DNA was quantified. The concentration of DNA in the soil samples was found very low. Optimization of PCR / nested PCR was done as above (similar as like that of in case of roots). The presence of *Phytophthora* spp. could be detected in four samples.

3 Host-pathogen interaction

In order to understand the mechanism of host tolerance / resistance in citrus against *P. nicotianae*, three rootstock genotypes, namely trifoliate orange (selected as tolerant), rough lemon and rangpur lime are grown in the screenhouse. The bark and roots of these rootstock seedlings were artificially inoculated with *P. nicotianae*. Six weeks after inoculation, plants were removed from the potting mix, roots and leaves were washed thoroughly with tap water, blotted dry and used for phenolic analysis. Results indicate that total soluble phenolics of roots were increased in all rootstock genotypes, but their relative increase after infection were lowest in roots of trifoliate orange (tolerant) seedlings. Leaf pheno-

lic levels were not changed upon infection and did not differ significantly between tolerant and susceptible plants.

Amplification of elicitin genes was obtained in *P. nicotianae* (isolate nos. NRCPh 47-64) using specific primers designed from conserved sequences.

a. *Phytophthora* -nematode interaction studies

Pot inoculation studies are being conducted using one-year old rough lemon rootstock seedlings. Individual and concomitant inoculation of *P. nicotianae* and *Tylenculus semipenetrans* was done in one litre polybags in the glasshouse. These bags were kept in glasshouse benches for taking observations related to root rot and other growth parameters.

4 Host resistance

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

Eleven rootstock seedlings viz. Rough lemon B 204, CRH-12, Citrumelo, CRH-47, Smooth Flat Seville (SFS), Troyer citrange (Chethali), Soh Nairiange, IPS – 147, sour orange, SFS x Argentina T. O .and Citrus hystrix are being raised in polybags for screening against *Phytophthora*. These rootstock seedlings were inoculated with *P. nicotianae* (chlamydospores and freshly released zoospores). The bags were kept on glasshouse benches lined with polythene sheet with 1" water. Observations related to root rot, shoot weight and *Phytophthora* population have been recorded. Analysis of data is in progress.

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

Work has been initiated to identify markers associated with citrus *Phytophthora* disease resistance using RAPD analysis. The following 13 citrus rootstocks/ cultivars (tolerant and susceptible) viz. Sour orange, Pomeroy trifoliate, Smooth Flat Seville (SFS), Rangpur lime (chethali), Rough lemon (rahuri), Alemow, Nagpur Mandarin, Cleopatra Mandarin, Sweet orange, Rangpur lime, Rough lemon, Citrus volkamarina and Swingle citrumelo were selected. All the plant materials were obtained from NRC for Citrus Experimental Research Farm. Total genomic DNA from young citrus leaf samples was extracted using Qiagen DNeasy Plant mini kit according to manufacturer's directions and DNA concentrations were estimated using a Eppendorf Biophotometer.

The decamer random primers (OPA series) were purchased from Operon Technologies, CA, USA. A total of three decamer oligonucleotides, of arbitrary sequence (kits OP-A,) were tested for PCR amplification. The PCR reactions were optimized in a thermal cycler. PCR was performed in a total volume of 25 ul, containing 25 ng of template DNA, 0.4 uM of a single primer, 1 U Taq DNA polymerase, 0.2 mM each dNTP, 2.5 mM MgCl2, and 1X PCR buffer. An initial denaturation cycle of 5 min at 94°C was followed by 45 cycles comprising 1 min at 94°C, 1 min at 35°C and 2 min at 72°C. An additional cycle of 10 min at 72°C was used for final extension. The PCR products were separated by electrophoresis in 1.5% agarose gels and stained in ethidium bromide (Fig. 28). Reproducibility of the patterns was tested by running the gel twice. DNA band scoring and interpretation of RAPD data are in progress.

5 Disease management

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

A total of 54 fungal biocontrol agents and 28 bacterial bioagents collected earlier were maintained for studying their antagonistic activities against *Phytophthora* spp.

b. *In vivo* efficacy studies

This year, nine isolates of *Trichoderma* NRCfBA 8, 14, 23, 25, 28, 29, 31, 33 and 37 were selected for *in vivo* efficacy studies of antagonism against *Phytophthora*. Six-month old seedlings of rough lemon seedlings, were raised in polybags and inoculated with 1 % (w/v) culture of *Trichoderma* spp. thoroughly mixed with the potting mixture. After one month of establishment of the bioagent inoculum, 25 ml zoospore suspension of *P. nicotianae* was added to each polybag. Six plants per treatment were kept in a RBD. The initial population of *Phytophthora* was assessed to establish the propagule density 20-30/ cc pot mix. The population of bioagents was maintained through out the experiment at 10^6 in rough lemon seedlings. After four months, the treated seedlings were uprooted. The roots were washed and screened for total root length and rotted roots by using a root scanner (Biovis, Mumbai). The results are given in Table 32.

c. Molecular identification of promising *Trichoderma* spp. isolates by ITS sequencing

Nineteen promising Trichoderma spp. isolates were cultured in potato-dextrose broth at 27°C for 48 h on a rotary shaker at 120 rpm. Mycelia were harvested by filtration, washed with distilled water, and ground in liquid nitrogen. Genomic DNA was isolated from the ground mycelia using DNeasy Plant mini kit (Qiagen, Hilden, Germany). Amplification of the ITS region was carried out with the oligonucleotide primers ITS 5 and ITS 4. PCR was conducted under the following conditions: initial denaturation at 94°C for 3 min, followed by 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 1 min. A final extension at 72°C for 10 min followed. The amplification products (amplicons) were purified using QiaQuick PCR purification kit (Qiagen), according to the manufacturer's protocol. The products were given for sequencing (bidirectionally).

d. Multilocational testing of *Trichoderma* isolates obtained from PhytoFuRa centres

Fifteen *Trichoderma* isolates (Phytofura 1 – 15, obtained from different PhytoFuRa centers) were received from IISR, Calicut on Nov. 2011. All the isolates were transferred to PDA and MRBA plates. Growth rate of the 15 isolates was estimated on PDA at 250 C. The dual culture plating and inhibition studies are in progress.

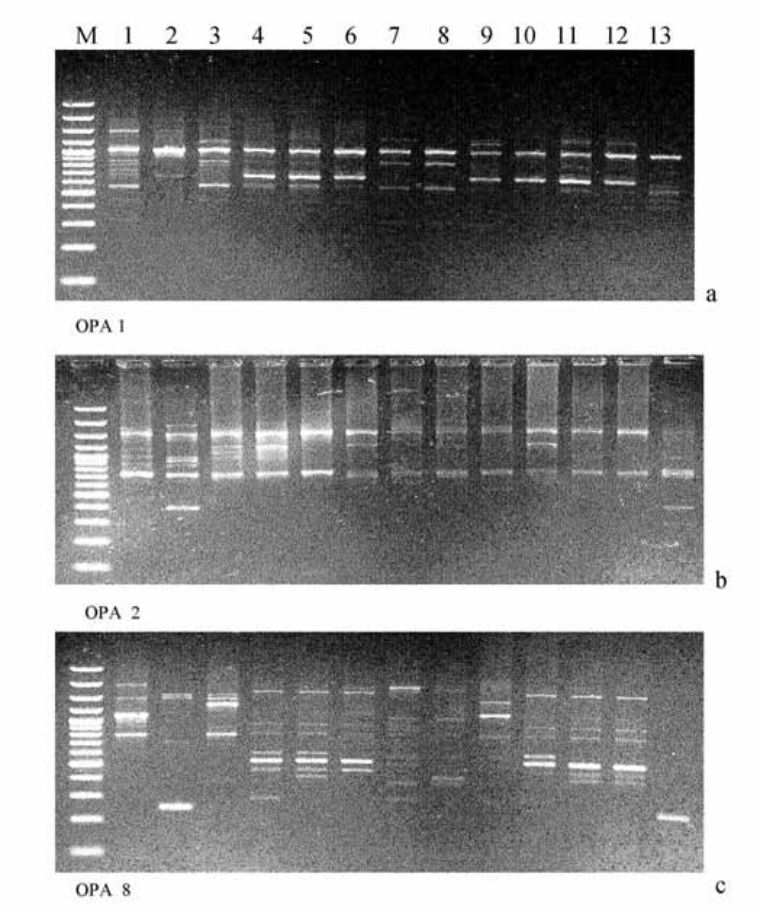

Fig. 28. RAPD profile generated by random primers in different citrus genotypes. (a) OPA 1, (b) OPA 2 and (c) OPA 8. Lane 1-13: Sour orange, Pomeroy trifoliate, Smooth Flat Seville, Rangpur lime (Chethali), Rough lemon (Rahuri), Alemow, Nagpur Mandarin, Cleopatra Mandarin, Sweet orange, Rangpur lime, Rough lemon, Citrus volkamarina and Swingle citrumelo.

Table 32. Effect of bioagents on growth of rough lemon seedlings and root rot due to P. nicotianae

Treatment	Root length (cm)	Dry wt. of root (g)	T Dry wt. of shoot (g) reat ment	Total shoot length (cm)	Bioagent popln		Phytoph. popln./cc soil	
					initial x 10^7	at end x 10^6	Initial/cc soilment	at end
NRCfBA -8	352.93	2.22	7.18	53.83	0.8	2.6	20-30	92
NRCfBA -14	321.62	2.10	7.30	58.81	1.2	3.0	”	86
NRCfBA -23	385.77	1.85	6.45	58.03	0.8	4.8	”	63
NRCfBA -25	367.21	1.38	4.52	44.86	1.6	12	”	46
NRCfBA -28	406.09	1.58	5.66	48.78	2.8	3.4	”	39
NRCfBA -29	514.84	2.56	7.60	66.11	5.2	3.1	”	27
NRCfBA-31	425.43	1.77	6.12	52.96	11.2	1.7	”	46
NRCfBA- 33	480.46	1.53	5.37	52.83	0.4	3.0	”	42
NRCfBA -37	498.13	2.02	6.50	65.28	5.2	1.6	”	34
Control	361.55	2.00	5.97	49.06	-	-	-	-
Control infested	190.45	1.37	3.55	44.03	-	-	20-30	190
CD (P=0.05)	9.8	0.26	1.17	3.8				

Sub project - 2

FUSARIUM

Wilt caused by species of Fusarium is one of the most serious disease problems of several agricultural, vegetable and fruit crops. Since, the disease results in complete mortality of the affected plants, the loss is total leading to huge monitory losses especially in perennial trees like guava. Annual yield losses severity up to 10% in chickpea, 97000 t in pigeon pea, 25% each in safflower and chilli and 30% in banana have been reported depending upon the disease severity and crop stage. One control method is to improve soil conditions because Fusarium spreads faster through soils that have high moisture and bad drainage. Other methods include planting resistant varieties, removing infected plant tissue to prevent overwintering of the disease, using soil and systemic fungicides to eradicate the disease from the soil, flood fallowing, and using clean seeds each year. Applying fungicides depends on the field environment. It is difficult to find a biological control method because research in a greenhouse can have different effects than testing in the field. So it become imperative to study in detail about the characteristic of the pathogen and its relationship to the host and environment which predisposes for infection and also its genetic behavior to explore the possibility of developing a resistance source as well as to control the pathogen by chemical or biological means. Hence the present project is initiated in a network mode involving eight different centers representing major crops like guava, banana, chilli, chickpea, pigeon pea, tomato etc. The species studied are F. oxysporum f. sp. carthami (safflower), F. oxysporum f. sp. ciceris (chickpea), F. oxysporum f.sp. cubense (banana), F. oxysporum f. sp. lycopersici (tomato), F. oxysporum f. sp. psidii (guava), F. solani (chilli) and F. udum (pigeon pea).

CENTRAL INSTITUTE FOR SUBTROPICAL HORTICULTURE LUCKNOW

Principal Investigator
Dr. B. K. PANDEY

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1 Biodiversity

a. Enumeration of total microbial population from guava orchards from different locations

Soil samples from different locations were collected before the onset of rains and analysed for the total microbial populations in the guava. *Bacillus*, *Pseudomonas* and *Fusarium solani* were the major populations recorded and one isolate each of fungi and bacteria showed antagonism against both *Fusarium* and *Trichoderma* (Fig. 29 a).

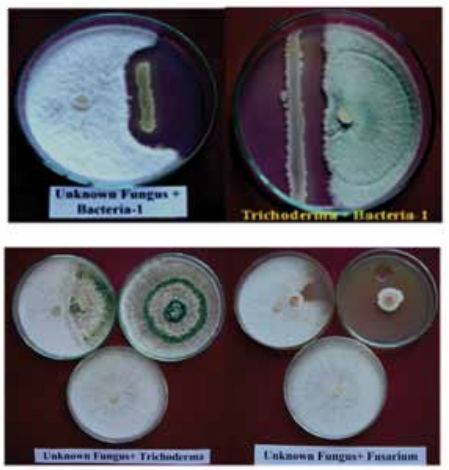

Fig. 29 b. Antagonistic effect of unknown fungi and bacteria towards *Fusarium* and *Trichoderma*

2 Diagnostics

a. Detection of *Fusarium oxysporum* f. sp. *psidii* with species specific primer

The DNA sequences of 28S ribosomal RNA and 28S-18S ribosomal RNA intergenic spacer partial sequences (GenBank: HM102500.1) were analyzed using the program PRIMER3. Each sequence was compared with the rest of the DNA sequences and one unique sequence was kept for primer design. Primer pair BKP1 (AAAACGGTCTCGGAGGGTAT) and BKP2 (GGGAAGGTCGATGACCACTA) detected *Fusarium oxysporum* f. sp. *psidii*. All isolates amplified a common 183 bp PCR product. No cross amplification with other species was observed (Fig. 29 b).

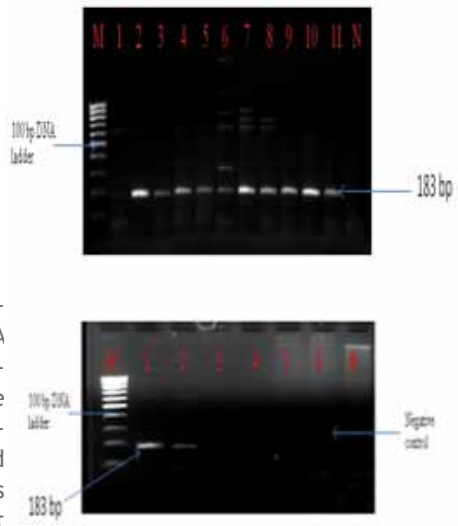

Fig. 29 a. Detection of *Fusarium oxysporum* f. sp. *psidii*. (top) M: 100 bp DNA Ruler, Lane1-11: *F. oxysporum* isolates, Lane N: Negative control; (bottom) M: 100 bp DNA ruler, Lane1-2: *F. oxysporum* isolates, Lane 3-4: *F.solani*, Lane5-6: *Colletotrichum gloeosporioides*, Lane N: Negative control.

b. PCR-RFLP of 28S-18S ribosomal regions

For all *Fusarium* isolates, DNA fragments of approximately 1400 bp were amplified using NS1 (GTAGTCATATGCTTGTCTC) and NS6 (GCATCACAGACCTGTTATTGCCTC) primer pair. No length variation was observed for the amplified 28S-18S ribosomal regions. Digestion of this using Taq I and HaeIII indicated that the ITS regions of the *Fusarium* isolates contain recognition sites for these enzymes.

c. Rapid identification of pathogenic *Fusarium sp*. using multiplex PCR

Three primer pairs viz. Fungal universal primer ITS1 and ITS4, *F. oxysporum* chitin synthase gene degenerate primer CS1F (TCGACTTCCTCCGATCTGAT) and CS1R (CCTTGTTCTTGAAGATGTCTG) and

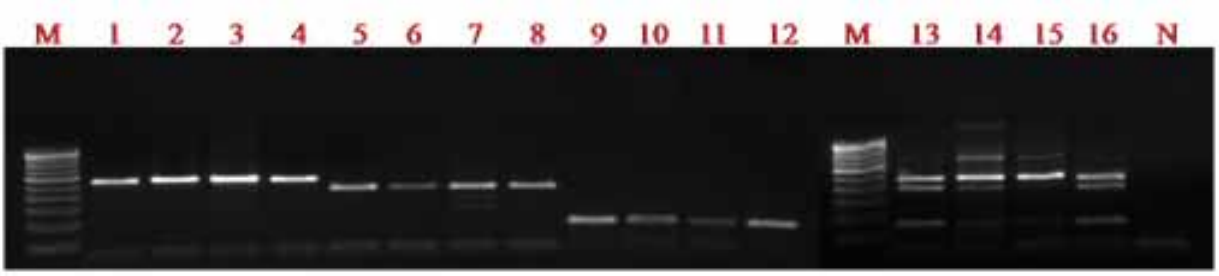

Fig. 30: PCR amplification using three primer pairs ITS 1- ITS 4, CHS1F- CHS1R and BKP1- BKP2, Lane M: 100 bp DNA ladder, Lane 1-4: 570 bp with ITSI & ITS4, Lane 5-7: 460 bp with CS1F & CS1R: Lane 9-12 : 183 bp with BKP1 & BKP2: Lane: 13-16 Multiplex PCR using the three primer pairs

F. oxysporum f. sp. *psidii* species specific ITS primer BKP1 (AAAAC-GGTCTCGGAGGGTAT) and BKP2 (GGGAAGGTCGATGACCACTA) were used to amplify *Fusarium* sp. by multiplex PCR. A product size of 570 bp with ITSI & ITS4, 460 bp with CS1F & CS1R and 183 bp with BKP1 & BKP2 were obtained (Fig. 30).

d. Community DNA extraction and estimation

Community DNA was extracted from soil sample collected from rhizospheric region of wilted guava plant. DNA purity was evaluated as 1.72 – 1.80 by measuring the absorbance at A260/280.

e. 18s rDNA PCR amplification of uncultured fungi

Amplification of 18S rDNA region was performed using the primer pair 18sF 5'-ATTG-GAGGGCAAGTCTGGTG-3'and 18sR 5'-CGATCCCTAGTCGGCATAG-3', and the PCR was performed by 34 cycles of denaturation at 94^{0}C for 60 s, annealing at 53^{0}C for 60 s, and extension at 72^{0}C for 1.5 min with an initial denaturation of 5 min at 94^{0}C before cycling and final extension of 5 min at 72^{0}C after cycling. All the samples amplified a common product of 500 bp. No length variation was observed among the 18s regions of the samples (uncultured fungi).

f. Specific primer designing and validation

The primers ITS1F (5'-CCAGAG-GACCCCCTAACTCT-3') and ITS1R (5'-GCCTGAGGGTTGTAATGACG-3') detected an amplicon of the approximately 230 bp for the genus *Fusarium*. The specific primer was tested with both fungal and soil sample i.e. uncultured samples. This assay has proved that it is useful for rapid identification of *Fusarium* spp., suggesting its efficiency in early detection of pathogen which will lead to timely management of wilt disease of guava.

3 Host resistance

Different *Psidium* species, varieties and hybrid (*Psidium molle* X *P. guajava*) were inoculated using stem hole inoculation technique. Except hybrid (*Psidium molle* X *P. guajava*) symptoms were developed in 20 to 70 days time.

4 Disease management

A field experiment was initiated for managing guava wilt with five treatments viz. FYM enriched with *Aspergillus niger*, *Trichoderma harzianum*, *T. viridae*, *Pseudomonas fluorescence* and control. The results for the first year were not satisfactory in biocontrol treatments due to the suppression of biocontrol agents by certain unidentified fungi and bacteria.

To manage the unidentified fungi and bacteria, another experiment was started with nine treatment of three soil fumigants viz., Sumid 98 G (Dazomet), Supam 40 EC (Metham Sodium) and Suzone 40 WS (Sodium Tetrathiocarbonate), at three concentrations. The total soil microflora was analyzed before and after one month of treatment imposition to find out the effect of soil fumigants. The result showed that all the microbes were killed after application of soil fumigants. The biocontrol treatments as mentioned above were again imposed in the same plot. Out of three fumigants only Suzone 40 WS was found effective for standing crop. New plantation of guava cv. Allahabad Safed has been done. Suzone 40 WS has also been applied in standing crop to find out its effect on wilt incidence. In control plot four plants died. The evaluation of soil revealed the presence of *F. oxysporum* f. sp. *psidii*.

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1 Biodiversity

a. Diversity analysis of *Fusarium oxysporum* f. sp. *carthami* isolates

Diversity among 54 isolates of *F oxysporum* f. sp. *carthami*, collected from different geographical areas was studied using TEF-1α gene specific primers. After PCR amplification the product obtained for different isolates was cloned and sequenced. The sequence information obtained will be used for differentiating four races that are identified based on their reaction to host differentials.

b. Pathogenic variability in *F. oxysporum* f. sp. *carthami* isolates

Based on reaction of 54 isolates of *F. oxysporum* f. sp. *carthami* to four host differentials, four races were identified. To reconfirm these results, a greenhouse study was carried out using all the wilt pathogen isolates against four host differential lines viz., 96-508-2-90, A1, DSF-4 and DSF-6 and existence of four races among Indian isolates of *F. oxysporum* f. sp. *carthami* has been confirmed.

2 Diagnostics

a. Diagnosis and detection of *F. oxysporum* f. sp. *carthami* by molecular methods

Three ITS primers viz. ITS1, ITS4 & ITS5 were used for amplification of *F. oxysporum* f. sp. *carthami* genomic DNA and a SCAR marker was developed based on the amplification product. ITS reactions were carried out and single sharp band of expected size of 580 - 600 bp was obtained (Fig. 31A). Amplified DNA fragments were purified from the gels and cloned into pTZ57R T/A cloning vector. Ligations, transformations of *E.coli* and plasmid amplification were performed following standard procedures. The transformation was confirmed by colony PCR (Fig. 31 B). The 660 and 680 bp product was cloned in a T/A cloning vector. DNA sequencing was done using M13 forward primer. SCAR marker of 162 bp was developed and used for identification of *F*. f. sp. *carthami* isolates In addition to that the SCAR showed specific amplification product of 162bp of *F*. f. sp. *carthami* isolates, which was not amplified in other *Fusarium* species (Fig. 31C). The work is under progress to identify the variants among the *F*. f. sp. *carthami* isolates by using ITS based SCAR markers.

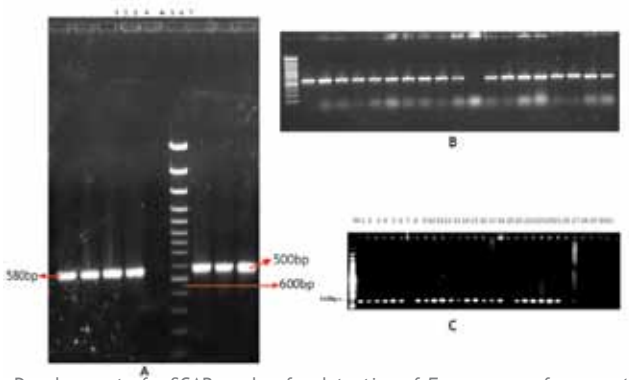


Fig. 31. Development of a SCAR marker for detection of *F. oxysporum* f. sp. *carthami*. A. PCR amplification of ITS region. Lane 1-4: Foc-1, M: Marker 100 bp and Lane 5-7: Foc-2. B. Colony PCR to select positive transformants C. SCAR amplification product of 162 bp fragment of *F.* f. sp. *carthami* but not other fusarial species Lane 1-25: Isolates of *F*. f.sp. *carthami* from safflower, Lane 26-31: *F.oxysporum*, *F. moniliforme*, *F. solani*, *F.o.ciceri*, *F.o. lentis* and *F.o. ricni*

b. Detection of *Fusarium* in early and mixed infections by RT-PCR

The TEF-1α gene specific primers were used for detection of *F. oxysporum* f. sp. *carthami* and the two root pathogens of safflower viz., *Macrophomina phaseolina* and *Rhizoctonia solani*. Three primer combinations 983F & EF-gr, EF-df & EF-gr and Ef-df & 2218R showed different sized amplification products in *F. oxysporum* f. sp. *carthami* compared with *M. phaseolina* and *R. solani*.

3 Host resistance

a. Quantification of defense molecules induced by bioagents

To study the role of defense enzymes viz., peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) in safflower induced by biocontrol agents viz., *Trichoderma harzianum* Th4d, *T. viride* Tv5 and *T. viride* T7316, a greenhouse study was conducted. The bioagents were selected based on their disease suppression against safflower pathogens (Table 33). The isolate

T. harzianum (Th4d) was found to be more effective in producing more quantities of PAL, PPO and PO in treated safflower seedlings compared to pathogen and untreated checks (Table 33). The experiment is under progress.

Table 33. Estimation of Phenylalanine Ammonia Lyase (PAL), Polyphenol Oxidase (PPO) and Peroxidase (PO) activity in safflower treated with biocontrol agents.

Treatment	PAL (g-1 min)	PPO (g-1 dry wt.)	PO (g-1 Protein/ min)
T. harzianum (Th4d)	0.37	39.00	12.07
T. viride (Tv5)	0.26	30.51	11.79
T. viride (T7316)	0.33	34.30	12.51
Carbendazim	0.27	29.83	10.31
Th4d + *Fusarium*	0.31	26.81	10.00
Tv5 + *Fusarium*	0.20	21.05	9.71
T7316 + *Fusarium*	0.29	27.05	11.30
Carbendazim + *Fusarium*	0.24	25.50	8.94
Pathogen check	0.18	16.05	7.14
Untreated Control	0.20	22.50	7.20

b. Marker assisted selection and molecular breeding

Planted five wilt resistant wild species viz., *C. creticus*, *C. oxyacantha*, *C. glaucus*, *C. lanatus* and *C. turkestanicus* along with susceptible cultivated species *C. tinctorius* ('Nira' Susceptible variety) and F1 generations of the crosses Nira x *C. glaucus*, Nira x *C. oxyacantha*, Nira x *C. oxyacantha*, Nira x *C. tinctorius*, Nira x *C. turkestanicus*, Nira x C. *lanatus* and Nira x *C. creticus* were planted in wilt sick plot.

F1 of the cross between susceptible cultivated species (Nira) and resistant cultivated species genotype (96-508-2-90) was also planted along with the above crosses. DNA isolation from all the above crosses is being undertaken and wilt incidence in parents and F1 generations was recorded four times from 15 days after sowing at 15 days interval.

As a prelude of present work, the wilt resistant wild species and susceptible cultivated species, *C. tinctorius* (A1 susceptible variety) and the already available following F1 generations viz: *C. tinctorius* x *C. glaucus*, C. *tinctoriusx* C. *oxyacantha*, *C. tinctorius* x *C tinctorius*, *C. tinctorius* x *C. turkestanicus*, *C. tinctorius* x *C. lanatus* and C. *tinctorius* x *C. creticus* screened in wilt sick plot during 2009-10.

All F1s of the above crosses and resistant parents exhibited resistant reaction (0% wilt incidence) in wilt sick plot. Their F2s were planted in wilt sick plot and wilt incidence in various F2 generations was recorded four times from 15 days after sowing with 15 days interval.

Total 777 RILs in F6 generation of the cross between susceptible cultivated species (*C. tinctorius*) variety' Nira' and resistant line 96-508-2-90 planted in wilt sick plot during 2009-10 exhibited high resistance to wilt (% disease incidence). DNA samples from RILs were extracted to validate the all ready identified RAPDs linked to *Fusarium* wilt resistant gene in *C. tinctorius* as well for SCAR development. SCAR development work is in progress.

c. Identification of EST-SSR markers polymorphic to cultivated and wild species

A total of 250 EST-SSR markers developed at DOR have been used to identify EST-SSRs which differentiate susceptible *C. tinctorius* from five wilt resistant wild species. Of these, 83 markers could differentiate the *C. tinctorius* (Nira) and *C. oxyacantha*, 21 were identified as polymorphic to parents *C. tinctorius* (Manjira) and *C. turkestanicus* while 23 EST-SSRs were found to be polymorphic between *C. tinctorius* (A1) and *C. glaucus* and 12 between susceptible *C. tinctorius* (Nira) and resistant *C. tinctorius* (96-508-2-90). Finally, 139 out of 250 were identified as polymorpmic markers. These would be used in F2 populations to identify species-specific EST-SSR markers flanked to wilt resistant genes (Fig. 32).

d. Validation of RAPDs flanked to wilt resistance in *C. tinctorius*

The already identified RAPDs linked to *Fusarium* wilt resistant gene in *C. tinctorius* have been validated in

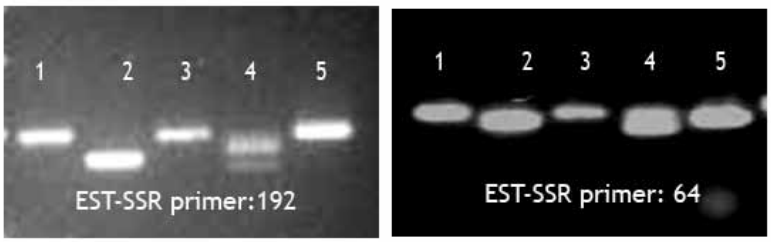
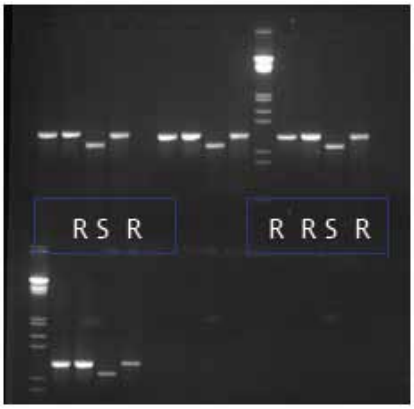


Fig. 32. EST-SSR markers polymorphic to cultivated and wild species of salflwer . Lanes: 1: Nira (*C.tinctorious*); 2. *C.oxyacantha*; 3. Manjira (*C.tinctorious*); 4. *C. turkistanicus*;5. 96-508-2-90 (*C.tinctorious*)



RILs (F6) of a cross between susceptible 'Nira' (*C. tinctorius*) and a resistant parent 96-508-2-90 (*C. tinctorius*). CAPS with NcoI R.E which identifies susceptible genotype was developed (Fig. 33).

Fig. 33. CAPS markers with NcoI R.E for identifying susceptible genotypes of *C. tinctorius*

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Co- Investigator
Dr. Parimal Sinha

1 Bio-diversity

a. Alpha translation elongation factor (TEF) analysis

The genetic diversity of 70 isolates of *Fusarium oxysporum* f.sp. *ciceris* representing various races collected from 13 major chickpea growing states of India was determined by using a set of translation elongation factor 1-alpha (TEF) primers (EF1-ATGGGTAAGGAAGGACAAGAC) and EF2- GGAGAGTACCAGTGCATCATGTT).

The genomic DNA of the isolates was successfully amplified with ~720bp amplicons. The isolates shared more than 90% sequence similarity with the TEF of *F. oxysporum* available in GenBank. Phylogeny tree constructed using bootstrap neighbor-joining analysis indicated the existence of high level of genetic variability among the isolates and grouped them into two major clusters. The first major cluster was further sub-divided into two sub-clusters. First sub-cluster consisted of 34 isolates including 24 isolates from the present study and 10 isolates from other parts of the world. Second sub-cluster had 10 Indian isolates along with one isolate from USA. Second major cluster consisted of 14 Indian isolates originating from different states. Each cluster had the isolates from different states of India indicating the existence of high variability among the population of Foc. Interestingly, majority of the foreign isolates were clustered in one group with Indian isolates representing nine states (Fig. 34).

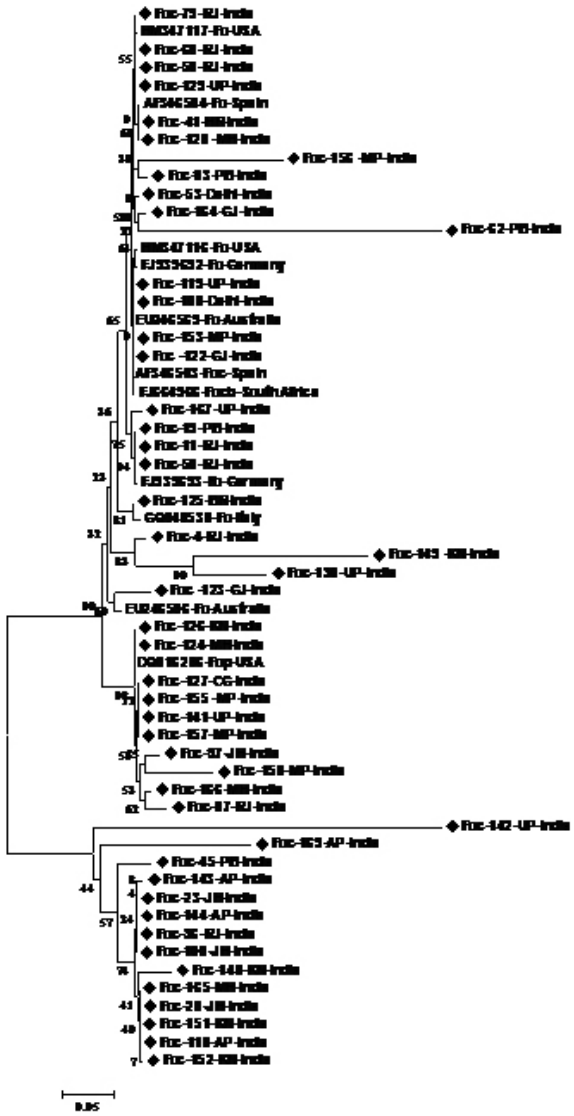

Fig. 34. Neighbor joining tree showing the phylogenetic relationships among isolates of *F. oxysporum* based on their TEF sequences. The sequences generated during this study were labeled (labeled diamond).
Fo- *F. oxysporum*, Foc- *F oxysporum* f. sp. *ciceris*, Focb- *F. oxysporum*. f. sp. *cubense*, Fop- *F. oxysporum* f. sp. *phaseol*

b. ß -tubulin gene analysis

The genomic DNA of 70 isolates was amplified with ~500bp amplicons using ß-tubulin primers, Bt1-TCCCCCGTCTCCACTTCTTCATG and Bt2-GACGAGATCGTTCATGTTGAACTC. The phylogeny tree constructed by bootstrap neighbor-joining grouped the isolates in two major clusters. All Indian isolates included in the present study were grouped in first cluster, whereas other *F. oxysporum* isolates representing different countries were grouped in the second cluster (Fig. 35).

c. Internal transcribed spacers (ITS) analysis

PCR amplification with specific primers ITS1 and ITS4 yielded a single DNA fragment ~550 bp in all isolates. The phylogeny tree (Fig. 36) grouped the isolates into two major clusters. All Indian isolates placed in first major cluster along with the isolates of other countries. Second major cluster had only two isolates, one each from Japan and Greece. First cluster was further sub-divided into two subclusters. One isolate each from Punjab, Karnataka, Andhra Pradesh and Jharkhand belonging to three different virulence groups were grouped in second sub-clusters, whereas all other isolates were grouped in first sub-cluster.

d. Calmodulin gene analysis

The genomic DNA of 70 isolates was amplified with ~500 bp amplicons by using a set of calmodulin primers CAL-737R (5'CATCTTTCTGGCCATCATGG 3') CAL-228F (5'GACTTCAAGGAGGCCTTCTC 3'). The products have been given for sequencing.

e. Universal rice primers (URPs)-PCR analysis

The genetic diversity of 70 isolates of Foc collected from different states of India representing various races were determined using universal rice primers (URPs). All URPs showed good amplification in all the isolates. The isolates were highly variable and the dendrogram derived from UPGMA analysis with 12 URPs grouped the isolates into eight clusters at 40% genetic similarity. Three clusters (I, VI and VIII) had single isolates in each from different states whereas, two clusters (II and VII) had two isolates from two different states. The cluster II consisted of 13 isolates from six states and cluster IV had maximum 35 isolates from 10 states, whereas 16 isolates from 8 states were grouped in cluster V (Fig. 37).

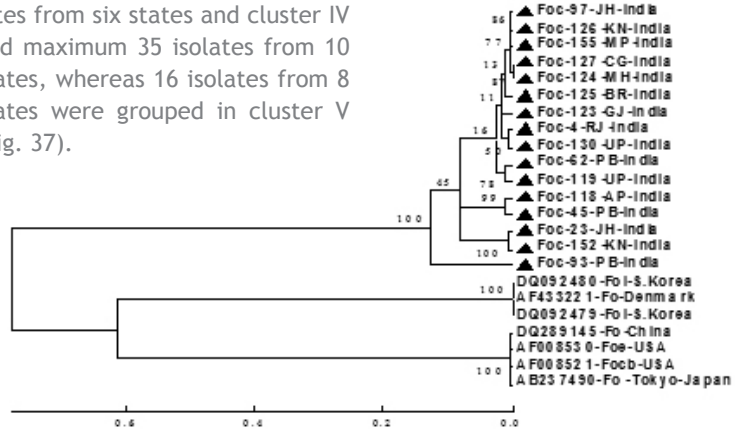

Fig. 35. Neighbor joining tree showing the phylogenetic relationships among isolates of *F. oxysporum* based on their Beta-tublin sequences. The sequences generated during this study were labeled (labeled Pyramid) Fol-*F. oxysporum*. f. sp. *lycopersici*, Fo- *F. oxysporum* Foe- *F. oxysporum*. f. sp. *erythroxyli*, Focb- *F. oxysporum* f. sp. *cubense*

The primers URP 13R and URP 2R amplified all the isolates and majority of the isolates showed similar DNA banding patterns. The dendrogram generated from URP 13R grouped the isolates into eight clusters. Majority of the isolates (49) from 13 states grouped together in a single cluster. The primer URP 2R grouped the isolates into seven clusters. About 90% isolates of a state grouped together and they were corresponding to the virulence groups.

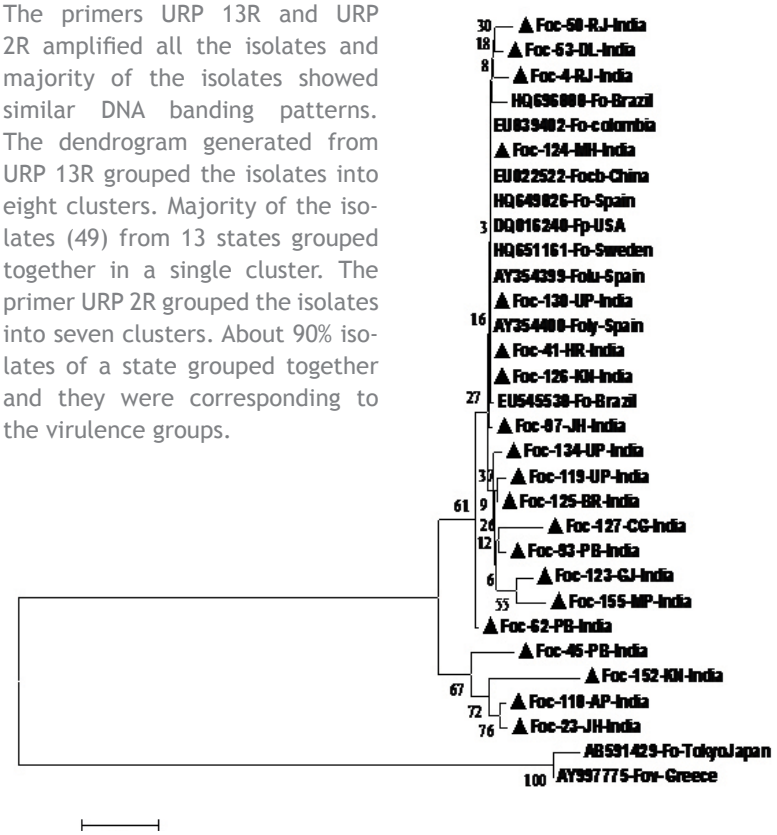

Fig. 36. Neighbor joining tree showing the phylogenetic relationships among isolates of *F. oxysporum* based on their ITS sequences. The sequences generated during this study were labeled (labeled Pyramid) Fo - *Fusarium oxysporum*, Focb - *Fusarium oxysporum*. f.sp. *cubense*, Fp- *Fusarium proliferatum*, Folu- *Fusarium oxysporum* f. sp. *luffaeFoly- Fusarium oxysporum* f. sp. *lycopersici*, Fov-*Fusarium oxysporum* f. sp. *vasinfectum*.

f. Simple sequence repeats (SSR) analysis

Four SSR primers were screened against 70 isolates of the pathogen and all of them showed good amplification (Fig. 38). The dendrogram grouped the isolates into six clusters at 20% genetic similarities. While the first cluster contained 14 isolates from eight states, the second one contained 27 isolates from 14 states, third 21 isolates from six states, fourth two isolates from two states, fifth five isolates from three states and the sixth one had a single isolate from Madhya Pradesh. Majority of the isolates obtained from a state were sub-clustered separately.

g. Random amplified polymorphic DNA analysis

The genetic diversity of 70 isolates of Foc collected from different states of India representing various races were determined by using RAPD. All RAPD primers showed good amplification of the isolates (Fig. 39). Relatedness among the isolates was estimated by means of scorable DNA bands.

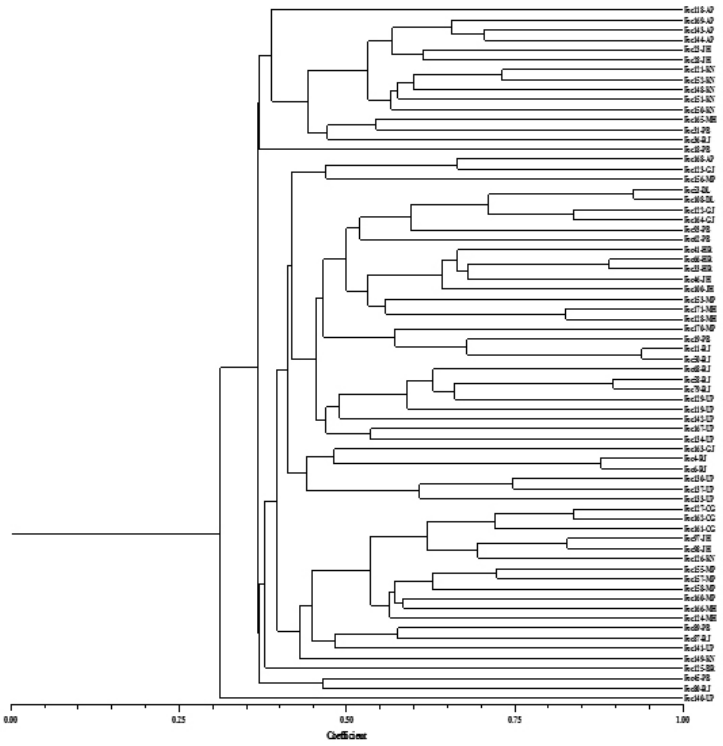

Fig. 37. Dendrogram derived from polymorphic DNA analysis of 70 isolates of *Fusarium oxysporum* f. sp. *ciceris* with 12 URP primers by UPGMA. The bottom scale is the percentage of Jaccard's similarity coefficient.

2 Diagnostics

ITS region based markers (FOC F1: 5'-AAGGAGACAACTCCCAAACCC CC) and FOC R1: 5'-CTTGCCG-CATAGGGCTCGC-3') were developed for detection of race/area specific (Rajasthan isolates-race 4) isolates of the *F. oxysporum* f. sp. *ciceris* causing wilt of chickpea. The markers were validated against 25 different Rajasthan isolates of the pathogen and it was able to amplify 322 bp size of amplicon (Fig. 40).

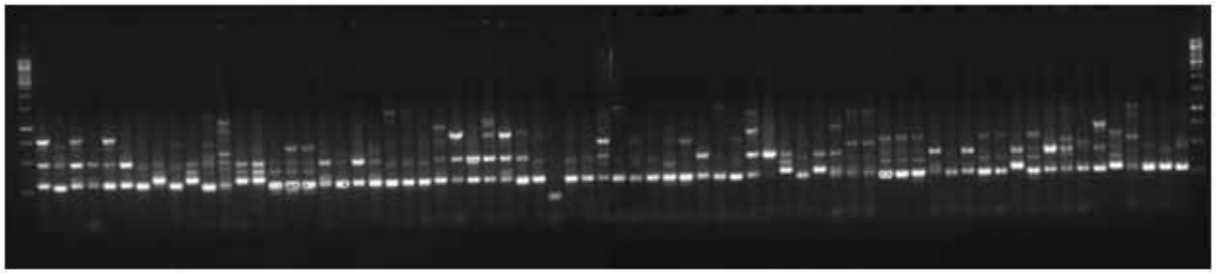

Fig. 38. DNA profile generated by SSR primer MB 18; M= marker- 1kb; Lanes 1-5 (Andhra Pradesh), 6-8 (Chhatisgarh), 9-10 (Delhi), 11-14 (Gujrat), 15-17 (Haryana), 18-23 (Jharkhand), 24-30 (Karnataka), 31-37 (Madhya Pradesh), 38-42 (Maharashtra), 43-49 (Punjab), 50-59 (Rajasthan), 60-69 (Uttar Pradesh) and 70 (Bihar) isolates of Foc.

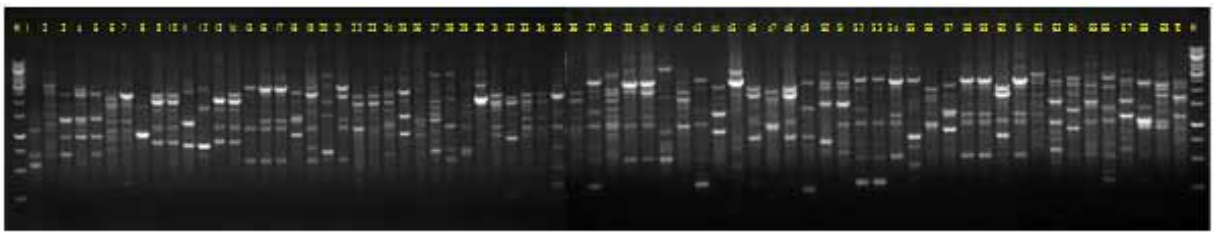

Fig.39. DNA profile generated by RAPD primer OPF-16 ; M= marker- 1kb; Lanes 1-5 (Andhra Pradesh), 6-8 (Chhatisgarh), 9-10 (Delhi), 11-14 (Gujrat), 15-17 (Haryana), 18-23 (Jharkhand), 24-30 (Karnataka), 31-37 (Madhya Pradesh), 38-42 (Maharashtra), 43-49 (Punjab), 50-59 (Rajasthan), 60-69 (Uttar Pradesh) and 70 (Bihar) isolates of Foc.

3 Host resistance

a. Evaluation of chickpea differentials against isolates of the pathogen

Virulence of 66 isolates of the pathogen was tested on a set of 10 differential cultivars of chickpea, namely, C 104, JG 74, CPS 1, BG 212, WR 315, KWR 108, GPF 2, DCP 92-3, Chaffa and JG 62 in net. The wilt incidence was recorded at 15 days interval up to maturity of the crop and the reactions were graded as resistant (0-20% wilt), moderately susceptible (>20 to 50% wilt) and susceptible (>50% wilt). The isolates were highly variable in causing wilt incidence ranging from 0-100% in various varieties and the incidences were mentioned into their reactions. Differential cultivars for the isolates originating from different states were identified (Table 34). Hence, based on differential responses, the isolates were categorized in to eight groups and those may be considered as new races or pathotypes of the pathogen in India.

4 Host pathogen interaction

a. Suppression subtractive hybridization (SSH)

Chickpea cultivar JG62 was selected for SSH library preparation. Plants were grown under glass house conditions and were inoculated with Foc 53 isolate and the plant material collected at 2, 3 and 5 days after inoculation as tester in SSH library preparation.

Fig. 40. Amplified product of 25 different isolates collected from different areas (Jaipur-FOC 4, 6, 7, 47, 48; Sardargarh-FOC 68, 69, 70, 71; Suratgarh-FOC 58, 73, 78; Churu-FOC 84, 85, 87; Sikar-FOC 2, 36; Alwar-FOC 5, 11; Jetsar-FOC 59, 79; Udaipur-FOC 50; Tonk-FOC 3; Sriganganagar-FOC 42, 60) of Rajasthan state with the Primer FOC F1 and FOC R1 and M - 100bp ladder at right side)

Table 34. Pathogenic groups and their differential cultivars of chickpea

Group	Differential cultivars	States
1	C 104 & GPF 2	Andhra Pradesh and Punjab
2	C 104 & KWR 108	Chhattisgarh, Jharkhand and Madhya Pradesh
3	BG 212 & GPF 2	Gujarat, Karnataka, Madhya Pradesh and Uttar Pradesh
4	JG 74	Madhya Pradesh and Uttar Pradesh
5	BG 212 & KWR 108	Delhi and Maharashtra
6	WR 315 & GPF 2	Rajasthan
7	WR 315 & KWR 108	Haryana
8	GPF 2 & DCP 92-3	Rajasthan

Un-inoculated plants used as a driver material in the study. Total RNA was isolated using SIGMA kit following manufacturer's instructions. The mRNA was purified using Qiagen kit. The cDNA was synthesized by using PCR-Select cDNA subtraction kit from Clontech (Palo Alto, CA, USA). The cDNAs were reverse transcribed from 2 µg mRNA from each sample pools separately and digested with RsaI and ligated to different adaptors. Two rounds of hybridization and PCR amplification were performed to enrich the differentially expressed sequences. The samples were electrophoresed on a 2.0% agarose gel to check the subtraction efficiency and the products were cloned with the suitable pGMT vector. Transformed colonies were selected and confirmed through colony PCR by using adapter specific primers (Fig. 41).

5 Disease management

a. Determination of tolerance in *Pseudomonas fluorescens* and *Rhizobium ciceri* to fungicides

Two sets of experiments were conducted for evaluation of fungicides against *P. fluorescens* (Pf-80) and *R. ciceri* (Cp-66) *in vitro* using paper disc plate method. Twelve fungicides, viz, Carboxin 37.5% + TMTD 37.5% WS (Vitavax power), metalaxyl 8% + mancozeb 64% WP (Ridomil MZ-72), captan 50% WP (Captaf), iprodione 25% + carbendazim 25% WP (Quintal), carbendazin 50% WP (Basatin), mancozeb 75 % WP (Indofil M-45), tetramethyl thiuram disulphide (TMTD) 75% WS (Thiram), copper oxychloride 50% WP (Blitox-50), carboxin 75% WP (Vitavax), metalaxyl 35% WS (Ri-

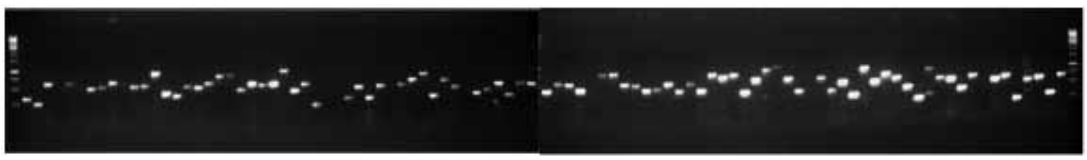

Fig. 41. Agarose gel showing amplified cDNA inserts from subtractive cDNA library with 1 kb molecular marker in chick pea inoculated with Foc 53 isolate

doxyl), thiophanate-methyl 70% WP (Topsin M) and propiconazole 35% EC (Result) at three concentrations were evaluated. The results clearly indicated that none of the fungicides was inhibitory to the growth of both the bacteria in plates as no inhibition zone was recorded.

b. *In vitro* evaluation of various seed treatments against the pathogen

Pusa 5SD a seed dressing formulation developed from a potential isolate of *T. harzianum* (IARI P 4) was selected for seed treatment along with bacterial antagonist *P. fluorescens* (PGPR), *Rhizobium ciceri* and fungicide vitavax power. In vitro experiment was conducted to evaluate the performance of these treatments alone and in combinations as seed treatment against the pathogen. The treatments were 1- *T.harzianum* (Pusa 5SD), 2- talc formulation of *P. fluorescens* (Pf-80), 3- talc formulation of *R. ciceri* (Cp-66), 4- vitavax power, 5- *T. harzianum* + *P. fluorescens*, 6- *T. harzianum*+ *R. ciceri*, 7- *T. harzianum* + vitavax power, 8- *P. fluorescens* + *R. ciceri*, 9- *P. fluorescens* + vitavax power, 10- *R. ciceri* + vitavax power, 11- *T. harzianum*+ *P. fluorescens* + *R. ciceri*, 12- *T. harzianum* + *P. fluorescens* + vitavax power, 13- *T. harzianum* + *R. ciceri* + vitavax power, 14- *P. fluorescens* + *R. ciceri* + vitavax power, 15- *T. harzianum* + *P. fluorescens* + *R. ciceri* + vitavax power and 16- control . Seeds were treated with fungicide at 2 g kg^{-1} seed while Pusa 5SD and talc based formulations were used at 4 g kg-Trebuchet MS of seed (10^8cfu g^{-1}) separately and for integrated treatment with half doses of fungicide (1g kg^{-1}) followed by bioformulations. Seeds of susceptible chickpea cultivar JG-62 were used in the experiment.

The result indicated that all the treatments evaluated provided protection to germinating seeds either by covering with the growth of *Trichoderma* or by creating inhibition zone around seeds (Fig. 42). The treatments that had Pusa 5SD (*T. harzianum*) as seed treatment either alone or in combination with others provided highest protection by covering the treated seeds with its growth. Pusa 5SD followed by Pusa 5SD + *Rhizobium*, Pusa 5SD + *P. fluorescens* + *Rhizobium* and Pusa 5SD + *P. fluorescens* showed maximum growth of *Trichoderma* around the seeds. The combination of Pusa 5SD + *P. fluorescens* + *Rhizobium* + vitavax power provided highest inhibition zone along with growth of *Trichoderma* around the seeds.

c. *In vitro* evaluation of PhytoFuRa isolates of *Trichoderma* species against *Fusarium oxysporum* f. sp. *ciceris*

The isolates of *Trichoderma* spp. were evaluated *in vitro* for volatile inhibition. Maximum growth inhibition was recorded in PhytoFuRa-5 followed by PhytoFuRa-11, PhytoFuRa-12, PhytoFuRa-15 and PhytoFuRa-7. The effect of non-volatile substances produced by the *Trichoderma* spp. against *Fusarium oxysporum* f. sp. ciceris revealed that all the isolates of *Trichoderma* spp. significantly inhibited the growth of Foc over control (Table 35). The maximum inhibition was observed in PhytoFuRa-10 followed by PhytoFuRa-3. PhytoFuRa-11 caused minimum growth inhibition. The culture filtrate of PhytoFuRa-5 caused the maximum growth inhibition of *F. oxysporum* f. sp. *ciceris* followed by PhytoFuRa-4, PhytoFuRa-8, PhytoFuRa-3 and PhytoFuRa-14. Considering over all performance, the isolates, PhytoFuRa-2, PhytoFuRa-3, PhytoFuRa-4, PhytoFuRa-10 and PhytoFuRa-13 may be recommended for the management of Foc.

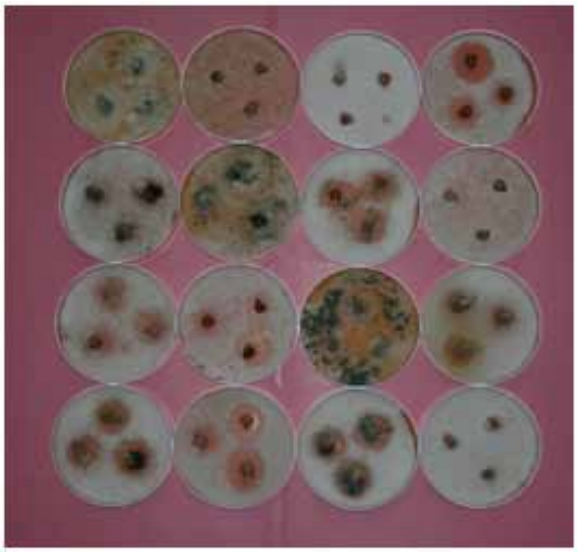

Fig. 42. Efficacy of seed treatment with Pusa 5SD (*Trichoderma harzianum* IARI P 4) and talc based formulation of *Pseudomonas fluorescens* (Pf 80), fungicide carboxin + TMTD (vitavax power) and *Rhizobium ciceris* alone and in combinations against *F. oxysporum* f. sp. *ciceris*.

Table 35. Effect of different isolates of *Trichoderma species* on radial growth inhibition of Delhi isolate of *Fusarium oxysporum* f. *sp. ciceris* incubation at 25+1°C

Treatment	Mean growth inhibition (%) of *F. oxysporum* f. sp. *ciceris* in		
	Dual culture (6 days)	Volatile com pounds (7 days)	Culture filterate (10 days)
T1 PhytoFuRa-1	45.4 (42.3)	19.7 (26.3)	17.8 (25.0)
T2 PhytoFuRa-2	44.2 (41.7)	05.5 (13.2)	19.9 (26.4)
T3 PhytoFuRa-3	53.8 (47.1)	20.3 (26.8)	24.0 (29.3)
T4 PhytoFuRa-4	52.6 (46.6)	18.9 (26.6)	26.4 (30.8)
T5 PhytoFuRa-5	45.0 (42.1)	23.2 (28.7)	27.7 (31.5)
T6 PhytoFuRa-6	42.6 (40.7)	18.9 (25.7)	14.6 (22.4)
T7 PhytoFuRa-7	39.0 (38.6)	21.6 (27.6)	19.3 (26.0)
T8 PhytoFuRa-8	40.2 (39.4)	8.6 (17.0)	25.0 (30.0)
T9 PhytoFuRa-9	38.4 (38.9)	07.0 (14.8)	14.9 (22.7)
T10 PhytoFuRa-10	54.1 (47.4)	08.0 (16.3)	18.1 (25.1)
T11 PhytoFuRa-11	29.8 (33.1)	22.8 (28.5)	21.9 (27.9)
T12 PhytoFuRa-12	38.6 (38.4)	22.4 (28.2)	13.5 (21.5)
T13 PhytoFuRa-13	47.4 (43.5)	04.3 (12.0)	17.8 (24.9)
T14 PhytoFuRa-14	40.7 (39.6)	18.9 (25.7)	23.6 (29.0)
T15 PhytoFuRa-15	42.2 (40.5)	22.0 (28.0)	13.1 (21.2)
SEm +	(0 .7)	(1.4)	(1.0)
CD (p=0.05)	(2.0)	(4.0)	(2.7)

The figures in parentheses are transformed angular values

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Co- investigators
Dr. R. G. Chaudhary
Dr. S. Datta

1 Bio-diversity

a. Identification and confirmation of variants of *Fusarium udum* and *F.oxysporum* f.sp. *ciceri*

Forty six isolates of *F.udum* from Uttar Pradesh, Bihar, Jharkhand, West Bengal, Madhya Pradesh, Maharashtra, Andhra Pradesh, Karnataka, Tamilnadu, Haryana, Rajasthan, Punjab and Delhi were inoculated on seven differential genotypes by sick soil inoculation method. Among the differentials, reaction of genotypes C11, ICP 8863, ICP 9174 and Bahar could differentiate the isolates as five variants (variants 1, 2, 3, 4 and 5) (Table 36).

Fifty nine isolates of *F. oxysporum* f.sp. *ciceri* from U.P., M.P., Chattishgarh, Gujarat, Maharashtra, Karnataka, Andhra Pradesh, Haryana, Punjab, Rajasthan, Jharkhand, and Delhi have been inoculated on 10 differential genotypes by sick soil method for pathogenic variability and the experiment is in progress.

Table 36. Pathogenic variability in isolates of *Fusarium udum* from different states in India

Differential	Wilt reaction and variants identified				
	Variant 1	Variant 2	Variant 3	Varian 4	Variant 5
S.ch (Bahar)	S	S	S	S	S
C 11	R	S	S	R	S
ICP 8863	R	R	S	R	R
ICP 9174	R	R	R	S	S
States (2010-11)	A.P., M.S Karnataka M.P., U.P.	A.P. T.N., Karnataka, M.P., U.P	Bihar, Karnataka, M.P., U.P.	Bihar, M.S. U.P.	Karnataka U.P.
Isolates unidentified (Susceptible reaction on all 4 differentials)	Bihar (1), Andhra Pradesh (1), Maharashtra (1), M.P. (1), Rajasthan (2), Haryana (3), Punjab (1), Delhi (1), U.P. (1 isolate)				

2 Diagnostics

Fifty isolates of *F. udum* representing distinct cultural, morphological and diverse ecological niches from different pigeonpea growing areas were studied for their diversity and development of diagnostic markers. Twenty Nine *Fusarium* specific primers selected on the basis of their diagnostic potential were also screened on these 50 isolates to identify specific amplicons of diagnostic value in *F.udum*. Out of 29, only four primers viz. FDP3 (5'CAGCAGTGAGGAATATTGGTCAATG3', 3'GCGGATCATCGAATTAAATAACAT5'-730-739bp); FDP4 (5'ATGGGTAAGGAAGACAAGAC3', 5'GGAAGTACCAGTGATCATGTT3'-709-721bp), FDP25 (5'ATGGGTAAGGA(A/G)GACAAGAC3', 3'GGA(G/A)GTACCAGT(G/C)ATCATGTT5'-750bp) and FDP29 (5'ATGGGTAAGGAGGACAAGAC3',5'GGAAGTACCAGTGATCATGTT3'-750bp) produced good amplification in all the 50 isolates of *F.udum* and the amplicon size ranged from 730-750 bp.

The sequencing and homology search from fourteen isolates amplified with primer FDP 29 showed maximum similarity with Translation Elongation Factor 1-alpha of different species of *Fusarium*. The similarity was as high as 99% for isolate Fu 88 with *F. udum* strain NRRL22949 translation elongation factor 1 alpha gene, partial cds. In general, most of the sequences showed more than 90% homology and the 'e' value in BLAST was as low as 1e-180 indicating the robustness of the results.

The phylogram of the sequences amplified by FDP 29 primer in the selected 14 isolates was also subjected to cladistic analysis and a phylogram of the sequences were generated (Fig. 43). The phylogram which is indicative of the relatedness of the sequences and therefore implies on genetic relatedness among the isolates, grouped the 14 isolates into two major groups I and II. Group I was further subdivided into two subcluster IA comprising of four isolates Fu 37, Fu 43, Fu 49 and Fu 61 and subcluster IB comprising of isolates F 8 and I 9. Cluster II had four isolates F3, I 3, NF 3 and NF15. One isolate F 17 did not group with any other isolate and fell outside the clusters.

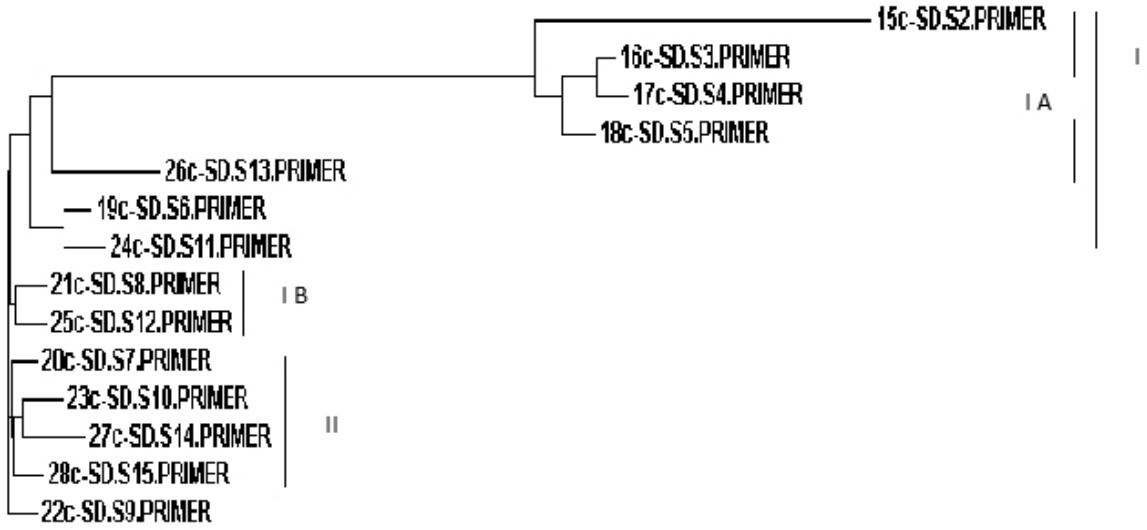

Fig. 43. Phylogram of sequences amplified by FDP 29 primer in fourteen isolates of *Fusarium udum*.

3 Host resistance

a. Resistance screening in wilt sick plot

Four hundred and fiftyfive genotypes of pigeonpea comprising of germplasm lines (250), breeding lines (29), promising lines (66), national elite varieties (17), disease resistant genetic stock (13), wilt differential genotypes (11), hybrid pigeonpea lines (09), wilt resistant donors (30), and multiple disease resistant lines (14) have been sown in wilt sick plot, predominantly infected with variant 2 of *F.udum* for resistance screening to wilt.

b. Resistance screening against different variants/races

Pigeonpea: Seventeen wilt resistant donors identified at IIPR Kanpur and under AICRP programme viz., AWR 74/15, BDN 1, BDN 2, Banda palera, MA 3, JA 4, ICP 8858, ICP 8859, ICP8863, ICP7035, ICP 9174, KPL 43, KPL 44, PI 397430, IPF 9, IPA 38 and IPA 203 have been grown in large cement pots inoculated with variants 1, 2, 3, 4, and 5 of *F. udum* for evaluating their resistance against these variants.

Chickpea: Twenty nine wilt resistant genotypes viz., IPC nos. 2004-3, 2004-8, 2004-34, 2005-5, 2005-15, 2005-18, 2005-19, 2005-24, 2005-26, 2005-27, 2005-30, 2005-34, 2005-35, 2005-37, 2005-41A, 2005-41B, 2005-43, 2005-44, 2005-45, 2005-46, 2005-52, 2005-59, 2005-62, 2005-64, 2007-4, 2007-9, 2007-68, KOD 1255, and GNG 1861 have been sown in pots inoculated with races 1,2,3,4,5 and 6 of *F. oxysporum* f.sp.*ciceri* for resistance screening.

4 Disease management

Experiment on the efficacy of seven strains of *Trichoderma viride*, *T.virens* and *T.harzianum* in controlling pigeonpea wilt is in progress. A pot experiment with soil application and seed soaking of salicylic acid, culture filtrate of *T. viride* (IIPRT 11), *T.virens* (TVS 12) and *T. harzianum* (IIPRT 31) has been conducted to evaluate efficacy of these treatments in imparting resistance to pigeonpea plants against wilt disease is in progress.

Twenty isolates each of *F. udum* and *F. oxysporum* f. sp. ciceri from different pigeonpea and chickpea growing areas of the country have been deposited at NBAIM, Mau for long term storage and maintenance.

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1 Biodiversity

Grouping of different isolates of wilt pathogens was done based on pathogenicity. Among 85 *F. oxysporum* f. sp. *lycopersici* (FOL) isolates tested, six were highly virulent, 38 virulent and two avirulent. In case of 54 FS isolates tested, 11 were highly virulent and 43 were virulent.

2 Diagnostics

DNA was isolated from 30 isolates of *Fusarium* spp. and amplified the ITS region with universal primer ITS1 (5'-TCCGTTGGTGAACCAGCG G-3') and ITS4 (5'-TCCTCCGCT-TATTGATATGC-3') which resulted in amplification of approximately 550 bp of fragment in all the *Fusarium* spp. selected.

Four of the 27 primers screened, produced clear bands on the RAPD amplifications and were used for analysis. The primers (OPC-13, OPC-14, OPC-18 and OPD-18) amplified a total of 72 markers. The total number of clear bands obtained from each primers ranged from 2 (OPC-13) to 6 (OPC-14), with an average of 6.4 bands per primer. An UPGMA dendrogram based on Jaccard's similarity coefficient for the sixteen samples of FOL indicates four major clusters viz., A, B, C and D.

3 Disease management

a. Screening fungal and bacterial bioagents

Among 89 antagonistic isolates screened against FOL and FS, 24 isolates showed significantly high level of inhibition of the pathogens apart from improving the germination of chilli and tomato (Table 38 & 39). Hence the selected isolates were forwarded to evaluate the efficacy against the diseases under greenhouse conditions.

Among the 24 different bio-agents evaluated for their efficacy against wilt incidence and plant growth (seed germination and plant height) in chilli and tomato, 8 isolates (BATF-39-1, BATF-43-1, BATB-4, BATB-20, BACF-6, BACF-13-4, BACB-5 and BACB-9) were found highly effective in reducing the wilt incidence and plant health improvement among chilli and tomato in vivo (Table 40 & 41)

Table 37. Screening of chilli and tomato germplasm lines for resistance against wilt disease

Reaction	No. of lines	Name of the germplasm
		Chilli
Resistant	8	Local colle.-35, PBC-904-UP, CV-1, BS-5, COO-713, COO-304, PDC-24 and LCA-335
Moderately resistant	19	Local collection-3, Ajeet, Arka abhir, 97-71-25-2, LCA-437, 0337-7065, CV-2, PBC-1439, 97-7116, LCA-414, LCA-402, EC-587050, Chandra Mohan Colle., Numex Teulight, DSL-2, SBT-12626, AKC-89/38, Kalyanpur Chanchal and PC-1
		Tomato
Resistant	3	IIVR-61, IIVR-40 and IIVR-28
Moderately resistant	8	IIVR-1, IIVR-10, IIVR-14, IIVR-18, IIVR-27, IIVR-29, IIVR-49 and IIVR-67

Table 38. *In vitro* efficacy of fungal and bacterial bio-agents on mycelial inhibition of FOL and tomato seed germination

Fungus	*Mycelial inhibition (%)	*Seed germination (%)	Bacterium	*Mycelial inhibition (%)	*Seed germination (%)
BAT F - 39 -1	92.86	100.0	BATB - 1	45.65	30.00
BATF - 35	43.30	100.0	BATB - 2	41.70	50.00
BAT F- 36 - 2	43.30	100.0	BATB - 3	1.00	50.00
BAT F- 25 - 1	79.46	100.0	BATB - 4	47.96	83.30
BAT F- 34 - 1	83.48	100.0	BATB - 5	55.18	40.00
BAT F- 7	58.93	100.0	BATB - 6	4.57	63.30
BAT F- 14	37.50	100.0	BATB - 7	4.98	50.00
BAT F- 22	66.00	100.0	BATB - 8	2.08	70.00
BAT F- 40 - 3	24.09	100.0	BATB - 9	43.98	40.00
BAT F- 42 - 1	79.90	100.0	BATB - 10	12.86	60.00
BAT F- 43 - 1	92.86	100.0	BATB - 11	50.63	36.60
BAT F- 27 - 2	23.21	90.00	BATB - 12	57.26	30.00
BAT F- 26 - 2	58.48	90.00	BATB - 13	00.00	80.00
BAT F- 40 - 4	23.21	90.00	BATB - 14	46.47	46.60
BAT F- 39 - 3	24.56	90.00	BATB - 15	48.96	50.00
BAT F- 4	78.12	86.60	BATB - 16	36.76	63.30
BAT F- 29	59.81	86.60	BATB - 17	8.30	30.00
BAT F- 25 - 1	79.46	80.00	BATB - 18	37.34	30.00
BAT F- 36 - 1a	92.86	80.00	BATB - 19	56.42	56.60
BAT F- 23 - 1	24.56	80.00	BATB - 20	50.20	70.00
BAT F- 40 - 1	24.09	80.00	BATB - 21	24.89	50.00
BAT F- 11	83.48	73.30	BATB - 22	51.45	23.00
BAT F- 36 - 1b	92.86	73.30	Control	00.00	40.00
BAT -F-17	86.60	70.00	CD (5%)	1.52	3.1
BAT F- 32	24.56	73.30			
BAT F- 25 - 4	24.09	63.30			
BAT F- 37 - 1	18.75	46.60			
BAT F- 28 - 2	78.57	63.30			
BAT F- 20	18.29	66.60			
BAT F- 41 -1	62.95	66.60			
Control	00.00	70.00			
CD (5%)	1.2	3.6			

*Values were arc sine transformed before the analysis

b. Screening of fungicides

Out of eight different fungicides tested against the wilt pathogens and crop compatibility, flusilazole 40% EC, carbendazim 8% + mancozeb 64% and tricyclazole 75%WP were found to be effective in the inhibition of mycelial growth of the pathogens at 0.1% and compatible with the crops (Table 42 & 43).

c. Integrated management of wilt diseases of chilli and tomato

A field trial consisting of tested integrated components viz., eight bio-agents (BATF-39-1, BATF-43-1, BATB-4, BATB-20, BACF-6, BACF-13-4, BACB-5 and BACB-9), three fungicides (flusilazole 40% EC, carbendazim 8% + mancozeb 64% and tricyclazole 75%WP) and two botanicals (10% extract of datura and garlic) against wilt disease in tomato indicated the superiority of BATF-39-1 with lowest PDI (1.33), followed by BATF-43-1 (2.66), Datura (4.00), Carbendazim+Mancozeb (4.66) and Garlic (6.00), respectively, in comparison to control with PDI (16.00) after 60 DAT. In case of fruit yield similar result was observed in all treatments.

Chilli field experiment also showed the same result as tomato i.e. BATF-39-1 showed minimum wilt incidence (1.33) followed by BATF-43-1 (2.00) as compared to control having a maximum PDI (9.33). A significant observation in case of chilli is that there is no wilt symptom in botanical treated plots after 60th DAT. The final yield data is still under progress. Another field trial consisting of evaluation of 15 biocontrol agents (obtained from Network Project Co-ordinator) against chilli and tomato is also in progress.

Table 39. In vitro efficacy of fungal and bacterial bio-agents on the mycelial inhibition of FS and chilli seed germination

Fungus	*Mycelial inhibition (%)	*Seed germination (%)	Bacterium	*Mycelial inhibition (%)	*Seed germination (%)
BACF - 11 - 3	31.70	100.00	BACB - 5	50.41	66.60
BACF - 51	34.15	63.30	BACB - 9	35.52	73.30
BACF - 8 - 1	62.60	86.60	BACB - 26	26.83	73.30
BACF - 2 - 3	54.70	80.00	BACB - 1	29.87	53.30
BACF - 13 - 3	58.81	96.60	BACB - 41	25.60	66.60
BACF - 6	84.15	96.60	BACB - 42	19.52	63.60
BACF - 6 - 1	50.03	96.60	BACB - 13	26.43	56.60
BACF - 1-1	81.30	86.60	BACB - 25	23.17	53.30
BACF - 6 - 2	52.21	90.00	BACB - 6	27.65	30.0
BACF - 8	34.56	63.30	BACB - 22	23.17	40.0
BACF - 14 - 3	49.59	70.00	BACB - 26	19.93	40.00
BACF - 9 - 3	70.73	100.00	BACB - 40	23.58	66.60
BACF - 7 -1	69.51	90.00	BACB - 29	39.84	40.0
BACF - 9 - 2	63.41	80.00	BACB - 7	42.28	60.0
BACF - 6 - 3	73.98	96.60	BACB - 16	39.04	43.30
BACF - 5 - 4	75.61	83.30	BACB - 30	43.50	50.00
BACF - 13 - 3	35.12	60.00	BACB - 11	26.83	63.60
BACF - 4 - 4	57.73	66.60	Control	00.00	63.30
BACF - 13 - 4	80.48	96.60	CD (5%)	2.1	2.6
BACF - 14 - 1	53.26	86.60			
Control	00.00	83.30			
CD (5%)	1.42	280			

*Values were arc sine transformed before the analysis

Table 40. Efficacy of fungal and bacterial bio-agent against wilt disease of tomato invivov

Fungal bio-agent	DI (%)	PDI*	Bacterial bio-agent	DI (%)	PDI*
BATF-39-1+ Pathogen	2.3	0.00	BATB-4+ Pathogen	12.3	6.66
BATF-25-1+ Pathogen	22.3	10.66	BATB-20+ Pathogen	11.0	8.00
BATF-34-1+ Pathogen	29.3	13.33	BATB-19+ Pathogen	25.3	12.00
BATF-7+ Pathogen	42.3	22.66	BATB-16+ Pathogen	28.6	13.33
BATF-22+ Pathogen	32.3	13.33	BATF-15+ Pathogen	29.3	14.66
BATF-42-1+ Pathogen	30.0	9.33	BATB-4	0.00	0.00
BATF-43-1+ Pathogen	1.6	0.00	BATB-20	0.00	0.00
BATF-36-1+ Pathogen	22.6	18.66			
BATF-39-1	0.00	0.00			
BATF-25-1	0.00	0.00			
BATF-34-1	0.00	0.00			
BATF-7	0.00	0.00			
BATF-22	0.00	0.00			
BATF-42-1	0.00	0.00			
BATF-43-1	0.00	0.00			
BATF-36-1	0.00	0.00			
Carbendazim + Pathogen	17.3	6.66			
Pathogen inoculated Control	48.6	30.66			
Healthy Control	0.0	0.00			
CD (5%)	4.1	2.2			

Table 41. Effect of integrated treatments on wilt of chilli and tomato under field conditions

Treatment	Chilli DI (%)	Chilli PDI*	Tomato DI (%)	Tomato PDI*	Tomato Yield** (Q/ha)
BATF-39-1	6.66	1.33	6.66	1.33	190.88
BATF-43-1	10.00	2.00	13.33	2.66	154.88
BATB-4	20.00	4.66	50.00	13.33	140.27
BATB-20	36.66	8.00	60.00	17.33	128.16
BACF-6	26.66	6.00	40.00	10.00	128.61
BACF-13-4	20.00	4.00	43.33	10.66	127.44
BACB-5	20.00	4.66	46.66	12.00	115.44
BACB-9	20.00	4.66	60.00	16.00	137.11
Flusilazole	30.00	6.66	40.00	10.66	38.38
Carbendazim + Mancozeb	10.00	2.66	23.33	4.66	107.27
Tricyclazole	10.00	2.00	33.33	7.33	121.22
Carbendazim	23.33	4.66	50.00	10.66	176.16
Datura	0.00	0.00	20.00	4.00	115.00
Garlic	0.00	0.00	30.00	6.00	123.72
Control	33.33	9.33	56.66	16.00	64.16
CD (5%)	6.7	2.3	5.63	2.6	10.2

*Values were arc sine transformed before the analysis

** Yield upto two harvests

Table 42. *In vitro* efficacy of fungicides on the inhibition of mycelium of FS and FOL

Fungicide	*Mycelial inhibition FS (Chilli) 0.05 %	FS (Chilli) 0.1 %	FOL (Tomato) 0.05%	FOL (Tomato) 0.1%	Germination and growth at 0.1 % Chilli	Tomato
Flusilazole 40% EC	74.77	78.57	90.10	91.67	+	+
Carbendazim 8% + Mancozeb 64%	92.85	92.85	80.73	89.07	+	+
Penconazole 10% EC	64.77	68.10	79.68	84.64	-	-
Tebuconazole 250 EC	92.85	92.85	92.18	92.18	+	+
Tricyclazole 75% WP	66.20	90.00	84.90	90.63	+	+
Triadimephon 25% WP	15.24	8.10	71.87	81.77	-	-
Propiconazole 25% EC	4.77	7.62	77.60	80.73	+	+
Carbendazim 50% WP	31.91	35.71	78.12	82.30	+	+
Control	0.00	0.00	0.00	0.00	+	+
CD (5%)	2.2	1.9	3.1	1.1		

*Values were arc sine transformed before the analysis.+ germination and growth were not affected - germination and growth were affected.

Table 43. Efficacy of fungal and bacterial bio-agentes against wilt disease of chilli *in vivo*

Fungal bio-agent	DI (%)	PDI*	Bacterial bio-agent	DI (%)	PDI*
BACF-6+ Pathogen	23.3	8.00	BACB-5+ Pathogen	12.3	8.00
BACF-13-4+ Pathogen	18.6	6.66	BACB-9+ Pathogen	11.0	6.66
BACF-9-3+ Pathogen	42.3	12.00	BACB-7+ Pathogen	29.6	13.33
BACF-6-3+ Pathogen	50.3	12.00	BACB-26+ Pathogen	37.2	13.33
BACF-1-1+ Pathogen	52.3	13.33	BACB-30+ Pathogen	40.2	12.00
BACF-5-4+ Pathogen	46.6	13.33	BACB-5	0.00	0.00
BACF-6	0.00	0.00	BACB-9	0.00	0.00
BACF-13-4	0.00	0.00	BACB-7	0.00	0.00
BACF-9-3	0.00	0.00	BACB-26	0.00	0.00
BACF-6-3	0.00	0.00	BACB-30	0.00	0.00
BACF-1-1	0.00	0.00	Carbendazim+pathogen	12.6	6.66
BACF-5-4	0.00	0.00	Pathogen control	53.2	29.33
Carbendazim+pathogen	25.3	9.33	Healthy control	0.00	0.00
Pathogen inoculated control	68.6	32.00	CD (5 %)	3.0	1.65
Healthy control	0.0	0.00			
CD (5 %)	4.2	1.9			

DI- disease incidence

*Values were arc sine transformed before the analysis.

NATIONAL RESEARCH CENTRE FOR BANANA TIRUCHIRAPALLY

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1 Biodiversity

Genetic diversity analysis for 91 isolates of Foc, which includes 66 representatives isolates from different parts of India, 15 isolates of VCG from Australia (for comparison) and 10 isolates of non-pathogenic *Fusarium oxysporum*, was carried out by RFLP analysis of elongation factor 1α region using 4 different restriction enzymes viz., Hha I, Msp I, Rsa I and Taq I. The banding pattern indicated the presence 15 different groups. Among these, the group 1 has 80% of the Indian Foc isolates and grouped under race-1 and race-1 VCGs obtained from Australia. Interestingly, this RFLP analysis has clearly distinguished the VCGs of race-1 and race-2 from the VCGs of race-4 isolates (Table 44). Besides, this RFLP analysis has grouped all the non pathogenic Fo isolates in the group 1 but failed to distinguish the pathogenic Fo from the non pathogenic Fo.

Validation of Foc specific molecular marker was done for detection from the plant under field condition. For this, DNA was isolated from root, corm and pseudostem of both Foc infected plants and non infected plants and subjected to PCR using Foc specific SCAR marker which has already been tested successfully under *in vitro* conditions. The SCAR marker detected the pathogen present in corm and pseudostem of infected plants (Fig. 44).

Table 44. Genetic diversity of Foc isolates of India and VCGs by RFLP analysis of elongation factor α 1

Group	Isolates	Variety	Collection site	TEF Genotype			
				Hha I	Msp I	Rsa I	Taq I
1.	Thai-7- 2 4192, W93/395, W93/404 ,Thai -6-1, Aus 23997, Thai 1-1, India 6,1, Aus 22408, 33, 34, 35, 40, 41, 43,44, 45, 46, 47, 51, 52, 53, 55, 56, 58, 82, 85, 76, 89, Sir, Dar, 120, 199, 97, 98, 99, 100, 121, 129, 130, 132, 136, 137, 142, 143, 146,149, 151, 153, 157, 167, 170,171, 172,175,177, 181, 182, 183, 184, 186,191, NP1, NP2, NP4, NP5,NP7, NP8, NP9, NP10, NP12,	Monthan , Neypoovan, Rasthali, Karpuravallli, Nattu Monthan	Thailand, Australia, India	A	A	A	A
2.	Mal 58 Phil 8	VCG 01217 VCG 0123	Malaysia Philippines	A	A	A	B
3.	Mal 42 23746	VCG 01216 VCG 0120/01211	Malaysia	A	A	B	B
4.	Phil 6	VCG 0126	Australia				
5.	36	Rasthali	Philippines	B	A	B	B
6.	73 42	Karpuravalli Neypoovan	TN TN	A C	B A	C A	C A
7.	54	Monthan	TN	A	A	E	A

2 Diversity

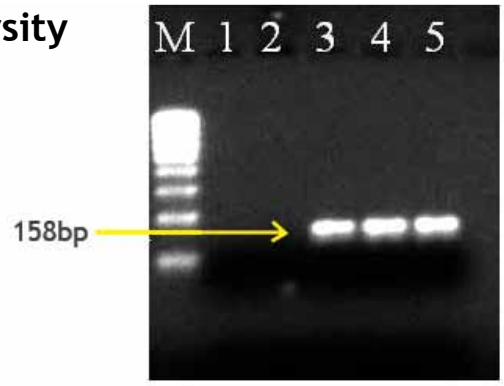

Fig. 44. Validation of scar marker for detection of Foc from banana tissues. M – Marker (100 bp) 1-Control (without infection), 2- Root, 3-Corm,4-Pseudostem, 5- Foc culture

For detecting from the soil, an easiest method of DNA extraction from just 2 g of soil without PCR inhibitors was standardized. The DNA extracted from infested and non-infested soils have been subjected for PCR amplification using Foc specific SCAR marker which has generated expected amplicon only from the Foc infested soil indicating that the SCAR marker can be used to detect the presence of pathogenic Foc present in the soil.

3 Disease management

a. Evaluation of rhizospheric and endophytic *Trichoderma* isolates

The evaluation of six rhizospheric isolates of *Trichoderma* against Foc individually and in different combinations was carried out under pot culture condition in cv. Grand Naine. Among the treatments the soil application of combination of *Trichoderma* spp. viz. K2T5 + *T.harzianum*, K2T5 + *T. pseudokoningii*, *Trichoderma* spp.-poovan + *T. harzianum*, *Trichoderma* spp. + *T .pseudokoningii*, recorded lowest disease score of 1.2 to 1.5 when compared to control (5.5%). Besides, there was a substantial increase (18 to 31%) in plant height, girth and total number of leaves also.

Among the 11 endophytic *Trichoderma* spp. evaluated against *Fusarium* wilt by root dipping method in cv. Grand naine, the isolate Bc2 and Prr2 has given 100% protection against Fusarium wilt disease under pot culture condition. Besides, these isolates have increased the height (169%), girth (205%), number of leaves (88%) and number of roots (230%) when compared to Foc alone inoculated plants.

The evaluation of combined application of 12 endophytic *Trichoderma* spp. isolates and two rhizospheric *Trichoderma* spp isolates viz. *T. koningii* and *T. harzianum* isolates applied in different combinations @ 30g/ plant as rice chaffy grain formulation in cv. Grand Naine under pot culture condition indicated that the application of endophytic *Trichoderma* strain BC2 + rhizospheric *T. koningii*, endophytic *Trichoderma* spp. strain Dsr1 + rhizospheric *T. koningii* and endophytic Trichoderma strain prr2 + rhizospheric *T. harzianum* isolate completely controlled the *Fusarium* wilt disease as there was no expression of either external or internal symptoms of the disease even after six months of planting.

b. Evaluation of rhizospheric and endophytic bacterial isolates

Soil application of five isolates of bacteria against Foc indicated that the application of Jrb1 recorded lowest disease score (2.00) as against Foc alone inoculated plants (5.0) and significantly increased the growth. Among the six endophytic bacterial isolates, evaluated against *Fusarium* wilt disease in cv. Grand naine, the root dipping of TC plants with Klr4 and Tvpr1 recorded lowest disease score of 1.4 as against the disease score of 5.0 in the Foc alone inoculated plants with increased height (61%), girth (84%), number of leaves (21%) and number of roots (150%).

The evaluation of combined application of six isolates of endophytic bacteria and four isolates of rhizospheric bacteria applied as soil drench in different combinations @30g/ plant as talc cum powder formulation in cv. Grand Naine under pot culture condition indicated that the application of endophytic bacteria *Pseudomonas putida* C4r4 + rhizospheric *Bacillus* spp. Jrb1, endophytic *Achromobacter* sp.Gcr1 + Rhizospheric *Bacillus cereus* strain Jrb5, endophytic *Rhizobium* sp. Lpr2 + rhizospheric *Bacillus* sp. Jrb1, endophytic *Bacillus* spp. Tvpr1 + rhizospheric *Bacillus* sp., endophytic *Bacillus* spp Tvpr1 + rhizospheric P. *putida* Jrb2 isolate completely controlled the *Fusarium* wilt disease (Score-1.0 -helathy) as there was no expression of either external or internal symptoms of the disease even after six months after planting.

c. Evaluation of effective endophytic actinomycetes

Bio-priming of banana plants cv. Grand Naine with 11 different endophytic *Actinomycete*s spp. isolates individually under pot culture condition indicated that out of 11 isolates, four strains viz. Dsc1, Enr1, Pjr1 and Gcc2 recorded 80% reduction in disease incidence com pared to control.

d. Compatibility of effective endophytic bacterial and actinomycetes isolates with certain effective fungicides

The compatibility of six effective endophytic bacterial antagonists and five endophytic actinomycetes with three different fungicides (Carbendazim, Difenaconazole and Propiconazole) effective against *Fusarium* wilt disease was assessed at five different concentrations (0.01 to 1%) by poison food technique in KB broth. The spectrophotometric reading of the bacterial suspension showed that there was no reduction in OD value due to the addition fungicides at all its

concentration tested indicating that all these effective bacterial and actinomycetes are compatible with all these three effective fungicides. This study gives scope that the antagonists can be applied along with fungicides for an efficient and effective management of the disease.

e. Evaluation of botanicals against *Fusarium* wilt disease

Six effective botanicals were evaluated against *Fusarium* wilt disease under pot culture conditions. Extracts from species such as *Alpinia* spp., Hibiscus spp. and Zimmu applied as dipping of plants for 11/2 hrs. + soil drench at @ 250 ml/ pot recorded 100% reduction of wilt incidence compared to control. Evaluation of *Zimmu* at different concentration from 5% to 100% applied as drenching and dipping against Foc in cv. Grand Naine indicated that either drenching with zimmu extract at 50 or 100% or dipping the roots at 50 or 100% completely contained the disease. Besides, the treatment of Zimmu leaf extract has increased the height (72%), girth (59%), number of leaves (39%) and number of roots (70%) compared to Foc alone inoculated plants.

f. Genetic diversity of effective *Trichoderma* spp.

Genetic diversity analysis carried out for 27 endophytic and 16 rhizhospheric *Trichoderma* spp. effective against *Fusarium* wilt pathogen by rDNA-ITS-RFLP analysis using six restriction enzymes viz. EcoRI, HhaI, HinfI, TaqI, MspI, grouped all these 43 isolates into two major clusters viz. A & B. The cluster A contains 20 isolates of which 14 are rhizospheric and six are endophytic. The cluster B contains 23 isolates of which 21 are endophytic and only two are rhizospheric and it was concluded that the rDNA-ITS-RFLP analysis separated all the *Trichoderma* isolates in to two major groups based on their habitat as endophytic and rhizospheric. However, this phylogenetic analysis could not differentiate the Foc effective from Foc ineffective *Trichoderma* spp. isolates (Fig. 45).

g. Development of fungicide resistant *Trichoderma* spp. mutant by UV-irradiation

To reduce the fungicide applications as well as to achieve the complete control of the disease, an attempt has been made to develop fungicide resistant *Trichoderma* spp. All the effective *Trichoderma* isolates when tested with different concentration of fungicides viz. carbendazim and difenaconazole (1% to 0.01%) *in vitro* showed complete suppression. All the isolates were then exposed to UV light at different intervals from 10 min. to 50 min and tested for their survival and multifunction characters. The *invitro* analysis made for both wild and UV mutated isolates of both rhizosphere and endophytic *Trichoderma* spp. indicated that all the antagonistic and growth promoting characters drastically increased which ranged from 4% to 253% in the mutated strains compared to wild strains. In certain cases, the strains which failed to produce IAA and HCN, were activated/ mutated to have these activities. This study gives a scope to improve the characters of bio-control agents.

4 Host resistance

Screening for identifying the highly resistant and susceptible cultivars under pot culture conditions revealed Pisang berlin as highly resistant and Grand Naine as susceptible. Cv. Rose was found moderately susceptible. Specific RGA primers were designed from the RGAs of cv. Rose and found no polymorphism. But when PCR product were digested with restriction enzymes, it was found that RGA2-1 (specific primer) and HinfI are showing polymorphic bands between the cultivars and can be used as CAP marker in identifying the *Fusarium* resistance lines.

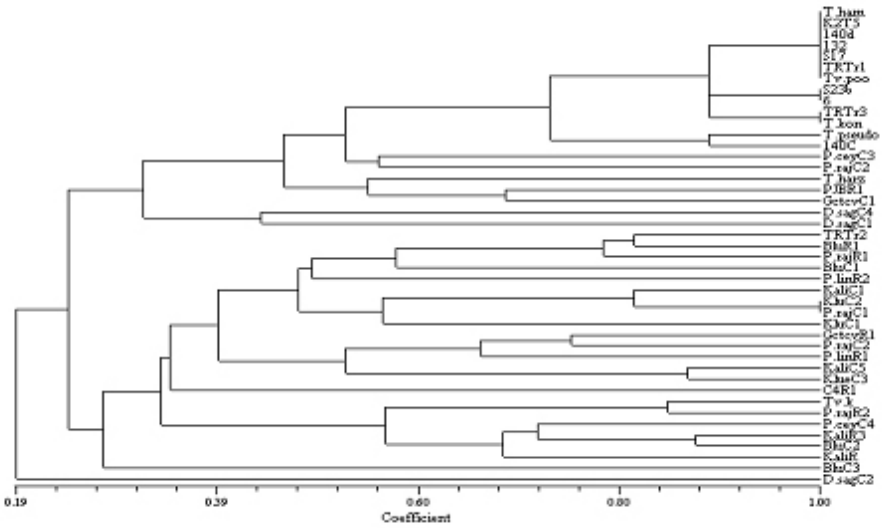

Fig. 45. Dendrogram showing diversity of effective *Trichoderma* spp. isolates (43 nos) by rDNA-ITS-RFLP

NATIONAL BUREAU OF AGRICULTURALLY IMPORTANT MICROORGANISMS, MAU

Principal Investigator
Dr. SUDHEER KUMAR

Co-investigators
Dr. Alok Srivastava

1 Biodiversity

One hundred and two *Fusarium* isolates including 62 isolates of *F. oxysporum* f. sp *lycopersici* from IIVR, 20 isolates of *F. udum* and 20 isolates of *F. oxysporum* f. sp. *ciceri* from IIPR were maintained. All the isolates were characterized for mycelium colour, colony growth pattern, growth rate and size of macroconidia and were conserved in mineral oil for short term conservation and in glycerol at -80^{0}C and lyophilized for long term conservation. Digitization of all the available information is under process to develop the database of Indian isolates of *Fusarium*. *F oxysporum* f. sp. *ciceri* isolates were characterized for morphological variability on the basis of pigmentation, growth pattern, colony colour, mycelial colour, shape and size of micro conidia and macro conidia.

The colony growth on PDA ranged from 38 to 84.0 mm on the 7th day. *F.oxysporum* f. sp. *ciceri* produced both micro and macro-conidia that in size from 4.9 – 15.0 x 1.2 – 3.8 µm and from 15.1 – 20.8 x 2.1 – 4.2 µm, respectively. Between different isolates of *Fusarium oxysporum* f. sp. *ciceri* considerable variation were recorded in conidial size as well as growth and pigmentation. The PCR-based characterization of *F oxysporum* f. sp. *ciceri* using ITS1 and ITS4 is given in Fig. 46.

Fig. 46. The PCR-based characterization of F oxysporum f. sp. ciceri using ITS1 and ITS4 (Around 550 bp)

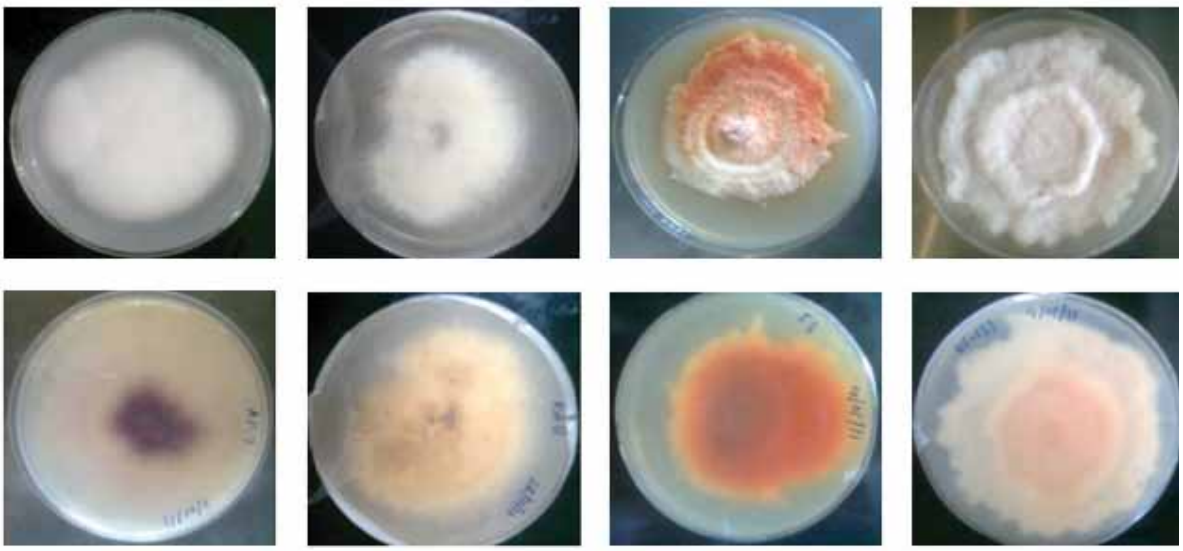

Fig. 47. Morphological diversity of F. udum

NATIONAL BUREAU OF AGRICULTURALLY IMPORTANT INSECTS, BANGALORE

Principal Investigator
Dr. S. SRIRAM

Co-investigators
Dr. R. Rangeshwaran
Dr. B.Ramanujam

1 Biodiversity

a. Host range studies

The pathogenicity of *Fusarium* isolates were tested on a range of crops and differentiated the isolates into non-pathogenic pathogenic isolates and also confirmed the distinctness using molecular tools. Chick pea, pigeon pea, safflower seeds and also seeds of bold seeded crops like groundnut, castor and water melon were treated with formulations and sown in aluminium trays/ plastic pots containing sand and tested for pre-emergence/ post-emergence wilt symptoms. Besides, tissue cultured banana plants were also inoculated by drenching and injection. No wilt symptoms were observed in any of the crops for up to one month after inoculation with any of the seven selected non-pathogenic isolates, while the plants inoculated with respective pathogens showed wilt symptoms.

b. Molecular confirmation of non-pathogenic nature *Fusarium* isolates

DNA extraction method for *Fusrium* was standardized using plant DNA isolation kit (CTAB method). The TEF-1á has been used as a single-locus identification tool in the assembled FUSARIUM-ID v.1.0 database. The tef1 specific primers EF1 (5'ATGGGTAAGGAAGACAAGAC3') and EF2 (5'GGAGGTACC AGTGATCATGTT3') were used to amplify the tef1 region. DNA amplification was done with seven isolates and the amplified products showed bands of size around 700 bp.

Table 45. Identification of non-pathogenic Fusarium isolates based on ITS and tef genes

Isolates (nbaii)	ITS identification	Tef identification
NBAII NPFu – 1	*Fusarium sp.* (99%)	*F. oxysporum f. sp. vasin fectum (98%)*
NBAII NPFu - 2	*F. oxysporum* (99%)	*F. oxysporum* f. sp. *lini* (98%)
NBAII NPFu - 3	*F. oxysporum* (95%)	*F. oxysporum a*(98%)
NBAII NPFu - 4	*F. solani* (98%)	*F. solani* (100%)
NBAII NPFu - 7	*F. solani* (99%)	*F. solani* (100%)
NBAII NPFu - 24	*F. solani* (97%)	*F. solani* (100%)
NBAII NPFu - 25	*F. solani* (98%)	*F. solani* (98%)

The PCR eluted product was sequenced through outsource (Bangalore Genie Pvt. Ltd). DNA sequences of selected isolates were compared using bioinformatics tools like NCBI and *Fusarium* data base using NCBI blast programme. Based on sequence comparison, the identification of *Fusarium* isolates was confirmed (Table 45).

2 Disease management

a. Mass multiplication and formulation of selected strains of non-pathogenic fusaria

A simple talc formulation was prepared for the selected non-pathogenic *Fusarium* isolates (NPFu1, NPFu2, NPFu3, NPFu4, NPFu7, NPFu24, NPFu25). The isolates were grown in potato dextrose broth in 5 L Sartorius fermentor and the biomass obtained after five days was mixed with talc at 1:2 ratio and dried to 12% moisture content. These formulations were used for the pot culture and field experiments.

b. Field testing of the selected non-pathogenic fusaria in chickpea and tomato

The talc formulations of the non-pathogenic *Fusarium* isolates NPFu1, NPFu2, NPFu3, NPFu4, NPFu7, NPFu24, NPFu25 along with talc formulation of *Trichoderma harzianum* (NBAII Th10) and chemical control (carbendazim) were tested against fusarium wilt on tomato at Attur Research Farm of NBAII, Bangalore. The formulations were applied as seedling dip (@10 g liter^{-1}) and soil application (@ 1 kg acre^{-1}). The formulation was mixed with completely dried FYM at 1 kg per 100 kg and FYM enriched with the antagonist was applied at the time of transplanting. No significant wilt incidence was observed in the field. Yield in tomato was high with Fu24 followed by Fu4, Fu25, Fu7 and Th10 (Fig. 48). After 90 days of plant-

ing, root bits were collected from tomato plants treated with *Fusarium* isolates and plated on *Fusarium* selective media (PCNB media) and calculated the percentage root colonization. Except Fu2 all other isolates showed good root colonization (>60%) even after 90 days with the maximum in Fu7 and Fu24 (Fig. 49).

The selected isolates were tested under pot and field condition for the performance of these non-pathogenic *Fusarium* isolates against chickpea wilt. For chickpea field trial 10 treatments were maintained with four rows each containing 10 plants per row. Treatments used were- seven non pathogenic *Fusarium* isolates, *T. harzianum*-NBAII10, Bavistin and control. Seeds were surface sterilized with sodium hypochlorite (0.01%) solution and treated with talc formulations (10g/kg of seeds) of all isolates containing $2x10^6$ spores/g of talc and bavistin 2 g/kg of seeds and control without any treatments. The germination was higher in NPF treated plots (except Fu25) where the maximum germination obtained was 85% (Fu3) when compared to control (Fig. 50).

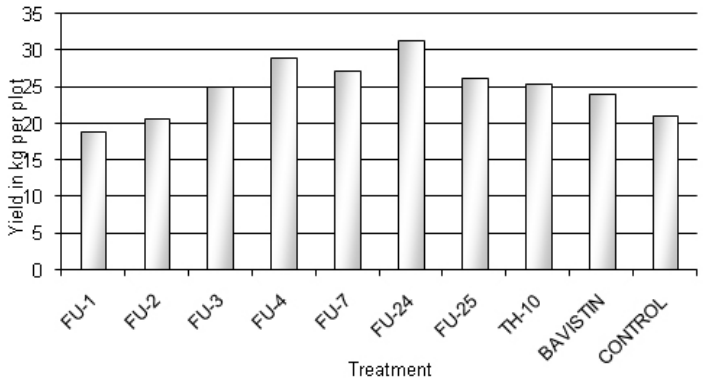

Fig. 48: Effect of non-pathogenic *Fusarium* isolates on yield in tomato.

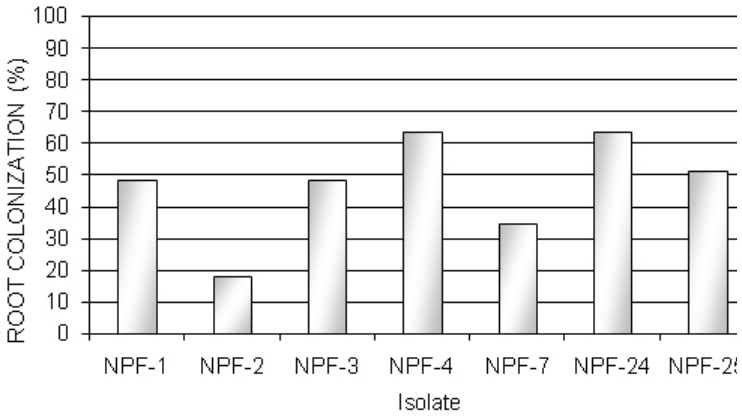

Fig. 49. Root colonization (%) by non-pathogenic *Fusarium* isolates in tomato plants 90 days after planting

c. Screening of selected isolates of NPF for their potential to induce systemic resistance in chillies

Secondary screening of selected isolates (after molecular identification) NPF-4, NPF-7, NPF-24 and NPF-25 and its elicitors were done based on the ISR studies i.e. biochemical analysis of expression of genes in plants in response to treatment with nonpathogenic *Fusarium* and its elicitors. Biochemical analysis includes: Estimation of total proteins, total phenols, and activities of peroxidase, polyphenol oxidase and PAL. The changes in total phenol content, protein, peroxidase and PAL were noticed only upto six days after treatment. After six days, there were no significant differences in these biochemical changes (Fig. 51).

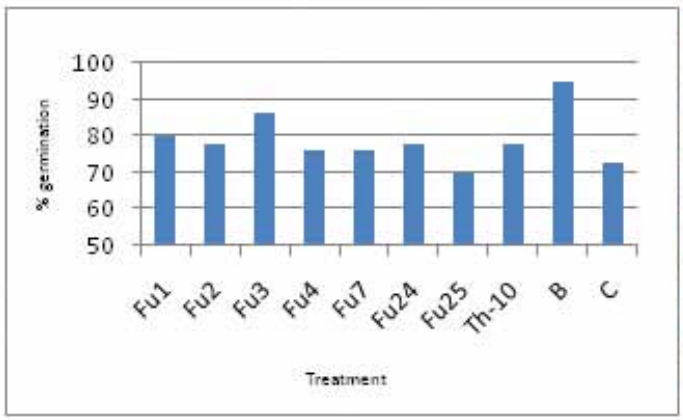

Fig. 50. Field evaluation of non-pathogenic *Fusarium* isolates against chickpea wilt, A. Germination (%) in chickpea, B. View of the trial plot

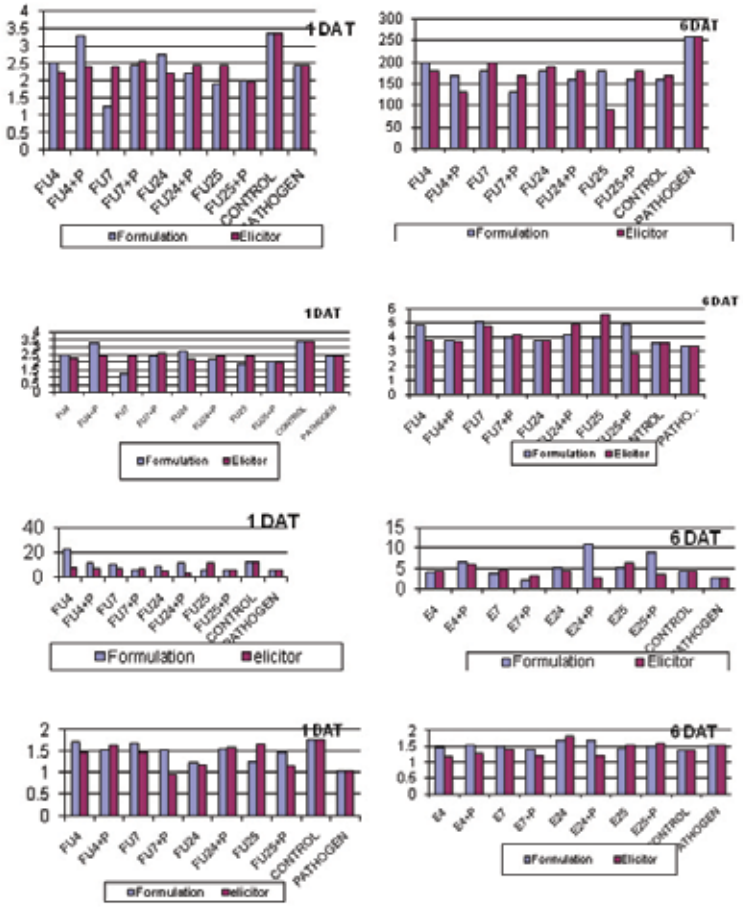

Fig. 51. Biochemical analysis of non pathogenic *Fusarium* isolates. (From top to bottom) Total phenol content, Total protein content, Peroxidase activity and Phenylalanine ammonia lyase activity in plants treated with non pathogenic *Fusarium* isolates and its elicitors with pathogen and without pathogen treatment (change in OD/min/gram of plant tissue).

Sub project - 3

RALSTONIA

Bacterial wilt caused by *Ralstonia solanacearum* is an important soil-borne disease that spreads worldwide. It belongs to the ß-proteobacteria and is considered a “species complex”. It has an unusually broad host range which comprises over 200 plant species, representing over 50 botanical families and covering both monocots and dicots extending from annual plants to trees and shrubs. The pathogen has a wide geographical distribution especially in tropical, subtropical, and some temperate regions. It has effective pathogenicity determinants to invade and colonize host plants but, also exhibits successful strategies for survival in harsh conditions. Under PhytoFuRa, bacterial wilt problems of ginger and vegetables are intensively studied.

INDIAN INSTITUTE OF SPICES RESEARCH, CALICUT

Principal Investigator
Dr. A. KUMAR *(upto 19 Nov. 2011)*
Dr. D. PRASATH *(from 20 Nov. 2011 onwards)*

Co-investigator
Dr. R. Suseela Bhai

1 Biodiversity

a. New collection of *Ralstonia solanacearum* from bacterial wilt affected plants

Six new isolates of *R.solanacearum* isolates collected from bacterial wilt affected ginger plants from Wyanad and Calicut districts of Kerala were added to the existing collection.

b. Phenotypic characterization and pathogenicity testing

According to Hayward's biovar testing based on utilization of disaccharides and sugar alcohols, the isolates were found to be biovar 3. The isolates were tested for their pathogenicity in healthy ginger plants. Up on soil inoculation the plants showed the typical wilting symptoms within 10-14 days.

c. Diversity analysis of *Ralstonia solanacearum* using molecular methods

Natural occurrence of bacterial wilt in small cardamom was observed in Wyanad, Kerala. The causative agent could be identified as *R. solanacearum* by colony morphology, Biolog based identification scheme and also by proving the pathogenicity on cardamom plants. During pathogenicity testing in cardamom the wilting symptoms could be observed after 20 days of soil inoculation. The symptoms include typical wilting of plants wherein all the leaves rolled or curled upward toward the center of midrib, turned yellow, and the whole plant finally died; the collar region of the plants showed water soaked lesions initially and turned dark brown eventually. Copious quantity of bacterial exudates could be observed on the cut end of the pseudostem.

Phenotypic and genetic characterization revealed that the *R. solanacearum* was biovar 3 and phylotype 1 confirming its Asian origin. Genotypic methods such as BOX-PCR fingerprinting, Multiplex-PCR based phylotyping, 16s rDNA & recN gene sequence based comparison and MLST based comparative genetic analysis further revealed that the strain is 100% similar to ginger strain of *R. solanacearum* which is widely prevalent in southern and north-eastern states of Indian subcontinent. Perusal of records on *Ralstonia* induced bacterial wilt in crop plants particularly among the Zingiberaceae family reveal that this is a new report of bacterial wilt disease in small cardamom.

d. 16s rDNA sequence comparison of *R. solanacearum* isolates

The 16s ribosomal DNA was amplified from10 strains of *R. solanacearum* representing host such as tomato, chilly, eggplant, potato, ginger, cardamom, eupatorium using universal primers pAF (5'-AGAGTTTGATCCTGGCTCAG-3') and pHR (5'-AAGGAGGTGATCCAGCCGCA-3'), sequenced and the sequences were compared. When the assembled sequences were aligned using CLC sequence viewer, the isolates from ginger and cardamom were clustered together except one isolate from Palavayal, Waynad.

e. RecN sequence comparison of *R. solanacearum* strains

RecN is a DNA repair protein. Primers for amplifying the gene were designed, and the gene was amplified using primers RecN3F (5'-GATTTCGTCATCGTCCATGC-3') and RecN5R (5'-ATCACCGAGATCGCTAGGC-3'), product length was 1300 bp (Fig. 52). The PCR product was sequenced from 21 strains of *R.solanacearum* representing host such as tomato, chilly, eggplant, potato, ginger, cardamom, eupatorium. The sequences have to be compared with the existing recN sequences from the database and conclusions have to be made.

f. Phylotyping of *R. solana cearum* isolates

This phylogenetic analysis classified *R. solanacearum* species into four phylotypes which reflects the geographical origin of the strains. Phylotype I and II are composed of Asian and American strains, respectively, whereas phylotype III consists of African strains and phylotype IV comprised isolates from Indonesia, Japan and Australia. This was done by a multiplex reaction and found all of the strains belongs to phylotype I indicat-

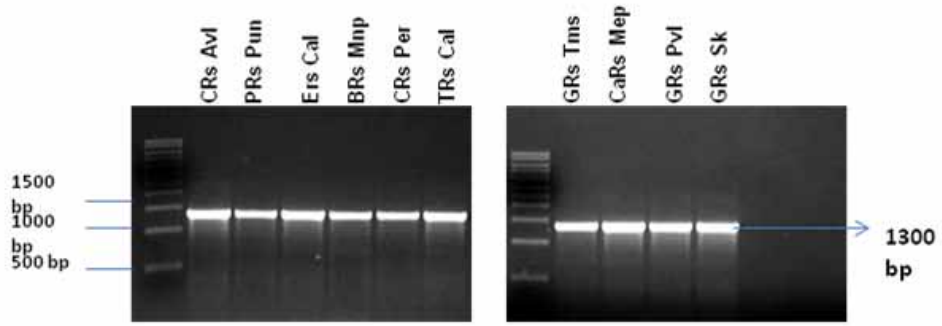

Fig. 52. Gel picture showing rec N gene amplification of R.solanacaerum

ing Asian origin except one strain from potato which is phylotype II indicating American origin. So it reveals the predominance of phylotype I in India.

g. Box PCR based DNA profiling

Box PCR using the universal primer BOX AIR (CTACGGCAAGGCGACGCTGACG) revealed close similarity among the ginger strains of *R. solanacearum* (Fig. 53).

h. Multilocus sequence typing of *Ralstonia solanacearum* (MLST)

Multilocus sequence typing (MLST) is nucleotide sequence based approach to uncover allelic variants in conserved genes for the purpose of characterizing, subtyping, and classifying members of bacterial populations. Based on this approach five housekeeping genes, dispersed in the chromosome (ppsA, phosphoenolpyruvate synthase; gyrB, DNA gyrase, subunit B; adk,adenylate kinase; gdhA, glutamate dehydrogenase oxidoreductase; and gapA,glyceraldehyde 3-phosphate dehydrogenase oxidoreductase), and three virulence-related genes, located on the mega plasmid (hrpB, regulatory transcription regulator; fliC, encoding flagellin protein; and egl, endoglucanase precursor) were used to classify *R. solanacearum* which was adopted with slight modifications in the protocol.

In this the eight genes from different isolates of *R.solanacearum* were PCR amplified, eluted and sequenced. Allele numbers were obtained by sequence comparison with alleles documented in the database www.pamdb.org. Several novel alleles could be found in ginger strain of *R. solanacearum*.

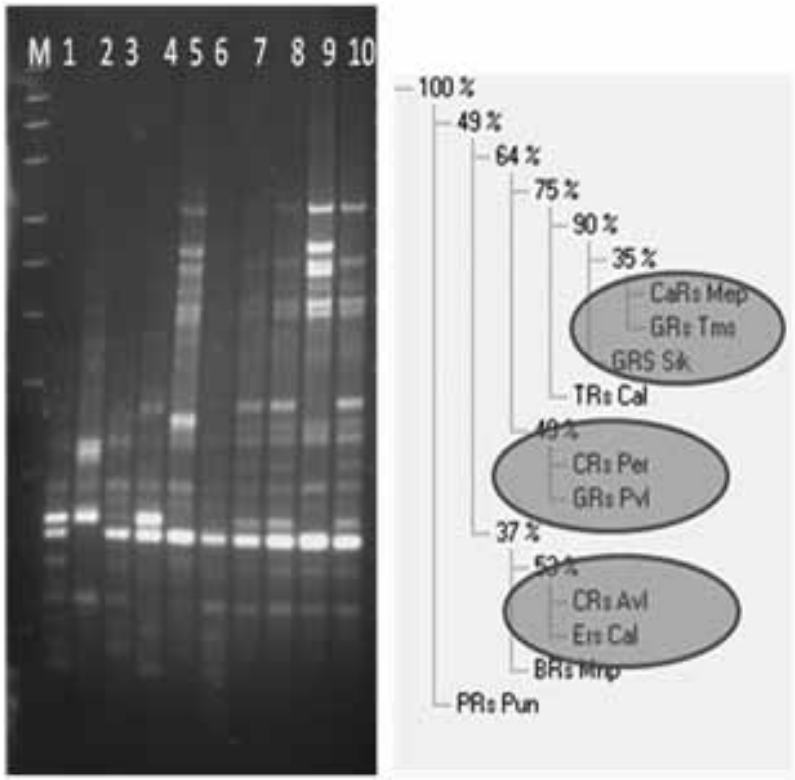

Fig. 53. Gel picture and dendrogram showing BOX PCR profiling of *R.solanacaerum* from different crops. M) 1 Kb ladder, 1) CRs Avl, 2) PRs Pun, 3) ERs Cal, 4) BRs Mnp, 5) CRs Per, 6) TRs Cal 7) GRs Tms, 8) CaRs Mep, 9) GRs Pv, 10) GRs Sikkim.

2 Diagnostics

Total soil DNA was extracted from soil collected from Wyanad, in which one was bacterial wilt affected and another one a fallow field using soil DNA extraction kit. A real time PCR was done using the universal primers 759 (5'-GTCGCCGTCAACTCACTTTCC-3') and 760 (5'- GTCGCCGTCAGCAATGCGGAATCG-3'). *R.solanacearum* was detected in all the samples in bacterial wilt affected field and fallow field is free from *R. solanacearum*.

3 Host resistance

Fifty six ginger mutants irradiated with gamma rays were challenge inoculated with *R. solanacearum* by soil inoculation. After three rounds of pathogen inoculation two accessions (HPO 5/15 and HPO 5/2) did not show any wilt symptoms. To assure the presence of pathogen in soil, the soil DNA was extracted and PCR was done using *R. solanacearum* specific primers 759 and 760. All soil was found to be positive for the pathogen. So these accessions will be further multiplied and screening has to be done in a sick field to develop a bacterial wilt resistant cultivar.

4 Disease management

a. Evaluation of antagonistic plants and biocontrol agents

A replicated trial with the following pre-plant treatment of ginger rhizomes was laid out in a greenhouse. In which biopriming of the rhizomes was done with *P. fluorescens* SBW25, *P. fluorescens* PF5, *P. fluorescens* Phz, *P. aeruginosa* IISR 51,*Stenotrophomonas maltophilia* GEB13, *Acinetobacter calcoaceticus* GEB 19, Unidentified Actinomycetes VC11and a chemical check, streptomycin sulphate (200ppm). The antagonistic crops were (*Tagetes* spp., *Ocimum sanctum*) pre-plantedbefore planting of rhizomes.The soil was inoculated with *R. solanacearum* and the microbial cadiates of biocontrol. Booster doses of biocontrol agents were applied in two subsequent months. After six months, yield was taken. Observation on yield as well as disease incidence showed that *Tagetus* spp. is a potential crop for reducing bacterial wilt incidence in ginger.

b. In vitro evaluation of rhizosphere actinomycetes against *Ralstonia solanacearum*

Ginger rhizosphere soil was collected from different places of Kerala, and Karnataka and also from the ginger germplasm accessions from experimental farm Peruvannamuzhi. The soil samples were serially diluted and plated on different culture media (potato dextrose agar, actinomycetes isolation agar). The isolated actinomycetes were differentiated based on morphological characters. Based on this 12 isolates were selected with different colony morphology and screened against *R.solanacearum in vitro*. Out of 12 cultures tested nine were showing antagonistic activity against *R. solanacearum*.

Another unidentified actinomycete isolated from vermicompost, VC 11, also showed *in vitro* antagonism against *R. solanacearum*. From this actinomycete culture filtrate was extracted with equal volume of ethyl acetate and the extract is dried under vacuum and dissolved in different solvents (DMSO, methanol and water). Bioassay was done against *R. solanacearum* by agar cup method with appropriate control. The extract which was dissolved in water showed inhibition against *R. solanacearum*.

Principal Investigator
Dr. R. RAMESH

Co-investigator
Dr. M. THANGAM

1 Biodiversity

a. Collection of *Ralstonia solanacearum* isolates

R. solanacearum isolates (212) were collected from different crops i.e. brinjal, chilli and tomato from Goa (70 different places, 147 isolates); Karnataka (15 different places, 18 isolates), Kerala (11 different places, 20 isolates), Andaman islands (26 places, 26 isolates). Isolation of the pathogen was done on TZC medium. All the isolates were preserved in water stocks and in 30 % glycerol stored at -70 °C.

Further, 72 more isolates are being maintained, which might be duplicates as they were collected from the same fields. However, difference in the virulence of these isolates was observed. All the *R. solanacearum* isolates are being maintained at our centre.

b. Phenotypic characterization of *R. solanacearum*

Morphological and cultural characteristics were recorded according to standard protocols. Virulent *R. solanacearum* colonies appeared white in colour with light pink centre with lot of EPS production. Avirulent forms of the bacterium developed butyrous, deep red colonies and developed less fluidal colonies which were completely pink to red on the medium. Biovar characterization using three disaccharides and three hexose alcohols indicated that the isolates belonged to biovar 3.

c. Pathogenicity of *R. solanacearum* isolates

Pathogenicity of the isolates collected from different regions was tested on brinjal seedlings by inoculating 4 ml of bacterial cells (approx. $\log_{10}8$ cfu/ml). The disease incidence was recorded as percentage of wilt starting from 7th day after inoculation. Further, the pathogenicity was tested on two other vegetable hosts, viz. tomato and chilli. Results indicated that more than 90 per cent of the isolates were pathogenic to all the three hosts. Incidence of wilt started very early in case of tomato (3-4 days after inoculation in case of some isolates). In chilli, only few isolates (27) caused typical wilting and more than 85 per cent of isolates produced chlorotic symptoms and stunted growth but not death of plant.

d. Confirmation of *R. solanacearum* using specific primers

Isolation of the genomic DNA was carried out by the modification of method suggested by Kumar et al (2004) DNA was isolated from the isolates, quality and quantity of genomic DNA was determined using Nano drop-1000, Thermo fisher scientific, USA. Polymerase chain reaction was done in DNA Thermal Cycler (Mastercycler Pro, Eppendorf, Germany) using Rs759 & Rs760 (280 bp). All the isolates were confirmed by PCR amplification.

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

From the total collection, 92 isolates were selected based on geographical distribution, pathogenicity and virulence for studying genetic diversity.

Phylotyping: Phylotyping of the isolates was carried out using five primers (Fegan and Prior, 2005). All 92 selected isolates belong to Phylotype 1.

BOX PCR: Ninty two isolates were studied for their diversity using BOX primer. All the fingerprints were determined and were found to produce scorable bands in the range of 300-2000 bp and these bands were scored and used for analysis by NTSYSpc software. A dendrogram was constructed by UPGMA clustering. Twelvd clusters of *R. solanacearum* formed when 75% similarity coefficient is considered. Isolates from Goa showed as many as 23 distinct patterns, Karnataka isolates showed seven patterns, Kerala isolates showed eight patterns, Andaman and Nicobar isolates showed five patterns. Bands were scored as 1 for presence of bands and 0 for absence of band. Based on the BOX-PCR analysis, there was significant similarity among all the strains of R. *solanacearum* collected from same geographical origin. BOX-PCR was not able to distinguish all the isolates distinctly based on region, host or phylotype.

f. Sequencing of virulance genes

Endoglucanase gene (*egl*): Specific primers were used to amplify the egl gene and the PCR reaction and conditions were standardized. Sequencing was carried out by using automated DNA sequencer (ABI PRISMTM 310 Genetic Analyser from Applied Biosystems Inc). Sequences were edited manually and aligned using Clustal W. All the sequences were deposited with NCBI, USA (Accession Nos. HQ244995 to HQ245086). Phylogenetic analysis was performed version 4.0 by using neighbor-joining (NJ) and the algorithm of Jukes and Can-

tor (1969) with 1000 bootstrap re-samplings. Results of 48 isolates from different clusters along with the sequences from Gene Bank are discussed.

Isolates from our collection cluster together with Phylotype I isolates described earlier from elsewhere (R292, O3, ZO4, PSS219, GMI1000, P11, JT523, M2 and PSS358). Isolates in the cluster could not be delineated according to host, origin or biovar characterization. Our isolates within the major cluster (Phylotype I) did not group based on the host/ geographical location. The only exception is clustering of isolates from Andaman and Nicobar islands in a different sub cluster. Based on the egl sequences, 20 of our isolates from the selected 92 are designated sequevar numbers. All other sequences constitute unknown sequevars.

Polygalacturonase precursor gene (*pga*): Specific primers (PehF and PehR- were used to amplify the pga gene and the PCR reaction and conditions were standardized. Sequencing was carried out by using automated DNA sequencer (ABI PRISMTM 310 Genetic Analyser from Applied Biosystems Inc). Sequences were edited manually and aligned using Clustal W All the sequences were deposited with NCBI (Accession Nos. HQ164450 to HQ164541). Phylogenetic analysis was performed using MEGA version 4.0 by using neighbor-joining (NJ) and the algorithm of Jukes and Cantor (1969) with 1000 bootstrap re-samplings.

Isolates from our collection cluster together in a single group along with the other *R. solanacearum* isolates described (M33692 and UW551 from Kenya). Sequences from the other isolates viz. IPO1609, GMI1000 and MolK2 cluster in a separate group from our isolates. Isolates in the cluster could not be delineated according to host, origin or biovar characterization. The grouping is not in consistent with Phylotyping classification as observed in *egl* sequences. Our isolates clustered in two major groups based on *pga* sequences. Forty-seven isolates were selected based on the *egl* and *pga* sequence data for further characterization using ITS, *mutS* and *hrpB* primers.

g. Characterization using Pulsed Field Gel Electrophoresis (PFGE)

PFGE was used to determine the diversity amongst the 92 *R. solanacearum* strains belonging to phylotype I biovar 3. Because of the G+C rich genome of *R. solanacearum,* rare cutting restriction enzymes Xba I and Dra I (Smith et al. 1995) recognizing AT rich sequences were used for determining the genetic diversity by restriction digestion of the whole genome. PFGE protocol for *R. solanacearum* was standardized in CHEF-DR-II drive module, Biorad, USA. The whole cell DNA was digested with XbaI and DraI; electrophoresis is carried out at 14°C, 6V/cm with pulse time of 4-50 sec for 20 h. All 92 isolates were subjected to the analysis.

Both the rare cutters were effective in elucidating the differences and yielded 12- 16 fragments ranging from 670 Kb to 20 Kb in size. Based on the number of bands and banding profiles, the isolates could be grouped into 36 pulsotypes as generated by Xba I and 28 pulsotypes as generated by Dra I within the 92 isolates. A total of 30 and 22 bands were scored in case of Xba I and Dra I, respectively. The binary data was entered in the NTSYSpc software and the dendrogram developed by UPGMA was analysed. Based on the dendrogram, at 80% similarity, Xba I divided the isolates into 29 different clusters whereas Dra I divided them into 21 different clusters.

By employing RC PFGE our phylotype I biovar 3 strains could be clustered into diverse pulsotypes representing the clonal lines of the *R. solanacearum* species complex. The pulsotypes obtained by both the restriction enzymes were difficult to be grouped based upon the geographical location, host crop or the year of isolation, with an exception being the isolates from Andaman and Nicobar Islands which clustered separately. Nevertheless, the technique proved useful for determining the number of different pulsotypes which could be present in a particular geographical location. Grouping of the isolates is presented in table 54.

Table. 46. Different pulsotypes in *Ralstonia solanacearum* phylotype

Location	No. of different pulsotypes	
	Xba I	Dra I
Goa	18	13
Karnataka	8	4
Kerala	8	8
Andaman	4	3
Bihar	1	1

2 Diagnostics

a. Sequencing of 16S-23S ribosomal RNA intergenic spacer (ITS)

Primers were designed to amplify the 16S-23S ribosomal RNA intergenic spacer (ITS) of *R. solanacearum*. Based on the sequence data available in NCBI, the primers (RS-ITS FP- AGTCGTAACAAGGTAGCCGTATCG; RS-ITS RP- GCCAAGGCATCCACCACATGC) were designed to amplify ITS region of the *R. solanacearum* isolates. The 47 isolates which were selected based on the *egl* and *pga* sequence data were used for ITS sequencing. Specific primers (RS-ITS FP and RS-ITS RP) were used to amplify ITS region; the PCR reaction and conditions were standardized. Sequencing was carried out by using automated DNA sequencer (ABI PRISMTM 310 Genetic Analyser. All the sequences were deposited with NCBI. Phylogenetic analysis was performed using MEGA version 4.0 by using neighbor-joining (NJ) and the algorithm of Jukes and Cantor (1969) with 1000 bootstrap re-samplings. A high level of sequence similarity was observed among our isolates. Based on the sequence similarity among our isolates, specific primers were designed for selective identification of our *R. solanacearum*. One forward primer and two reverse primers were designed for validation.

3 Host resistance

a. Screening of eggplant germplasm against *R. solanacearum*

Seeds (brinjal, tomato and chilli)

have been obtained from GBPUAT, KAU, OUAT, NBPGR and IIHR. *Solanum torvum* seeds have been obtained from NBPGR. The 44 local accessions were obtained for screening for resistance. Total of 57 varieties were screened in glasshouse. Three *R. solanacearum* isolates which differ in the virulence (highly, moderately and mildly virulent isolates) are used in the screening. Incidence of wilt was recorded regularly starting from seven days after inoculation and continued up to 45th day after inoculation. Local susceptible cultivar Agassaim was used as control along with the test varieties. Wilting started 7th day in most of the varieties whereas in resistant types no wilt was recorded till the end of the experiment. Since the purpose of screening is to identify a stable resistant donor for crossing, only varieties which were not showing any wilt incidence till the end of the period were selected. Out of 57 varieties, 13 cultivars were short listed for 2nd time screening under stringent conditions in glasshouse. In the second screening the same quantity of *R. solanacearum* inoculum (approx. 2.0 x 10^8 cfu ml^{-1}) was used as of initial screening. Incidence of wilt was not recorded in any of the test varieties up to 20 DAI. The same plants were again inoculated with the higher concentration of *R. solanacearum* inoculum (approx. 4.0 x 10^9 cfu ml^{-1}) after 20 days of the 1st inoculation.

Incidence of wilt was recorded starting from 7th day after inoculation. Typical wilting was observed only in Utkal Jyothi and accession no. 27 inoculated with Rs-08-17 and Rs-09-100 and Rs-09-109 in soil drenching and prick inoculation method. Surya, Swetha and Utkal Madhuri were healthy and did not show any wilting symptoms including chlororsis, stunted growth etc till the end of screening period. Though the other varieties did not wilt, symptoms such as mild chlorosis and stunted growth were observed in accession nos. 38, 28 and 15.

Based on the above screening it is concluded that Surya, Swetha and Utkal madhuri could be used as donor parent in resistant breeding as these varieties did not wilt and did not allow the pathogen to multiply in the tissue even under high inoculum concentration which is evident from the population analysis from the tissues of resistant and susceptible varieties. Anatomical studies indicated damaged vascular tissues in the susceptible plants and intact tissues in the resistant plants after pathogen inoculation. Development of F1 population between Agassaim and Surya is in progress. Crosses are made and seeds will be collected from the successful crosses for developing F2 population.

4 Disease management

a. Evaluation of microbial candidates for use against *R. solanacearum*

Glass house condition: Based on the previous results, five antagonistic bacteria were evaluated for their efficiency in reducing the incidence of wilt under glasshouse condition. Two experiments were conducted, one with the young plants (25 days old), other with grown up plants (50 days old). In each experiment two sets were maintained, one set without *R. solanacearum* inoculation and other set with inoculation. Height of plant and wilt incidence were recorded from the set I and set II plants, respectively, over a period of time. When younger plants were inoculated, there was no significant difference in the incidence of wilt due to antagonistic treatment compared to control. However, when the grown up plants were inoculated, the antagonistic treatments reduced wilt incidence compared to control. In control, incidence of wilt started seven days after inoculation and 100 per cent plants wilted on 21st day. However, EB69, Rs-08-72 and EC13 treatments incidence is reported only after 15 days and on 21st day it was only 50 per cent. Antagonists improved plant growth which is evident from the increased plant height in the treatments. These antagonistic bacteria are identified as *P. aeruginosa* (EB69, Rs-08-72) and *Bacillus* spp. (EC13). These antagonistic bacteria could be used in reducing the incidence of bacterial wilt in the field, for that large scale field trials need to be taken up with the formulations. Other promising antagonistic bacteria are being evaluated in the glasshouse for their efficiency to reduce wilt.

Field condition: Field experiments were laid out to evaluate the efficiency of the biocontrol agents in reducing bacterial wilt in eggplant. Nursery was treated with talc formulation of the above three biocontrol agents @ 50 g m^{-2} area. One treatment of consortium (EB69+ Rs-08-72) was also included based on the compatibility study. Seedlings raised in the treated nursery and control nursery (not treated with biocontrol agents) were planted in the field in January 2011. The plants were drenched with the biocontrol suspension (50 g lit^{-1}) @ 50 ml/plant. Appropriate controls were maintained. Further, nursery treated seedlings were planted in two other locations in the farmers field. Seedlings were drenched after transplanting in these fields also. All the three experiments are in progress and observations on wilt incidence, growth and yield will be recorded.

b. Study of xylem bacterial population

Vacuum extraction of the xylem sap from susceptible and resistant brinjal and tolerant chilli plants was carried out according to the standardized method. A total of 160 xylem residing bacteria including the isolates obtained earlier are being maintained. All the isolates were preserved in glycerol stocks and stored at -70^{0}C. All the isolates are being screened for their antagonism to *R. solanacearum*. 24 isolates inhibit *R. solanacearum* growth and out of which seven produced esterase. Promising xylem bacteria (species of *Pseudomonas*, *Burkholderia* and *Bacillus*) are being evaluated in glasshouse for possibility of using them as biocontrol agents because they share a common niche as of *R. solanacearum*.

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Principal Investigator
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1 Biodiversity

Surveys were conducted to Meghalaya, Mizoram, Manipur, Arunachal Pradesh, Sikkim and samples were collected from bacterial wilt affected tomato, brinjal, capsicum, chilli and ginger. From the 214 samples collected, 123 isolates of *R. solanacearum* were collected from tomato, capsicum, brinjal, chilli and ginger. However, 81 isolates could be revived from the preservation. The isolates have been allotted codes in correspondence place & date of collection, variety/genotype and names of the farmer, village, block, district and state. The revived isolates were categorized in TTC medium based on the intensity of colour produced by the isolates in reaction to the TZC. So far 43 representative isolates i.e. 12 from tomato, 7 from brinjal, 3 from capsicum, 6 from chilli and 15 from ginger were subjected to Biovar test for characterization using Lactose, Maltose (Carbohydrates sugars) Mannitol, Sorbitol (Alcohol sugars) and grouped under Biovar-3. The biovar testing for the rest of isolates is being completed. Characterization of representative *R. solanacearum* isolates using PCR based molecular tool is now being conducted using specific primers Y2 (5-CCCACTGCTGCCTCCCGTAGGAGT-3) & OLI 1(5-GGGGGTAGCTTGCTACCTGCC-3) and nine isolates of *R. solanacearum* have been identified using PCR based molecular tools from infected samples.

2 Diagnostics

The pathogenicity test of the above isolates were conducted. Tomato plants (25 days) were inoculated with 24 old *Ralstonia* cultures. *R. solanacearum* diagnosed with specific primers Y2 (5-CCCACTGCTGCCTCCCGTAGGAGT-3) & OLI 1(5-GGGGGTAGCTTGCTACCTGCC-3) and nine isolates of *R. solanacearum* have been identified using PCR based molecular tools from infected samples. Identification of *Bacillus subtilis* has been carried out using (5'-GCTGCGTTCTTCATCGATGC-3'), (5'-GCATCGATGAAGAACGCAGC-3') primers and three isolates have been identified as *B. subtilis*. However, other species of *Bacillus* are yet to be confirmed. Identification of *P. fluorescens* has also been tried using species specific primer 16 PSEflu and 16 PSE, (5'-TGCATTCAAAACTGACTG-3'), (5'-AATCACACCGTGGTAACCG-3') and few isolates of *P. fluorescens* have been confirmed by PCR based molecular tools.

3 Epidemiology

The data in field trials were recorded at 15 days interval in order to compute apparent rate of infection and area under disease progress curve.

4 Host resistance

Thirteen established genotypes of tomato were screened against bacterial wilt under artificial inoculated (*R. solanacearum*) conditions. Among the genotypes screened, MT-1 was found to be resistant and Pusa ruby as susceptible genotype under field condition.

5 Disease management

The bacterial bio-agent, *P. fluorescens* and *Bacillus* spp. from rhizosphere soils of healthy tomato, maize, soybean, groundnut, rice and ginger plants, were isolated. So far six *P. fluorescens* and seven *Bacillus* spp. cultures are being taken up for characterization. The *in vitro* bio-efficacy test of bio-agents was tested following agar diffusion method and paper disc method. *P. fluorescens* isolate 404, was found producing clear zone of inhibition against *R. solanacearum* followed by 403 and 408. Similarly *B. subtilis* isolates 507, was found producing clear zone of inhibition against *R. solanacearum* followed by 516 and 518.

Three field trials were also conducted using various treatments in order to manage the disease of ginger (local and Nadia variety), capsicum (Thai wonder) and tomato (Pusa ruby). In the first trial conducted at Soil Science field for management of bacterial wilt of ginger, NPK+mulching+Mz 72 was found best at par with NPK+lime+Agnee Plus followed by hot water treatment. In the second trial at plant pathology field, ATW was found best, followed by Agnee and Agnee Plus at par with hot water treatment. In the third trial at general field of ICAR, Bhoomika was found best, followed by Agnee Plus and hot water.

Two more trials were conducted to manage bacterial wilt in capsicum and tomato. In the Capsicum trial (Thai Wonder variety), Pig slurry was found best, followed by HEYC at par with Lantana seed. In the tomato trial (Pusa Ruby variety), HEYC was most promising followed by HEY and Lantana seed.

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1 Biodiversity

a Survey and biovar characterization

A survey was conducted in bacterial wilt prone area of West Bengal, Orissa and Jharkhand in 2010 -11. The wilted samples of brinjal, tomato, chilli, capsicum and potato were collected for isolation. In Jharkhand, the samples were collected from Ranchi District and wilt incidence in tomato was 5 - 20% (September 2010). In Orissa, survey was conducted in September 2010 and January 2011, wilt incidence varied from 5 - 35%. In West Bengal (Mohan, Kalyani), maximum wilt incidence in tomato and brinjal was recorded (15 -35%) followed by Birbhomi, (10 -30%) and Cooch Bihar (5- 15%). Twenty seven isolates of *Ralstonia solanacearum* were isolated from these hosts. The pathogenicity, morphological, biochemical and molecular characterization were done. The isolates were confirmed by using pathogenicity and hrp gene based amplification at 323 bp. Presently IARI center has 111 isolates of *R. solancearum* collected from tomato, brinjal, chilli, capsicum and potato.

Ninety two isolates of *R. solanacearum* were taken for biovar study, which represented six states viz. Himachal Pradesh, Jammu & Kashmir, Uttarakhand, Jharkhand, Orissa and West Bengal (Table 47). Out of 92 isolates, 83 were found to be biovar 3 and only nine were grouped under biovar 4. All the isolates of capsicum, chilli, and potato belonged to biovar 3, but in case of isolates from tomato and brinjal, they were biovar 3 and 4 in the states of Himachal Pradesh and Uttarakhand and Orissa. However, biovar 3 found to be most prominent (90.2%) in all the six states whereas biovar 4 was found only 9.8 per cent. It indicates that biovar 3 and 4 of *R. solanacearum* causing bacterial wilt in solanaceous crops belong to race 1. The isolates were differentiated into biovars, based on their ability to utilize carbon sources like dextrose, mannitol, sorbitol, dulcitol and trehalose and oxidized lactose, maltose and (D+) cellobiose. Although, biovar 4 also utilized these carbon sources but did not oxidize lactose, maltose and (D+) cellobiose. Deny and Hayward (2001) reported that biovar 3 and 4 found in Asia causing wilt diseases has wide host range including solanaceous crops.

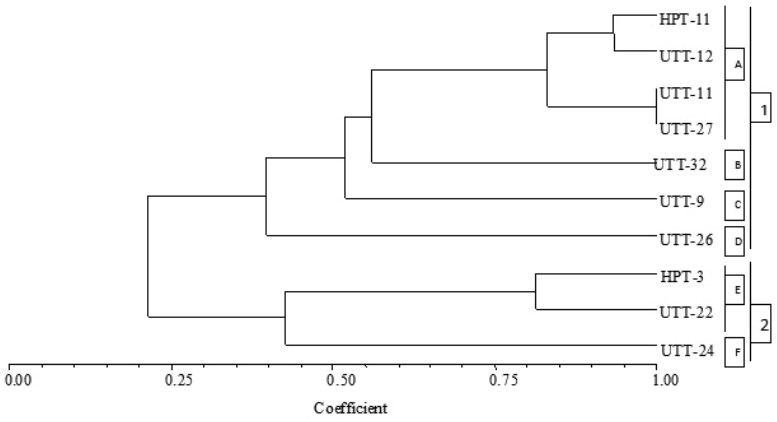

Fig. 54. Cluster analysis of Ralstonia solanacearum biovar 3 and unweighted paired group mathematical average (UPGMA) dendrograms were generated using Pearson's correlation coefficient. The minimum similarity coefficient of all isolates of *R. solanacearum* was used to define distinct groups which are labeled numerically. Distinct groups of band based genotypes are labeled alphabetically.

Table 47. Status of biovar / race and molecular detection of *Ralstonia solanacearum* isolates from Northern and Eastern states of India

State	Host	*R. solanacearum* isolates	Biovar	Race**
Himachal Pradesh	Potato (3)*	HPP-10, HPP-53, HPP-2	3	1
	Capsicum (4)	HPC-2,HPC-6, HPC-7, HPC-3	3	1
	Tomato (7)	HPT-3a, HPT-3b	4	1
		HPT-5,HPT-19a, HPT-19b, HPT-11a, HPT-11b	3	1
	Brinjal (1)	HPB-14	3	1
Jammu and Kashmir	Tomato (2)	JKT-1, JKT-2	3	1
Uttarakhand	Tomato (31)	UTT-1, UTT-2, UTT- 3, UTT-4, UTT- 5, UTT-6, UTT-7, UTT- 8, UTT-9, UTT- 11, UTT-12 , UTT- 13, UTT- 14, UTT-15, UTT-16, UTT-17, UTT- 18, UTT-19, UTT- 20, UTT-21, UTT-23, UTT-25, UTT-26, UTT-27, UTT-28, UTT-32	3	1
		UTT-24, UTT-10, UTT-19,UTT-29, UTT-22	4	1
Jharkhand	Tomato (9)	JHT-02, JHT-03, JHT-04, JHT-3b, JHT-01, JHT-1h, JHT-2P JHT-15 , JHT-16	3	1
	Chilli (3)	JHC-2 , JHC-16 , JHC-3	3	1
	Brinjal (4)	JHB-10, JHB-06, JHB-14, JHB-1	3	1
West Bengal	Tomato (3)	WBT-28, WBT-5, WBT-20	3	1
Orissa	Tomato (11)	ORT-1, ORT-2, ORT-3, ORT-4, ORT-5, ORT-6, ORT-7, ORT-8, ORT-9, ORT-10 , ORT-11	3	1
	Brinjal (10)	ORB-1,ORB-2,ORB-3, ORB-4 ORB-5, ORB-6, ORB-7, ORB-8, ORB-9	40	1
	Potato (4)	ORB-2	-	-
		ORP-01, ORP-02, ORP-03, ORP-04		

* The numbers in parenthesis indicates number of isolates of *Ralstonia solanacearum* used for biovar study

** Deny and Hayward, 2001

2 Diagnostics

a. Detection using species-specific primers

A pair of primers (Hrp_rs2F and Hrp_rs2R) was designed from locus of hrp gene between 7629 to 7952 bp of strain *R. solanacearum* (Accession No. AJ 245811.1). To study the specificity of the primer, 1 µl DNA of *R. solanacearum*, *Xanthomonas campestris* pv. *campestris*, *Pseudomonas fluorescens* and *Bacillus subtilis* was used as a template for PCR. To determine the detection threshold of primer, bacterial suspension of *R. solanacearum* strain was prepared by making a 10-fold dilution series

up to 10^{-8} from a liquid culture. Two and 5 µl bacterial suspensions were taken from 100 dilution containing 10^{9} CFU /ml and 5 µl from dilution 10^{-1} to 10^{-8} used as template for PCR. To determine the population of bacteria, 100 µl of bacterial suspension from dilution 10^{-6} to 10^{-8} was inoculated on the TZC medium and incubated for 72 h at 28^{0}C. Amplified PCR products were separated by electrophoresis and visualized. The primer was tested for differentiation of *R. solanacearum* colony from other saprophytic bacterial colonies on TZC medium through colony PCR. The bacterial colonies were isolated from wilted tomato plants after oozing in sterilized distilled water and further diluted up to 10^{-8}. The 100 µl of bacterial suspension from each dilution was inoculated on the TZC and incubated for 72 h at 28^{0}C. Two different types of colonies were taken from TZC plates and directly used as DNA templates for PCR. Amplified PCR products were separated by electrophoresis and visualized.

All 13 strains tested belong to *R. solanacearum* with an amplification of 323 bp where as no amplification was obtained with other group of bacteria like *B. subtilis*, *X. campestris* pv. *campestris* and *P. fluorescens*. Hence, the PCR based approach was mostly specific for *R. solalnacearum*. False positive reactions with bacterial strains outside the genus *Ralstonia* not likely to occur. The sequence similarity within the *hrp* gene fragment to the other bacteria was found low. To determine the sensitivity of this primer, dilutions of exponentially growing culture were diluted up to 10^{-8} dilution and each dilution of bacterial suspension was directly used as a template for PCR. Special emphasis was placed on the applicability of primer hrp_rs2F and hrp_rls2R on bacterial DNA. PCR applicability of all DNA samples directly extracted from bacteria was confirmed by the amplification of hrp genes fragments. The sensitivity of primer for detection of *R. solanacearum* was evaluated by making serial dilution up to 10^{-8}. The detection limit of the primer from bacterial suspension is 2.0×10^{2} cfu /ml. Since it was possible to detect the 323 bp hrp gene fragment down to a level of 2.0×10^{2} cfu /ml of *R. solanacearum*, it can be assumed that the pathogen may be detectable by *hrp* PCR even if no symptoms of wilt disease appear.

b. Colony PCR

The primer was tested for differentiation of *R. solanacearum* colony from other saprophytic bacterial colonies on TZC medium through colony PCR. The oozing material from wilt infected tomato plants were isolated on TTC medium by dilution techniques and two types of colonies appear on the medium as (i) round, circular, raised, dark pink and (ii) fluidal, irregular raised, pink in centre and whitish in periphery of the colony. Both types of colonies were used as template for PCR. Amplification of fragments of hrp gene was observed only in second type of colonies as fluidal, irregular raised, pink in centre and whitish in periphery, whereas no amplification was noted in round, circular, raised, dark pink. Hence, this set pair of primer clearly differentiated the *R. solanacearum* from other endophytic bacteria.

c. Detection of *R. solanacearum* bacteria from asymptomatic tomato plants

Tomato cv. Pusa Rubi was grown in semi- controlled conditions (Temp. 26± 2^{0}C at National Phytotron Facility, IARI, New Delhi. The 48 h old culture of *R. solanacearum* strain UTT- 24 was inoculated to the 30 days old plants at crown region. Asymptomatic plants were taken at 4th day after inoculation. The sap of macerated plant tissues was used as DNA template for PCR. The *R. solanacearum* was detected from wilted and asymptomatic plants but there was no amplification in uninoculated healthy plants.

3 Epidemiology

a. Population dynamics

An experiment was conducted to find out the inoculum potential of *R. solanacearum* to cause wilt disease in tomato. Nursery of tomato cv. Pusa Rubi was grown under Phytotron at 26 ± 2^{0}C and transplanted at 25 days. *R. solanacearum* strain UTT-25 was grown on CPG agar medium and harvested after 48 h and diluted culture upto 10^{-9}. The inoculum load of *R. solanacearum* maintained from 10^{1} to 10^{10} cfu/ml and inoculated at root zone of mechanically injured and uninjured tomato plants. The inoculum load of bacteria significantly influences disease and its incidence. The wilt disease appeared on 4th day of inoculation in both injured and uninjured roots of tomato in 10^{10} cfu/ml (Table 48) and wilt incidence was 30% and 5% in injured and uninjured roots of tomato. However, injured root of tomato, the disease appeared on 4th day even with the bacterial population of 10^{8} cfu/ml. There was no disease incidence at inoculums levels of 10^{2} cfu/ml in uninjured roots and 10^{1} cfu/ml in injured roots of tomato up to 11 days of inoculation. The data showed that injured roots accelerated wilt incidence even at low population of the bacteria.

b. Effect of temperature on wilt incidence on tomato

Nursery of tomato cvs. Pusa Rubi and N-5 were grown under Phytotron at 26 ± 2^{0}C and transplanted at 25 days. *R. solanacearum* strain UTT-25 was grown on CPG agar medium and harvested after 48 h and inoculum level 10^{10}cfu/ml was maintained. Five ml of bacterial culture was inoculated at root zone of mechanically injured and uninjured plants. The inoculated plants were kept at 20, 25, 30 and 35^{0}C. The appearance of diseases was recorded on every alternate day at 20^{0}C. Wilt incidence did not show more than 20 days of inoculation in both the tomato cultivars

N- 5 and Pusa Rubi either injured or uninjured roots (Fig. 55). However, the wilt incidence increased from 93.86 to 98. 53% in Pusa Rubi injured roots by increasing the temperature from 25 to 35^{0}C, uninjured root from 91.93 to 98.73%. The resistant N-5 showed lower wilt incidence than Pusa Rubi at all the temperatures. But at higher temperature (35^{0}C), wilt incidence in both the cultivars was at par in injured roots.

4 Host resistance

a. Screening of germplasm against wilt pathogen

Seventeen cultivars of tomato were evaluated for their performance against bacterial wilt caused by *R. solanacearum*. Out of 17 cultivars, three cultivars viz., N- 5, H- 24 and Feb-2 showed moderate resistance against this pathogen. Minimum wilt disease incidence was found in N- 5 (36.3%) after 10 days of inoculation followed by H- 24 (51.3%) and Feb- 2 (51.3%). There was a delay of two days in cultivar N- 5 to express the symptoms of wilt disease. However, no cultivar was found to be resistant against *R. solanacearum*. Pusa Rohini, N- 1, Blakan, Chikoo, Pusa Rubi were found highly susceptible (Table 49).

It was also observed that the population of *R. solanacearum* in moderately resistant cultivar was less as compared to susceptible cultivar Pusa Rubi. On 6th day, the disease was expressed in susceptible cultivar having 7.14 log value cfu/g of plant tissues, whereas in moderately resistant cultivar it was 4.62 log value cfu/g of plant tissues. The population of bacteria increased significantly with increasing period. The population of bacteria declined after complete wilting of the plant.

Table 48. Inoculum level of *R. solanacearum* causing bacterial wilt in tomato

Inoculum level of *R. solanacearum* (CFU/ ml	Disease incidence (%)							
	Injured root				Uninjured root			
	4th	7th	9th	11th	4th	7th	9th	11th
10^{10}	30.0 (33.1)	91.0 (76.2)	93.0 (77.6)	98.0 (85.6)	5.0 (7.6)	73.3 (59.0)	90.0 (74.9)	95.0 (82.3)
10^{9}	15.0 (22.5)	90.0 (74.9)	93.0 (77.6)	96.7 (83.8)	0	81.7 (65.1)	91.7 (76.2)	98.3 (85.6)
10^{8}	13.0 (21.3)	75.0 (60.0)	86.7 (68.8)	96.7 (83.8)	0	75.0 (60.0)	88.3 (70.0)	96.5 (83.8)
10^{7}	0	71.7 (57.9)	85.3 (67.3)	95.0 (79.5)	0	58.0 (49.8)	80.0 (63.5)	91.7 (76.2)
10^{6}	0	68.0 (55.8)	80.0 (63.7)	86.7 (68.3)	0	55.0 (47.8)	68.0 (55.7)	80.0 (63.5)
10^{5}	0	56.7 (48.8)	76.7 (61.1)	83.3 (66.2)	0	51.7 (45.9)	70.0 (56.9)	78.3 (62.5)
10^{4}	0	53.3 (46.9)	80.0 (63.9)	83.0 (66.1)	0	0	36.7 (37.2)	73.3 (58.9)
10^{3}	0	0	53.0 (46.8)	73.3 (58.9)	0	0	0	6.7 (8.9)
10^{2}	0	0	0	63.3 (52.7)	0	0	0	0
10^{1}	0	0	0	0	0	0	0	0

CD at 5% : Inoculums level: 3.4; Duration: 2.1; injury level: 1.5; Inoculums level X Duration: 6.7;

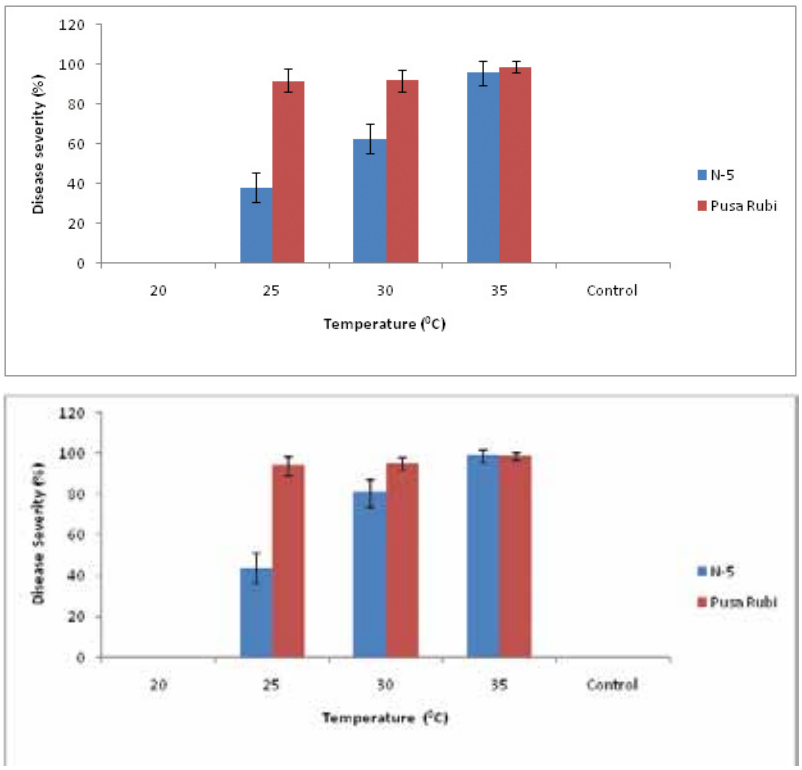

Fig. 55. Effect of temperature on wilt incidence caused by *R. solanacearum* on moderately resistant (N-5) and susceptible (Pusa Rubi) cultivars of tomato (A) uninjured roots (B) injured roots.

5 Disease management

a. Effect of chemicals on growth of *Ralstonia solanacearum* and antagonistic bacteria

An experiment was conducted to test the sensitivity of *R.Solanacearum* chemicals like bleaching powder, calcium chloride, sodium carbonate and sodium bicarbonate, which have antibacterial property. All four chemicals were added in the CPG and NSA medium @ 0.25, 0.50, 0.75, and 1.0 % concentration before sterilization. The pH of the medium was recorded after adding the chemicals separately. Minimum colony 5.89 log value cfu/ml) of *R. solanacearum* was found in bleaching powder (1.0%) followed by calcium chloride (1.0%). In case of *Pseudomonas fluorescens*, minimum colony (5.69 log value cfu/ml) was noted. These four chemicals were less effective against *Bacillus subtilis* (Table 50). The increase in concentration of chemicals from 0.25 to 1.0% decreased the population of both antagonistic and pathogenic bacteria. Bleaching powder (1.0%) suppressed the growth of *R. solanacearum* (5.89 log value cfu/ml) more as compared to *P. fluorescens* (6.37 log value cfu/ml) and B. subtilis (6.26 log value cfu/ml). Since these chemicals were found to reduce the population of *R. solanacearum* as well as antagonistic bacteria (*P. fluorescens* and *B. subtilis*) about 50 percent. For testing of compatibility of chemicals viz., bleaching powder, calcium chloride, sodium carbonate sodium bicarbonate @ 0.1 % concentration with bioagents, was taken up (Table 51). Maximum inhibition zone (7.23 cm^2) was formed by sodium carbonate (0.1%) and B. subtilis (10^{10} cfu/ml) followed by sodium bicarbonate and *B. subtilis* (10^{10} cfu/ml). Bio-efficacy of both the bio agents was enhanced by combining with chemicals. The compatibility of chemical bleaching powder, calcium chloride, sodium carbonate, sodium bicarbonate at the concentration of 0.1% was tested with *P. fluorescens* to improve their bio-efficacy against *R. solanacearum*. Maximum inhibition zone (10.4 cm^2) was formed by sodium carbonate with *P. fluorescens* (10^{10} cfu/ml) followed by sodium carbonate and *P. fluorescens* (Table 52). The affectivity of bioagent was reduced significantly by decreasing the population 10^{10}cfu/ ml to 10^{8}cfu/ ml to reduce formation of inhibition zone 7.08 to 4.90 cm^2 against *R. solanacearum* respectively. Improvement of bioefficacy of both the bioagents *B. subtilis* and *P. fluorescens* might be due to these chemicals have antibacterial prop-

Table 49: Evaluation of important cultivars of tomato against *Ralstonia solanacearum*

Cultivars of tomato	Wilt incidence (%)		
	5 Days	7 Days	10 Days
Pusa Rohini	27.5 (31.2*)	76.3 (61.0)	95.0 (80.8)
N-1	6.3 (7.5)	62.5 (52.4)	96.3 (84.3)
Feb-2	3.8 (5.7)	43.8 (37.5)	51.3 (45.6)
Balkan	30.0 (33.1)	53.8 (47.2)	93.8 (82.5)
Chikoo	13.8 (18.9)	24.0 (25.4)	93.8 (82.5)
H-88	12.5 (14.8)	41.3 (39.6)	70.0 (60.7)
Booster	12.5 (14.8)	43.8 (38.4)	71.3 (59.6)
Pusa - 120	3.75 (5.7)	36.3 (37.0)	80.0 (64.5)
H- 86	35.0 (36.1)	61.3 (51.6)	85.0 (73.4)
H- 24	12.5 (14.8)	42.5 (33.8)	51.3 (45.6)
Pusa Uphar	20.0 (23.2)	43.8 (41.3)	82.5 (73.3)
N- 5	0	17.5 (20.5)	36.3 (34.3)
Pusa Gaurav	3.8(5.7)	47.5 (43.5)	75.0 (60.6)
Pusa Sadabahar	6.3(7.5)	46.3 (42.8)	52.5 (46.3)
H - 36	10.0(13.2)	52.5 (46.4)	85.0 (70.3)
Pusa Rubi	8.8(12.3)	62.5 (52.2)	91.3 (75.3)
Pusa Sheetal	15.0(19.8)	48.8 (44.3)	85.0 (70.4)

Figures in parenthesi are arc sine transformed values.
CD at 5%: Cultivars: 9.0; Duration: 5.4; Cultivars X Duration: NS

Table 50. Effect of chemicals on growth of pathogenic and antagonistic bacteria.

Chemical	pH of media		Growth of bacteria (log value cfu/ ml)		
	CPG	NSA	*Ralstonia solanacearum*	*Pseudomonas fluorescens*	*Bacillus subtilis*
Bleaching powder - 0.25%	7.89	8.10	6.49	6.48	6.36
Bleaching powder - 0.50%	7.95	8.26	6.37	6.46	6.31
Bleaching .powder - 0.75%	8.10	8.81	6.19	6.43	6.28
Bleaching powder - 1.0%	8.20	8.82	5.89	6.37	6.26
Calcium chloride - 0.25%	7.37	6.84	6.38	6.37	6.33
Calcium chloride - 0.50%	7.40	6.89	6.25	6.33	6.31
Calcium chloride - 0.75%	7.59	6.99	6.07	6.31	6.27
Calcium chloride - 1.0%	7.89	7.55	5.91	6.25	6.23
Sodium carbonate - 0.25%	7.69	7.37	6.39	6.48	6.28
Sodium carbonate - 0.50%	7.80	7.40	6.28	6.45	6.23
Sodium carbonate - 0.75%	7.89	7.59	6.26	6.38	6.23
Sodium carbonate - 1.0%	8.10	7.89	6.23	6.32	6.22
Sodium bicarbonate - 0.25%	6.84	7.89	6.48	6.35	6.46
Sodium bicarbonate - 0.50%	6.89	7.69	6.45	6.04	6.34
Sodium bicarbonate - 0.75%	6.99	7.80	6.39	5.97	6.31
Sodium bicarbonate - 1.0%	7.55	7.89	6.33	5.69	6.31
Control	6.98	7.13	9.48	10.45	10.47
CD (5%)			0.039	0.24	0.13

erty, which reduces the inoculum of *R. solanacearum*, thus bioagents able to form more inhibition zone.

An experiment was conducted in Phytotran, IARI, New Delhi as pot culture. The pot was field with 1 kg. of soil and soil was treated with bleaching powder (@ 0.025% and 0.05%) and calcium chloride (@ 0.1 and 0.25%) based on results of in vitro study. *R. solanacearum* (10^9 cfu/ml) was inoculated at crown region. Minimum disease incidence (47.5%) was found in calcium chloride (0.1% conc.) followed by bleaching powder (0.025% conc.) after 9th day of inoculation. The wilt disease incidence was reduced by 50- 52.5 % by applying these chemicals. There was no significant variation was noted in wilt incidence by treating bleaching powder and calcium chloride (Table 53). However, the calcium chloride and bleaching powder both have potential to use as bactericide to control the disease in some extend. The results need to be confirmed by repeated experiments.

Table 51. Improvement of bio-efficacy of *Bacillus subtilis* against *R. solanacearum in vitro*.

Chemical	Inhibition zone (area cm^2)			Mean
	10^{10} (cfu/ ml)	10^9(cfu/ ml)	10^8 (cfu/ ml)	
Bleaching powder (0.1%)	3.96	2.93	2.52	3.13
Calcium chloride (0.1%)	4.16	3.14	2.05	3.12
Sodium carbonate (0.1%)	7.23	6.31	5.18	6.24
Sodium bicarbonate (0.1%)	5.45	2.18	2.04	3.23
Control	3.14	2.45	1.83	2.48
Mean	4.65	3.33	2.77	
CD (5%)	Treatment: 0.46; Population of bacterium: 0.36; Treatment x Population of bacteria: 0.80			

Table 52. Improvement of bio-efficacy of *Pseudomonas fluorescens* against *R. solanacearum* in vitro.

Chemical	Inhibition zone (area cm^2)			Mean
	10^{10} (cfu/ ml)	10^9(cfu/ ml)	10^8 (cfu/ ml)	
Bleaching powder (0.1%)	4.90	4.52	3.25	4.22
Calcium chloride (0.1%)	6.90	5.31	3.91	5.38
Sodium carbonate (0.1%)	9.25	8.05	7.39	8.23
Sodium bicarbonate (0.1%)	10.40	9.31	7.22	8.99
Control	3.96	3.14	2.74	3.28
Mean	7.08	6.07	4.90	
CD (5%)	Treatment: 0.46; Population of bacterium: 0.36; Treatment x Population of bacteria: 0.80			

Table 53. Effect of chemicals on artificially inoculated bacterial wilt of tomato under semi controlled conditions.

Treatment	Concentration (%)	pH of the soil	Wilt incidence (%)					Mean
			5th day	6th day	7th day	8th day	9th day	
Bleaching powder	0.025%	6.98	8.75 (14.91)	18.75 (24.86)	28.75 (31.16)	38.75 (37.63)	50.00 (44.98)	29.00 (30.70)
	0.05%	7.19	15.00 (22.63)	22.50 (28.21)	41.25 (39.88)	51.25 (45.73)	51.25 (45.71)	36.25 (30.84)
Calcium chloride	0.1%	6.86	15.00 (22.41)	28.75 (32.24)	32.50 (34.59)	38.75 (38.29)	47.50 (43.55)	32.50 (34.21)
	0.25%	7.13	11.25 (14.13)	25.00 (26.18)	35.00 (35.77)	42.50 (40.39)	51.25 (45.58)	33.00 (32.41)
Control		6.71	32.50 (34.77)	47.50 (43.54)	65.00 (53.91)	75.00 (60.21)	100.00 (89.96)	64.00 (56.41)
Mean			16.5 (21.71)	28.5 (31.0)	40.5 (39.06)	49.25 (45.45	60.43 (53.95)	

INDIAN INSTITUTE OF HORTICULTURAL RESEARCH BANGALORE

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Dr. C. GOPALAKRISHNAN

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1 Biodiversity

a. Collection of strains of *Ralstonia solanacearum* (RS)

One hundred thirteen *R. solanacearum* isolates were collected from wilted tomato, pepper, and eggplant at 90 different locations in five states. The 109 representative isolates used for further analysis using *R. solanacearum* specific primers for confirmation as *R. solanacearum* isolates. Genomic DNA from 113 representative isolates, representing different hosts and geographical origins was isolated. The isolates were identified by specific amplification of 370 bp DNA fragment. The samples which were confirmed by *R. solanacearum* specific primers were used for further characterization.

b. Molecular characterization of strains of *R. solanacearum* by PCR

The oligonucleotide primers for amplification of 16S rRNA gene of *R. solanacearum* used for the real-time amplification of were designed based on the sequence of 16S rRNA operon available in the NCBI database. Accordingly primer pair MKRSF1/MKRSR1 was designed which amplifies 1.5kb DNA fragment. The primers were selected based on highly conserved region of 16S RNA operon. The genomic DNA isolated from 35 *R. solanacearum* isolates was used for PCR amplification. The 16S rRNA genes of the 15 isolates were amplified by PCR (27mF: 5′-AGAGTTTGATCMTGGCTC AG-3′, 1492mR: 5′-GGYTACCTTGTT ACGACTT-3′). Of the 35 isolates, 15 isolates amplified a PCR DNA fragment of expected size of 1.5kb; where as in control and 5 isolates did not amplify any band of expected size. All the amplified PCR DNA fragments were purified and cloned in to pTZ56 cloning vector. The plasmids were extracted and 16S rRNA genes sequenced. Plasmid isolation and sequencing are in progress for the rest of the 13 isolates.

2 Diagnostics

a. PCR based diagnosis of *R. solanacearum* population in soil/host tissue

PCR based detection of *Ralstonia* was attempted using specific primer pair which amplifies 0.3kb DNA fragment from bacteria infected sample but not from other samples. Initial attempts for direct detection of *R. solanacearum* from plant samples gave very faint band whereas from soil samples multiple bands appeared.

3 Host-pathogen-environment interaction

a. Interaction between *Melodogyne incognita* and *Ralstonia solanacearum* in disease development

Experiment on interaction between nematode and *R. solanacearum* in disease development has been initiated. The nematode, *Melodogyne incognita* is being multiplied on live hosts. Thirty days old brinjal seedlings in pro trays have been inoculated with juveniles (30 juveniles/ml) and are being kept in the glasshouse for multiplication. Twenty days after incubation the infected seedlings will be transferred to earthen pots and kept for 45 to 60 days for large scale multiplication of nematodes. The interaction study will be carried out with simultaneous inoculation of nematode and bacteria on tomato.

4 Host resistance

a. Screening of germplasm lines of tomato for resistance to bacterial wilt

The experiment on tomato germplasm screening for bacterial wilt resistance has been carried out under glass house condition. A total of 39 entries have been screened for wilt resistance with ten replications of five plants in each pot. Eighteen days old tomato seedlings were inoculated with *R. solanacearum* suspension of 10^8 cfu/ml (0.3 OD at 600 nm) by both leaf clipping and axil puncturing method. Weekly observations were recorded on wilting symptoms and the disease was confirmed through ooze test. Results showed that of the 39 entries screened, eleven entries showed highly resistant reaction, three entries showed resistant reaction, seven showed moderately resistant reaction and eighteen entries showed highly susceptible reaction.

b. Screening of germplasm lines of brinjal for resistance to bacterial wilt

The experiment on brinjal germplasm screening for bacterial wilt resistance has been carried out under field condition in bacterial sick plot. A total of 28 entries have been screened for wilt resistance with forty plants for each entry. Forty-five days old plants were inoculated with *R. solanacearum* suspension of 10^8 cfu/ml (0.3 OD at 600 nm) by axil puncturing method. Weekly observations on wilting symptoms were recorded and the disease was confirmed through ooze test. Results showed that of the 28 entries screened, nine entries showed highly resistant reaction, fifteen entries showed resistant reaction and four entries showed highly susceptible reaction.

Table 54. Integrated management of bacterial wilt in tomato (% wilt)

Treatmen	Mean % wilt*	Yield (t/ha)*
1. FYM @ 20 t/ha	24.2	15.6
2. Green manure (sunhemp)	22.4	18.0
3. *Pseudomonas fluorescens* (soil application) @ 5kg/ha	18.8	18.8
4. *Pseudomonas fluorescens* (seedling root dip)	18.6	20.2
5. *Pseudomonas fluorescens* (soil drenching @ 1% solution at 15 days interval starting from 20 days of transplanting	15.2	24.0
6 Streptocycline 250 ppm + COC (3g/l)	32.6	14.6
7. Treatments of FYM + GM + Pf soil application + Pf seedling root dip + Pf drenching	10.2	26.6
8. Control	56.0	8.0

* Mean of four replications

5 Disease management

a. Isolation and characterization of bioagents of *Ralstonia solanacearum*

A total of 35 isolates of *Pseudomonas fluorescens* (Pf) and 15 isolates of *Bacillus subtilis* (Bs) have been isolated from the rhizosphere soils collected from tomato, brinjal and chilli growing areas of Karnataka, Andhra Pradesh and Orissa. Colony characteristics were recorded. Biochemical tests (Grams staining, catalase test, Vogas-Proskaeur test, utilsation of citrate, growth in NaCl, KOH test, pigment production, Levan formation, Starch hydrolysis, Arginine dihydrolysate reaction and liquefaction of gelatin) were carried out for Pf and Bs. The cultures of Pf and Bs were tested against *R. solanacearum* for antagonistic reaction by paper disc and well diffusion assay and the inhibition zone was measured after 72 h of inoculation. Of the 35 isolates of Pf tested for antagonistic reaction against *R. solanacearum*, 11 isolates showed positive reaction. The isolate IHRPf-24 showed the maximum inhibition zone of 70.0 mm. Similarly, of the 15 isolates of Bs tested for antagonistic reaction nine isolates showed positive reaction with inhibition zone ranging from 5.0 to 8.3 mm.

For PCR characterization, DNA extractions were made using standard protocols and PCR characterization was done using ITSI and ITS2 16S-23S rRNA intervening sequence specific primers to get an amplicon of size of 560bp. For Bs 16S rRNA intervening sequence specific primers BCF1 and BCR2 were used to get an amiplicon size of 546 bp.

b. Field evaluation of chemicals FYM, green manure and bioagent against bacterial wilt in tomato

A field trial was carried out in tomato variety Arka Vikas (Bacterial wilt susceptible) for the integrated management of bacterial wilt disease with various treatments and combinations. The treatments include; i) FYM @ 20 t/ha, ii) Green manure - Sunhemp (GM), iii) *Pseudomonas fluorescens* (Pf) (soil application) @ 5 kg/ha, iv) *P. fluorescens* (seedling root dip), v) *P. fluorescens* (soil drenching @ 1% solution at 15 days interval starting from 20 days of transplanting, vi) streptocycline 0.05 g/l + COC 3 g/l, vii) Treatments of FYM + GM + Pf soil application + seedling root dip + Pf drenching and viii) Untreated control. Each treatment was replicated four times with plot size 3 x 4 m in a randomized block design. The whole experiment was carried out in bacterial wilt sick plot. Observations were recorded on percent bacterial wilt incidence and yield.

Results showed that the wilt incidence was lowest (10.2%) in plots where combined treatment of FYM + GM + Pf soil application + Pf seedling root dip + Pf drenching was imposed. This was followed by the treatments, Pf - soil drenching (15.2%) and seedling root dip (18.6%). The treatments, soil application and green manure incorporation recorded 18.8 and 22.4 per cent. FYM and streptocycline + COC recorded 24.2 and 32.6 per cent wilting, respectively. The untreated control registered maximum per cent wilt of 56.0 (Table 46). Similarly, the yield was also significantly high (26.6 t/ha) in plots where treatment combinations of FYM + GM + Pf soil application + Pf seedling root dip + Pf drenching were imposed. The treatments soil drenching, seedling root dip.soil application and green manure incorporation recorded yield of 24.0, 20.2, 18.8 and 18.0 t/ha, respectively. Though the treatments with FYM and streptocycline + COC recorded low yield of 15.6 and 14.6 t/ha, respectively, but they were better than the untreated control which recorded the lowest yield of 8.0 t/ha.

NATIONAL BUREAU OF AGRICULTURALLY IMPORTANT INSECTS, BANGALORE

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1 Disease management

a. Identification and characterization of lytic phages

Electron microscopy: Since PEG method of phage precipitation was not effective in obtaining high titer phage and difficulty in removing PEG completely from the suspension, an alternative method was tried. Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-30 membrane (Millipore) were used to increase the titer of phage (>10^9 pfu/ml) suspension to take TEM image and genomic DNA isolation. One drop of the High-titer (10^9 pfu/ml) phage suspension was placed onto a copper grid coated with formvar and allowed to dry. The phages on the grid were negatively stained with 1% phosphotungstate and images were taken at 12000X using the TEM facility available at Neuropathology lab, NIMHANS, Bangalore. Further purification of phage samples using cesium chloride ultra centrifugation is necessary in order to remove bacterial debris that was also seen in the TEM images. The size of the phage sample ΦRSS5P1 was found to approximately 45.45 nm head, 51.4 nm tail and another phage sample ΦRSS19P1 was observed to be tailless having 16.5 nm head (Fig. 56).

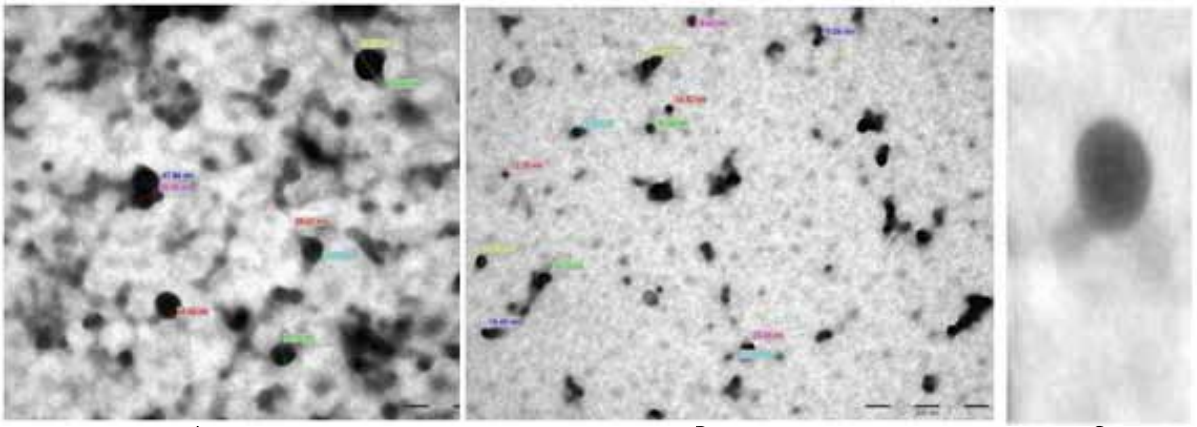
b. Bacteriocin production

Twelve *R. solanacearum* isolates were qualitatively analyzed for their ability to produce bacteriocin. Two days grown isolates were centrifuged to remove bacterial cells and the supernatant was filtered using 0.2 µm filter units. Twenty µl of the filtered supernatant was taken and checked against other strains by well diffusion method and disc method. The plates were incubated for overnight and observed for the presence of inhibition zone. But no inhibition was observed indicating that they do not produce bacteriocin that inhibits the growth of other pathogenic strains.

c. Molecular characterization of phages

Phage lysate concentrated using TFF was used to isolate the genomic material. Viral DNA extraction kit (Chromous Biotech) was used for this purpose. In this method, 200 µl of the concentrated phage isolate ΦRSS22P1 was used to give a final volume of 25 µl of the phage DNA sample. DNA sample (15 µl) was loaded onto 1.5% agarose gel and electrophoresed for 1 h. The molecular weight of the purified phage DNA was above 10 Kb. The exact size of the phage DNA will be known using pulse-field gel electrophoresis (PFGE).

The attP - L 5'-CAGTATGTGTCCTGGGTGTTTGTCTACCG-3' and attP - R 5'-CCTCTTATCAGAACGCCCCACCTCCC-3' primers were used to amplify attP region in the isolated phage genomic material as template. The PCR conditions included initial denaturation at 94°C for 3 min followed by 35 cycles each consisting of denaturation and 94°C for 1 min, primer annealing at 62°C for 1 min, primer extension at 72°C for 1 min and then



A B C

Fig 56. A phage sample ΦRSS5P1 at 120000X B phage sample ΦRSS19P1 at 120000X C ΦRSS19P1

Table 55. Effect of seedling dip treatment with different Pseudomonas isolates on the bacterial wilt incidence in brinjal

Treatment	%wilt		Shoot length$		Root length$		Shoot weight$		Root weight$	
	-P	+P	-P	+P	-P	+P	-P	+P	-P	+P
RP1	0	54.2	99.2	69.4	69.6	55.8	13	5.7	2	1.3
RP2	0	41.7	100.2	72.9	58.3	65.0	11.7	7.0	1.7	1.3
RP3	0	29.2	102.1	81.3	78.1	60.6	15.3	8.7	2	2
RP4	0	20.8	108.8	76.7	74.2	55.0	15.6	7.0	1.7	1.3
RP5	0	8.3	112.1	97.9	76.9	73.9	15	13.0	2.7	2
RP6	0	29.2	121.9	79.8	75.0	65.4	27.7	7.7	2.7	1.7
RP7	0	37.5	93.1	83.8	61.2	72.9	8.7	7.3	1.3	1.3
RP8	0	4.2	107.4	98.5	75.0	83.3	16.7	16.0	3	1.7
RP9	0	33.3	110.2	70.8	64.8	60.0	19	5.7	2.7	1
RP10	0	54.2	111.0	65.2	64.4	56.5	16.7	5.0	2.7	1.3
Control	0	100	112.5	62.5	102.5	39.1	30	0.6	4.7	0.8

RP1-RP10: Pseudomonas isolates, +P: with pathogen, -P: without pathogen, $Length in mm and weight in gram

a final extension of 72°C for 10 min. The amplified products were checked on 1.5% agarose gel. The amplified product was found to be about 300 bp. The presence of attP site indicates that the phage consists of the genes responsible for lysogenic life cycle. Hence they have the possibility to form prophage with the wilt pathogens and are identified as non-virulent or temperate phage. However high titer phage and clear plaques on plate assay against *R. solanacearum* isolate G3 revealed that this phage strain although having the lysogenic genes, do not integrate into the host chromosome of this isolate to form lysogen.

d. Identification of potential phages for biocontrol purpose and development of formulation

For phage mediated biocontrol, the pfu per g of the formulation has to be minimum of 10^{10} to 10^{12} pfu per g. The phages have to be purified from the fermentation biomass of *R. solanacearum*. Tangential Flow Filtration unit has been procured recently for the mass production of bacteriophages. Mass production of phages has been standardized and the potential phage isolates will be identified.

e. Bacterial antagonists for bacterial wilt management

As per the suggestion made during the annual meeting, work on identifying the efficient bacterial isolates for the management of bacterial wilt caused by *R. solanacearum* was started. Forty five effective PGPR isolates from the culture collection of NBAII Bangalore were checked for their biocontrol activity in controlling bacterial wilt. The selected isolates tested in the experiment include *Pseudomonas mosselli* isolates (CK8c and CK24C), *P. aeruginosa* isolates (CK19E, AFP3, AFP4, AFP6, RFP7, OTN8), *P. fluorescens* isolate (GR3ARS3), *P. plecoglossicida* isolate (BA11D1). *Pseudomonas* isolate RFP 8 was found to product the brinjal plants from bacterial wilt and increased the plant growth also. Brinjal plants were treated with the second set of 10 *Pseudomonas* isolates that includes *P. aeruginosa* isolates (PDB1, PDB8, AFP5, AFP7, AFP8, AFP9), *P. fluorescens* isolate (Pf-DWD), *P. putida* isolate (CK-24E, Pf-4K), and along with the phage sample ΦRSS22P1 for their ability to control wilt disease. This work is in progress under greenhouse conditions (Table 55).

LIST OF PUBLICATIONS

Research articles

1. Das, A.K., Ashok Kumar Ingle, A. and Nerkar, S. (2010). Molecular identification of *Phytophthora* spp. causing citrus decline in Vidarbha region of Maharashtra. *Indian Phytopathology* (communicated)

2. Dinesh Singh, Sinha, S., Yadav, D. K., Sharma, J. P., Srivastava, D. K., Lal, H. C., Mondal, K. K. and Jaiswal, R. K. (2010). Characterization of biovar/ races of *Ralstonia solanacearum*, the incitant of bacterial wilt in solanaceous crops. *Indian Phytopathology* 63 (3): 261 - 265.

3. Loganathan., M., Rai., A.B., Ramesh., R., Sharma, B.K., Rai, R.K. and Rai, M. (2009). Vascular wilt diseases-a menace in vegetable crops. Vegetable Science 36: 1-13.

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7. Sriram, S., Savitha, M.J. and Ramanujam, B. (2010). *Trichoderma*-enriched coco-peat for the management of *Phytopthora* and *Fusarium* diseases of chilli and tomato in nurseries. Journal of Biological Control 24(4): 310-314.

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9. Vishwa Dhar, Datta S., Chaudhary R.G., Upadhyay J.P., Saifulla M., Mishra S., Prajapati R.K. and Md. Shamim (2010). Pathogenic and molecular characterizations of pigeonpea wilt pathogen, *Fusarium udum*. Archieves of Phytopathology and Plant Protection (communicated).

Book chapters

10. Ramesh, R., Phadke, G. S. and Bhosle, S. (2010). Inhibition of *Ralstonia solanacearum* by antagonistic bacteria: mechanisms of antagonism. In: Plant growth promotion by rhizobacteria (Eds. Reddy, M.S., Desai, S., Sayyed, R.Z., Sarma, Y.R., Rao, V.K., Reddy, B.C., Reddy, K.R.K., Podile, A.R. and Kloepper, J.W.) Scientific Publishers (India) pp 136-141.

Papers presented in seminar/symposia

11. Dubey, S.C., Priyanka, K. and Singh, V (2010). Genetic diversity in Indian population of *F. oxysporum* f. sp. *ciceris* causing chickpea wilt by sequence analysis of translation elongation factor 1- alpha gene. Presented in National symposium on Perspective in the Plant Health management, December 14-16, 2010, Anand Agricultural University, Anand, p 115.

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BUDGET

Head	Actuals			BE 2011 - 12	Total
	2008-09	2009 - 10	2010 - 11		
A. Recurring					
Contingencies/Other charges	23.85	136.18	241.00	324.802	725.832
TA	1.00	Nil	17.00	106.00	124.00
HRD	Nil	Nil	Nil	159.00	159.00
B. Non Recurring					
Equipment	Nil	55.35	274.00	627.65	957.00
Total	24.85	191.53	532.00	1217.452	1965.832

(Rs in lakhs)