MM I 3.2.Development of Diagnostic Tools for differentiation of biotypes/races of pathogens and insect pests of cotton

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Investigations were done to study the diversity among races / biotypes of economically important pathogens and pests of cotton with the ultimate aim to develop diagnostic tools for their detection and differentiation. The differentiation of strains of pathogens and pests was attempted based on morphological, biochemical and molecular basis at five centers including CICR, Nagpur and its regional stations at Sirsa and Coimbatore, PAU, Ludhiana and UAS, Dharwad. The investigations on various pathogens were done with following major objectives at different centres:

1. Documentation of CLCuV disease syndrome and characterization of viral strains associated with different symptom types.
2. Expression of recombinant coat protein of CLCuV for immuno-detection.
3. Development of protocols for detection of major fungal pathogens of cotton.
4. Collection of strains of *Ramularia areola* and *Fusarium oxysporum* fsp. *vasinfectum* and determination of genetic variability among races/biotypes
5. Collection of strains of *Rhizoctonia solani* and *R. bataticola* and validation of PCR primer for species-specificity.

Work on pests was done mainly to:

1. Identify molecular markers for host-specificity and insecticide resistance in whitefly (*Bemisia tabaci*) and to,
2. **Characterize variability among cotton and non-cotton strains of *Helicoverpa armigera* population.**

Documentation of symptoms of CLCuV infection on cotton and strain variability

Six different symptoms types of cotton leaf curl virus disease viz., 1) upward and 2) downward curling of lamina, 3) severe and 4) mild curling, 5) vein-thickening and 6) enation, were documented based on a survey of disease in states of Punjab, Haryana and Rajasthan. The genome of CLCuV strain associated with each symptoms types were PCR amplified (Fig. 1), cloned and sequenced to determine variability in the strains of virus existing in North India and their role in causing different grades of severity and symptoms, if any. Comparison of the nucleotide sequences of DNA-A and β DNA components of CLCuV associated with different symptom types, revealed large variability that might have resulted by recombination of viral genomes of varying specificities.

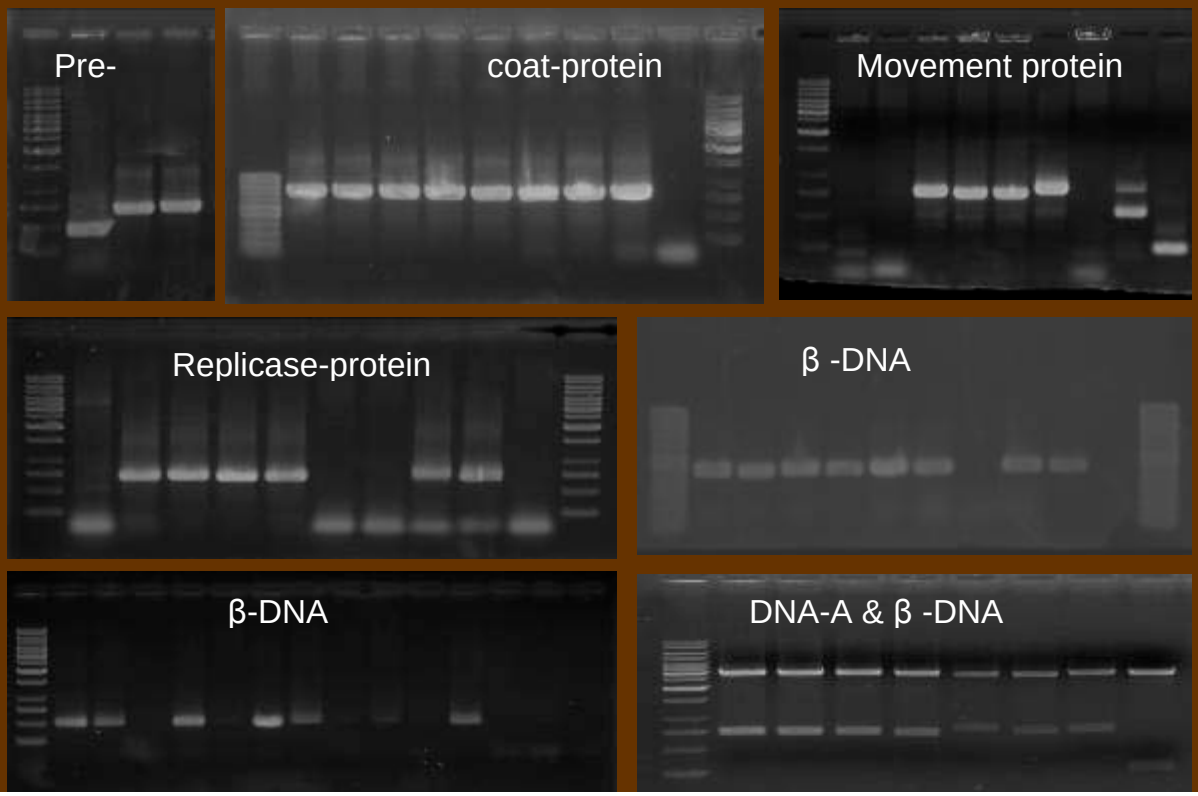

Fig.1. Amplified DNA-A and β-DNA genomic components of CLCuV strains associated with different symptom types.

Immunodiagnosis of CLCuV

The coat protein gene of CLCuV was cloned and expressed in prokaryotic expression vectors pET28b (Novagen) and pCALn (Stratagene). A 32 kD recombinant coat protein of CLCuV having a Calmodulin binding protein tag of pCaln, was used as antigen for the development of polyclonal antibody in rabbit. Western blotting of the expressed coat protein using anti-CLCuV antibody showed that the antibody could bind specifically to express CLCuV coat protein when used as antigen (Fig 2).

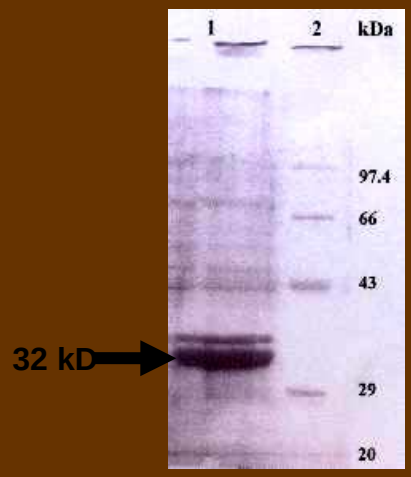

Fig. 2. Western blotting of recombinant CLCuV coat protein with anti-CLCuV antibody

Molecular detection of major fungal pathogens of cotton

PCR based protocols were developed and improvised for specific detection of four major foliar fungal pathogens of cotton viz., *R. areola*, *R. bataticola*, *R. solani* and *A. macrospora*. Primers were developed based on variable nucleotide sequences in the ITS 1 and ITS 4 of the ribosomal RNA genes of these pathogens. Four sets of primers viz., pRSol, pRBat, pAMac and pRare could specifically detect strains of *R. solani*, *R. bataticola*, *A. macrospora* and *R. areola* by amplification of rDNA fragments of 255, 400, 542 and 372 bp, respectively (Fig. 3).

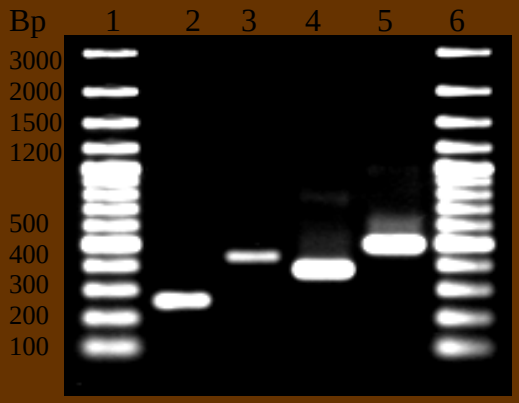

Fig. 3: Detection of cotton pathogens using species-specific primers. Lanes 1 and 6, 100 bp DNA ladder; 2-5, *Rhizoctonia solani*, *R. bataticola*, *Ramularia areola*, *Alternaria macrospora*.

A species-specific PCR primer capable of detecting all strains of *Rhizoctonia bataticola* was also developed and validated at Sirsa center. The new set of primer detects all strains of R. bataticola by amplification a fragment of 450 bp (Fig. 4).

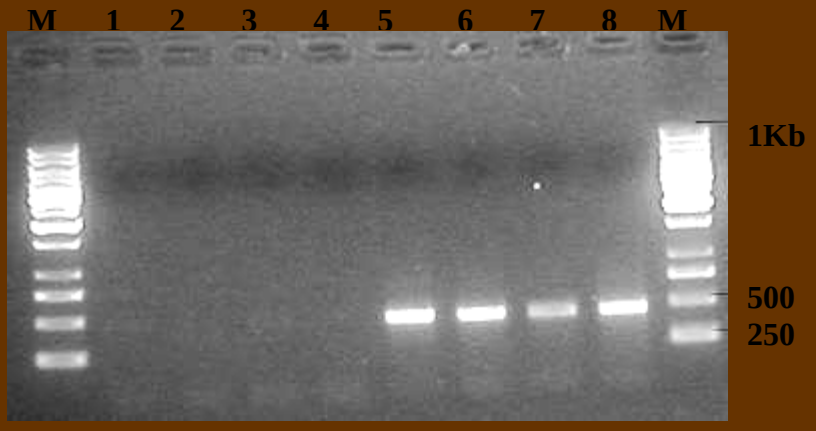

Fig. 4. Specificity of the PCR assay (ITS-Rb-F and ITS-Rb-R primer pairs) with genomic DNA from different isolates of *R.bataticola* and *Fusarium* species. Lanes 1-4 No DNA amplified from Fusarium spp isolates; Lanes 5-8 DNA amplification from *R bataticola* species; M: 1 Kb molecular weight markers.

Variability among strains of *R. areola*

Nine isolates of *R. areola*, three of *G. arboreum* and two each of *G. herbaceum* and *G. hirsutum*, were cultured on a newly formulated synthetic media. Comparison of cultural, morphological and pathogenic characteristics revealed wide variation among three strains isolated from three cultivated species of cotton. The isolates from *G. arboreum* and *G. herbaceum* grew faster on synthetic medium compared to those from *G. hirsutum* cultivars/hybrids, while conidiophores of *R. areola* from *G. arboreum* and *G. herbaceum* cultivars were comparatively smaller than those of *G. hirsutum* cultivars. The strains however did not differ with respect to their conidial size. Isolates of the pathogen from each species were able to infect more prominently the susceptible cultivars / germplasm lines of same species but could infect cultivars/ germplasm lines of other species rather poorly. All isolates however, failed to infect *G. barbadense* cultivars / germplasm lines.

At Dharwad center forty seven isolates of *Ramularia areola* infecting *G. herbaceum, G. arboreum* and *G. hirustum* varieties and *G. hirsutum* hybrids, were collected from cotton growing areas of Maharashtra, Karnataka and Tamilnadu. Based on colony morphology, thickness of mycelial mat, sizes of the perithecia, etc. they have been classified into four groups. The isolates also showed variability in their catalase and peroxidase isozyme profiles. The peroxidase isozyme pattern of the isolates supported existence of four groups identified earlier based upon morphological characteristics.

Based on reaction of *R. areola* isolates, a set of differentials comprising of different species of cotton were identified that is capable of differentially identifying the strains/ races of the pathogen. LRA 5166 is universally susceptible cultivar while GB 124 is universally resistant line in this set. Other cotton lines in this set can aid in identification of specific strains/ isolates. Ten *G. arboreum* genotypes namely, 30814, 30815, 30821, AKA4, AC 631, B-Desh, AK 235, AC 24, G-135-49 and T-1/57-1-1 have consistently shown high degree of resistance against grey mildew for several years and have been identified as potential source of resistance in breeding programme.

Differentiation of strains of Fusarium oxysporum f.sp. vasinfectum.

Eleven isolates of *F. oxysporum* f. sp. *vasinfectum* were obtained from wilt infected roots for characterization of morphological, physiological, pathogenic and molecular variability in the pathogen. Isolates of *F. oxysporum.* f. sp. *vasinfectum* isolated from different locations of the cotton growing areas exhibited distinct variation in their mycelial growth pattern, and

indicated existence of well documented genetic variants within pathogen population. The isolates of *F. oxysporum* f. sp. *vasinfectum* tested for their salt tolerance capacity showed existence of physiologic specialization among the pathogen isolates. Fusarium isolates from Gujarat showed highest levels of salt tolerance at all levels of concentration and time intervals. Eleven isolates of *F. oxysporum* f. sp. *vasinfectum* when tested for their pathogenic variability on a susceptible cultivar G-27 of *G. arboreum* exhibited significant variation in aggressiveness and pathogenic potential.

RAPD finger printing of Fusarium isolates

Analysis of RAPD profile grouped all isolates of the pathogen in two major clusters with several sub-clusters within each group showing varying degree of relatedness (Fig. 5). Correlation of RAPD profiles with virulence pattern of the isolates needs to be established.

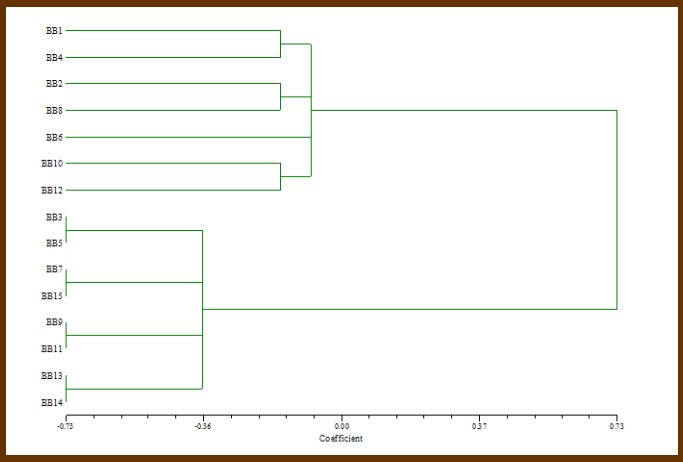

Fig 5 : Dendrogram showing genetic relatedness amongst different isolates of *Fusarium oxysporum* f. sp. *vasinfectum*.

Variability in *Helicoverpa armigera*

An inexpensive method using FTA card (Whatman) equivalent matrix was standardized to store DNA of a wide range of cotton insect pests for subsequent use and analysis. The membrane impregnated DNA would serve as repository for *H. armigera* or any other insect pests for analysis, manipulation and future study. *H. armigera* was a pest that occurred at a low key on cotton in 2006-07. Population of moth in pheromone trap was negligible during the cotton season. Thus diagnostic significance of cornutal spines remains inconclusive. Phylogenetic analysis based on mitochondrial DNA sequence showed existence of 19 haplotypes of *H. armigera* in North, South and Central India with Haplotype 17 as a founder haplotype. The strains can be identified by PCR –RFLP of mitochondrial CO I using *Bst* 2UI or *Bcn*1 (Fig. 6 a &b).

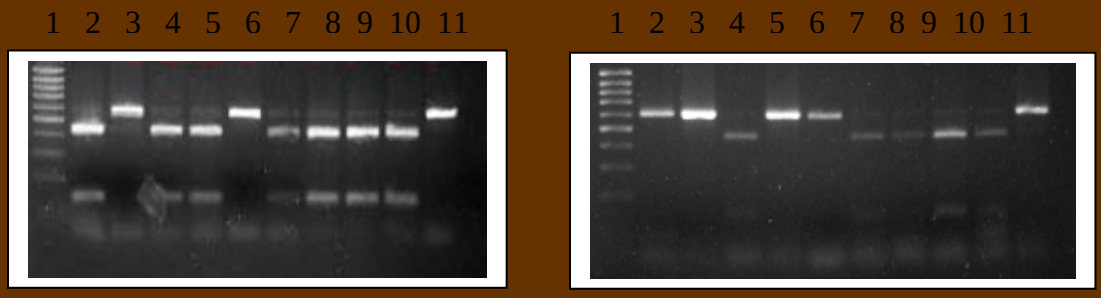

Fig. 6 a&b: PCR-RFLP of the partial CO-1 region to identify haplotype 17 of *H. armigera*. Partial CO-1 PCR product of Haplotype 17 specifically possesses *Bcn*I (a) and *Bst*UI (b) restriction sites. Lanes: 1, 100 bp DNA ladder; lane 11, undigested PCR product; lanes 3 and 6 in a, and 2-3, 5-6 in b are not strains of haplotype 17.

Molecular mapping of *H. armigera* populations from cotton and non-cotton hosts from different geographical locations using SSR markers done at Coimbatore center indicated significant genetic variability. The non-cotton population formed a group that is more or less distinct from the cotton strain. Similarly *H. armigera* strains from different locations of Maharashtra showed polymorphism based upon SSR marker-based fingerprinting. Though host-specific SSR based marker could not be established, distinct variability among *H. armigera* population was documented at different geographical locations. The multi-crop ecosystem in India might be facilitating *H. armigera* population for interbreeding, generating new specificities.

Molecular Characterization of Host-specificity in Whitefly

Genetically heritable markers for host-specificity (Fig. 7) and insecticide resistance were identified through comparative RAPD profiling of whitefly populations reared on specific plant hosts (cotton, brinjal, soybean, tomato, potato, *Sida* sp) or selected for insecticide resistance (Imidacloprid, Triazophos). Out of total 85 RAPD markers identified for host-specificity, 39 markers appeared to be specific for single-host with maximum markers identified for tomato (14) and cotton (13) followed by brinjal (5), *Sida* sp (4) and Soybean (3) with no single-host specificity marker for potato. Thirty-seven genetically stable markers for host-specificity were cloned and eight were sequenced and specific primers designed for subsequent screening and validation for specificity.

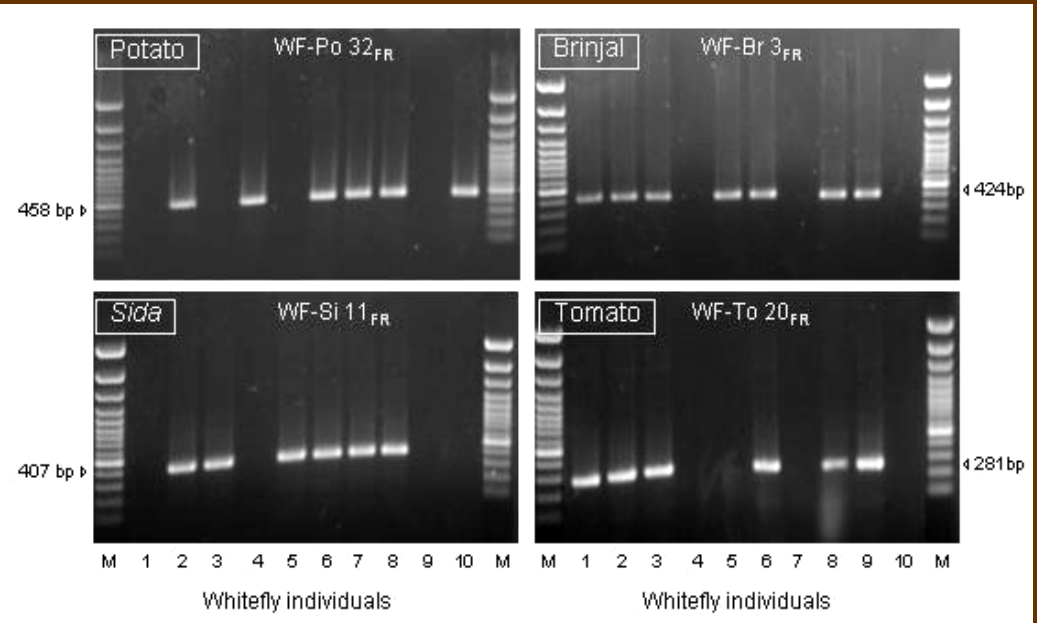

Fig 7. Validation of different SCAR markers for specificity in whitefly populations collected from fields of Potato, Brinjal, *Sida* and Tomato)

Besides host-specific markers nine heritable markers were identified for resistance against Triazophos (7) and Imidacloprid (2) in cotton whitefly.