MM 3.2 : Development of diagnostic tools for differentiation of biotypes/races of pathogen and insect pests of cotton

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Targets and achievements

Target / activity	Achievement
Xanthomonas axonopodis* pv. *Malvacearum	
Diagnostic tools for identification and detection of *Xam*	The PCR method has been further optimized to detect minimum of 25 bacterial cells
Differentiation of races and biotypes of Xam.	RFLP marker developed differentiated virulent race 18 isolates from less virulent races. At least 10 biotypes documented within race 18 based on native plasmid, RFLP, RAPD and Rep-PCR genomic finger printing of isolates of *Xam.*
***Rhizoctonia* species**	
Survey and collection of *R. solani* and *R. bataticola* isolates.	Isolates of *rhizoctonia* species were collected from different growing area of the country.
Differentiation of isolates	rDNA sequences of isolates of *Rhizoctonia* spp. were amplified using conserved ITS primers. Restriction enzymes analysis of the ITS region showed significant variability. PCR amplified ITS regions representative isolated under RAPD groups was cloned and sequenced.
Marker for B-Biotype	SCAR markers were developed for standard B- biotypes and Indian Whitefly. Using specific PCR primer the whitefly types were mapped in cotton growing areas of north, central and south India.
Host and Pesticide induced variability	RAPD amplicons were generated from whitefly populations collected from different hosts as well as insecticide induced resistant populations of cotton whitefly.
Pesticide induced variability and developments of SCAR markers for insecticide resistance monitoring	Could not be undertaken.

Target / activity	Achievement
Cotton leaf curl virus	
Detection protocol	PCR method developed detected latent infection of CLCuV and is routinely used for detection of the virus. The desi cotton is still safe to grow as proved by southern hybridization.
Characterization of the viral gene sequence for differentiation among CLCuV strains.	Could not be done last year
Immunodiagnostic detection by twig imprint blot method	The method has been developed to detect infection in cotton using anti-cabbage leaf curl virus antibody.
Grey Mildew	
Standardization of media for isolation of *Ramularia areola*	Two media, a synthetic and a non-synthetic were developed for culturing *R. areola.* The latter also supported development of perithecia in culture.
Morphological, physiological and molecular based diagnostic tools for identification and differentiation of races/biotypes of cotton pathogen *Ramularia areola*	The conidiophores of *hirsutum* and *barbadense* isolates *R. areola* were 2-3 times longer than the *herbaceum* and *arboreum* isolates. The arboreum isolates were more aggressive than other isolates. RAPD marker for different isolated of *R. areola* developed, which need validation at different centers.
Biochemical analysis of the host pathogen interaction	Zymogram developed based on peroxidase isozyme pattern of 25 isolates exhibited 3 distinct pattern of variability.
Fusarium wilt	
Cultural variation	The 13 isolates of FOV showed wide variability in their salt tolerance, pigment production in the medium and pathogenicity based on which race variability needs to be documented.
Diagnostic tool	The PCR based method for diagnosis of *Fusarium* isolates standardized using primer conserved to rDNA of fungal pathogens.

Target / activity	Achievement
Helicoverpa armigera	
Intra-specific variability-Morphological markers	Cornutal spines as equivocal morphological marker was not validated as strict correlation could not be observed between host specificity and spine numbers.
Biochemical markers	Unique esterase isozyme contemplated as biochemical marker for identification of cotton strain of *H. armigera* could not be validated during this year.
Molecular marker	PCR-RFLP of COI region with *Bcn*I and *Bst*IUI distinguished cotton strain from non-cotton strain of *H. armigera.*
Inter-specific variability	PCR-RFLP of COI region with *Rsa*I and *Alu*I distinguished *Helicoverpa armigera* and *H. assulta* species.

Progress of work

Investigations were done to study the diversity among races / biotypes of economically important pathogens and pests of cotton with the ultimate aim of developing 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 - CICR, Nagpur and its regional stations at Sirsa and Coimbatore, PAU Ludhiana and UAS Dharwad.

Bacterial Blight (Xanthomonas axonopodis pv. malvacearum)

Diagnostic tools for detection of *Xam*

Bacterial blight of cotton caused by *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*) is the only important bacterial disease of cotton of quarantine significance. African strains of the pathogen prevalent in USA and Africa are the most destructive and do not possess any commercial sources of resistance. A rapid protocol for detection of the seed borne infection of *Xam* will be helpful to prevent introduction of hitherto non-existent, African strains into our country. A PCR protocol was developed that enabled rapid diagnosis of strains of *Xam*. The protocol was simple, rapid and amplification can be achieved within 1.30h.

Besides, the amplification can be done using whole cells of bacterium without the need for extraction of DNA.

A minimum template threshold that could support successful amplification was determined by using diluted samples for PCR and simultaneously plating each dilution on the YGCA medium to ascertain bacterial population. Using this method it was observed that a minimum of 25 *Xam* cells were needed for successful amplification in PCR

The PCR detection method is routinely used in our lab to identify strains of *Xam* from other yellow colour saprophytes, which often exit as morphologically indistinguishable contaminants of *Xam*

Characterization of Biotype variability in race 18 of *Xam*

While identifying the races of *Xam* on differentials it was noticed that race 18 isolates showed apparent variability in their aggressiveness. This was evident from difference in their water soaking ability symptoms on cotton differential lines. On susceptible lines some of the race 18 isolates showed more confluent water soaking compared to others

On immune lines 101-102 and S295 the necrotic spots produced by some of them appeared moist rather than the typically dry HR reactions characteristic of immune host response. Growth curve analysis of race 18 isolates of *Xam* on bacterial blight immune and susceptible line had previously shown that the isolates varied significantly in their growth pattern.

Thirty new isolates of *Xam* were collected from farmers' fields in districts of Wardha, Nagpur and Amravati. Based on their reaction on cotton differentials all of them were found to belong to race 18. Genomic and plasmid DNA were extracted from all 30 isolates in order to delineate biotype variability based on their native plasmid profiles, RAPD, Rep-PCR and RFLP.

Rep-PCR genomic fingerprinting

Rep-PCR genomic fingerprinting makes use of DNA primers complementary to naturally occurring, highly conserved, repetitive DNA sequences, present in multiple copies in the genomes of most Gram-negative and several Gram-positive bacteria (Lupski and Weinstock 1992). Three families of repetitive sequences have been identified, including the 35-40 bp repetitive extragenic

palindromic (REP) sequence, the 124-127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence, and the 154 bp BOX element (Versalovic *et al.* 1994). These sequences appear to be located in distinct, intergenic positions around the genome. The use of these primer(s) and PCR leads to selective amplification of distinct genomic regions located between REP, ERIC or BOX elements. The corresponding protocols are referred to as REP-PCR, ERIC-PCR and BOX-PCR genomic fingerprinting respectively, and rep-PCR genomic fingerprinting collectively (Louws et al 1999). The rep-PCR genomic fingerprints generated from bacterial isolates permit differentiation to the species, subspecies and strain level.

Rep-PCR genomic finger printing of race 18 isolates did not yield conclusive pattern that could be of any diagnostic significance. However BOX and ERIC-PCR generated reproducible pattern of genomic fingerprint of race 18 isolates. Based on the BOX-PCR fingerprinting pattern 30 isolates were grouped in 10 major groups with group V, accommodating 13 isolates

Six groups were unique having one isolates each. However, ERIC-PCR was more sensitive as it further delineated variability within three isolates that were accommodated within group V, based on BOX-PCR (Fig.3.2.5a-c). Based on ERIC PCR, the isolates formed 11 groups (Fig. 3.2.5d). The isolates 19, 20 and 35 grouped together based on BOX-PCR fingerprinting formed separate groups on the basis of ERIC-PCR.

Randomly amplified Polymorphic DNA (RAPD) analysis

RAPD analysis of 30 race 18 isolates was done with primer OPA13 that was previously found to generate reproducible fingerprinting pattern for *Xam* strains. RAPD analysis with OPA 13 primer further showed variability among some group members that were grouped together based on Rep-PCR. Based on RAPD fingerprinting (Fig 3.2.6 a-c), 30 isolates were grouped in 16 groups **(Fig 3.2.6d**).

The primer generated amplicons ranging in size from 0.25 to 4.0 kb with minimum number of amplicons of 1 and maximum 10.

Comparison of Plasmid profiles

The Native plasmids of 30 race 18 isolates of *Xam* were extracted following the protocol developed in our Lab (Chakrabarty 2003) and compared for assessing intra-racial variability. All isolates possessed plasmids that varied in number from 1-3 among different race 18 isolates (**Fig 3.2.7a-c**).

Quite often isolates possessing same number of plasmids may vary in sizes of their plasmids. Plasmid profiles of *Xam* isolates categorised 30 isolates of race 18 in 12 groups with numbers ranging 1-3 .

Restriction fragment length polymorphism (RFLP) analysis

RFLP analysis was done to detect variability amongst 30 race 18 isolates of *Xam*. RFLP analysis exhibited distinct polymorphism between several race 18 isolates of *Xam.* RFLP analysis of 30 isolates of *Xam* showed scorable hybridising bands in 15 isolates.

Fifteen isolates of Xam. formed seven groups based on their RFLP patterns. Most of the race 18 isolates possessed two pthN hybridising fragments. Accordingly group I is the biggest and is comprised of 5 isolates each having two hybridising bands of ca. 14 and 5 kb. Maximum of six hybridising bands were present in a lone isolate that belonged to group VI.

Molecular analysis of 30 isolates based on Rep-PCR, RAPD, RFLP and plasmid profile showed existence of at least 10 biotypes within race 18 of *Xam* in three districts of Maharashtra.

Development of race 18 specific RFLP marker

RFLP of genomic and plasmid DNA of isolates belonging to six different races clearly exhibited polymorphism among the races (Fig 3.2.9). Number of bands that hybridised to pthN probe varied from 2-6 in different races. The numbers of pthN hybridising fragments were less (usually two) in highly virulent race 18 compared to more number of bands in less virulent races. In general race 18 isolates do not possess pthN hybridising bands between 5-14 kb while in less virulent races several bands exist between these two, race-18 specific RFLP markers (Fig.3.2.10).

rDNA PCR analysis

In order to differentiate biotypes of race 18 based on rDNA sequence, PCR analysis of six representative isolates, one from each RFLP group, was done with conserved primers. The primers amplified approximately 0.65 kb DNA fragment from six out of seven isolates representing seven RFLP groups.

The rDNA fragment amplified from isolate 6 was slightly bigger compared to rest of the six isolates. Variability in ITS region is being analysed by restriction digestion of the amplified rDNA fragment. The rDNA fragment amplified from seven representative isolates of *Xam* were cloned in pGEMT (Fig. 3.2.11) and will be sequenced using vector based primers.

Cotton leaf curl virus detection

Use of new PCR protocol for detection of infection

Rapid, PCR protocol was employed for detection of CLCuV infection in *G. hirsutum* cotton, weed hosts as well as from diploid cotton grown in the vicinity of CLCuV infected *G. hirsutum*. Besides, detecting infection in plants showing typical symptoms of disease, primers also detected infection in several asymptomatic cotton by amplification of a 0.7 kb DNA fragment. Surprisingly diploid cotton also generated some non-specific amplicons of size less than 0.7 kb with CP gne specific primer. Southern hybridisation of PCR amplicons generated from diploid cotton with CP gene probe showed that the it did not hybridise to the amplified fragments ruling out any possibility of diploid cotton serving the collateral host to the pathogen

Coat Protein Expression

The conditions for expression of CLCuV coat protein in *E. coli* were standardised. Protein expression and western blotting of several recombinant clones were done to evaluate the level of coat protein expression (Fig. 3.2.13 a).

Grey Mildew *(Ramularia areola)*

The work on physiological specialization of *Ramularia areola* was conducted independently at three centres viz., CICR, Nagpur, Coimbatore and UAS Dharwad with the following objective:

1. Morphological, pathological and molecular variability in isolates of *R. areola*

Survey and collection of diseased samples

The initial appearance of the grey mildew was observed with few scattered white powdery spots on the under surface of the lower leaves during the first week of August. Maximum growth of the pathogen with profuse sporulation was observed from the second week of September. Due to continuous dry spell the disease development was slow during the month of October and decline from November and onwards. However under irrigated conditions disease progressed slowly on the under surface of lower leaves even during the month of December 2004 and January-February 2005.

Mycelial growth of different isolates

Nine new isolates of *R. areola* were cultured from the samples collected from different cotton growing areas of Maharashtra. These isolates were compared based on their differences in growth rate on synthetic medium. Observation on radial growth was recorded at 72 hours after inoculation

The isolates from the cultivars of *G. arboreum* and *G. herbaceum* grew fast on the synthetic medium compared to the isolates from *G. hirsutum* cultivars/ hybrids. To further ascertain the differences in their growth pattern the cultures were grown in new synthetic broth for analysis of the dry weight. After culturing the isolates from different species under uniform growth conditions dry weight of mycelium was highest in case of *G. arboreum* isolates followed by the isolates from *G. herbaceum*. Minimum mycelium was recorded in case of the isolates obtained from *G. hirsutum* cultivars/hybrids.

Morphological differences in conidiophores

Freshly infected leaves were collected from 23 different cultivars of *G. arboreum*, *G. herbaceum* and *G. hirsutum* cultivars/hybrids for recording morphological differences in conidiophores. The size of the conidia from the infected leaves of the cultivars of *G. arboreum*, *G. herbaceum* and *G. hirsutum* cultivars/hybrids were almost similar and ranged from 2.4 –5.0 x 10 –31.0 µ. However, there was a significant difference in average size of conidiophores in freshly infected leaves of *G. arboreum, G. herbaceum* and *G. hirsutum* cultivars/hybrids (Table-3.2.1).

Table 3.2.1: Variability in *R. areola* isolates based on the sizes of conidiophore

S NO.	SPECIES	CULTIVAR	SIZE OF CONIDIOPHORES	
			Average	Range
1	*G. arboreum*	AKH-4	45.5 X 3.5 u	14 –80 X 3 –5.5 u
2	*G. arboreum*	B-185 NLL	45.9 X 3.5 u	14 –80 X 3 –5.5 u
3	*G. arboreum*	B-12 A	45.7 X 3.5 u	14 –80 X 3 –5.5 u
4	*G. arboreum*	AKA-8401	45.5 X 3.5 u	14 –80 X 3 –5.5 u
5	*G. arboreum*	BDN 5628	45.7 X 3.5 u	14 –80 X 3 –5.5 u
6	*G. arboreum*	G-27	45.5 X 3.5 u	14 –80 X 3 –5.5 u
7	*G. arboreum*	AC-25	45.6 X 3.5 u	14 –80 X 3 –5.5 u
8	*G. arboreum*	G-25	45.5 X 3.5 u	14 –80 X 3 –5.5 u
9	*G. arboreum*	AKA-5	45.5 X 3.5 u	14 –80 X 3 –5.5 u
10	*G. arboreum*	Chandrolla	45.9 X 3.5 u	14 –80 X 3 –5.5 u
11	*G. herbaceum*	Jayadhar	47.5 X 3.5 u	20 - 84 X 3 - 5.5 u
12	*G. herbaceum*	4711	47.5 X 3.5 u	20 - 84 X 3 - 5.5 u
13	*G. herbaceum*	DB-312	47.8 X 3.5 u	20 - 84 X 3 - 5.5 u
14	*G. herbaceum*	Digvijay	47.7 X 3.5 u	20 - 84 X 3 - 5.5 u
15	*G. hirsutum*	LRK-516	133.2 X 4.2 u	32-240 X 3 – 6 u
16	*G. hirsutum*	SRT-1	137.2 X 4.2 u	32-240 X 3 – 6 u
17	*G. hirsutum*	LRA-5166	137.2 X 4.2 u	32-240 X 3 – 6 u
18	*G. hirsutum*	4235 (BT)	117.4 X 4.1 u	28-160 X 3 – 6 u
19	*G. hirsutum*	4224 (BT)	110.2 X 4.1 u	28-190 X 3 - 6 u
20	*G. hirsutum*	4243 (BT)	118.1 X 4.1 u	28-165 X 3 – 6 u
21	*G. hirsutum*	4257 (BT)	117.2 X 4.1 u	28-155 X 3 – 6 u
22	*G. hirsutum*	4221 (BT)	117.4 X 4.1 u	28-160 X 3 – 6 u
23	*G. hirsutum*	4225 (BT)	110.2 X 4.1 u	28-190 X 3 - 6 u

The length of conidiophores of *R. areola* on *G. hirsutum* cultivars/hybrids was almost three times the size compared to that of the isolates growing on *G. arboreum* and *G. herbaceum* cultivars. Thus morphological variability in terms

of size of the conidiophores provided evidences of existence of physiological races/ biotypes of *R. areola*.

Cross inoculation test

During the year 2004-05, five new isolates along with eight isolates collected during 2003-04 were tested for their cross infectivity on twenty six cultivars/lines belonging to four cultivated species of cotton. The isolates from *G. herbaceum, G. arboreum* and *G. hirsutum* cottons were able to readily infect the respective host cultivars from which they were isolated. They could also infect several other cultivars belonging to the species from which the isolates were originally obtained causing different degree of severity. *While G arboreum* isolates could infect some cultivars of *G. herbaceum* as well as *G. hirsutum* with moderately resistant reaction none of the *G. herbaceum* isolates could infect *G. hirsutum* lines. Similarly none of the *G. hirsutum* isolates could infect *G. hirsutum* lines. Isolates from *G. hirsutum* and *G. herbaceum* cottons infected some highly susceptible lines of *G. arboreum* with moderately susceptible to moderately resistant reaction. The development of the disease was rather slow on these lines of *G. arboreum* exhibiting hypersensitive reactions. The observations indicated that arboreum isolates had a wider infectivity compared to other isolates. Although isolates from all the three cotton species showed some degree of cross-infectivity, but the isolates were best suited to the respective host cultivars and species from which they were originally cultured. None of the thirteen isolates tested could infect *G. barbadense* plants on artificial inoculation. Variability in reaction of thirteen isolates on twenty-six cultivars of four different cultivated species indicated existence of physiological specialization in *R. areola*.

In Coimbatore heavy incidence grey mildew was noticed on all four cultivated species of Gossypium (during 2004 – 05). For the first time, many germplasm lines of G. barbadense were found affected by this disease. However, the damage to the foliage was not to the extent as on other three cultivated species. In addition, Grey mildew like symptoms were also observed extensively on the common weed (*Euphorbia heterophylla*) found near the cotton fields. The *Ramularia areola* isolates were collected from the above named five hosts and utilized for the study.

Based on symptomatology and morphological features *Ramularia areola* Isolates were categorised into four groups :

***Arboreum and Herbaceum* isolate:** Irregular, angular/areolate spots with powdery growth on the under surface of the leaves with corresponding yellowish green lesions on the upper surface. In severe cases, the mildew also appeared on the upper surface (Fig. 14a & b). The conidiophores short, emerging through epidermal layer in clusters from the sub-epidermal stroma bearing 0 – 3 septate, cylindrical/oblong conidia singly on each conidiophore.

***Hirsutum* isolate:** Initially scattered greyish white powdery growth on the under surface of older leaves at the base of the plant with corresponding yellowish green spots on the upper surface. As the disease developed, the powdery growth spread on the entire leaf and also on the upper surface (Fig.3.2.15a & b).

The conidiophores long, emerging through the epidermis in clusters from a sub-epidermal stroma-bearing single 0 – 3 septate, cylindrical, oblong solitary conidium on each conidiophore.

***Barbadense* isolate:** Scattered greyish powdery growth mainly on the under surface of the leaves with yellowish/brownish spots seen on the upper surface. Necrotic spots are also seen on the upper side of leaves (Fig.3.2.16a & b).

Hyaline long conidiophores borne singly or in bunches emerging through epidermis from a sub-epidermal stroma bearing single celled or multi-septate, cylindrical or oblong conidia singly on each conidiophore.

***Euphorbia* isolate:** Large areolate powdery spots seen on the under surface of the leaves with visible chlorotic (yellowish green discolouration) on the upper surface. Under severe disease development, powdery growth seen on the upper surface also The conidiophores hyaline, single, long and flexuous; emerging through epidermal layer and bearing a single cylindrical, club or spear shaped conidia; the conidia are single celled.

Pathogenic variability on different hosts

Spores of *R. areola* were extracted washed and obtained from the leaves and used for inoculation of host plants. Plants of six lines of *G. hirstutum*, nine of *G. arboreum*, four of *G. herbaceum*, five of *G. barbadense* (identified from earlier studies) and the weed host (*E. heterophylla*) raised in pots were inoculated with

spore suspensions of the pathogen collected from various hosts. Based on observations made from these experiments, few hosts have been identified which could be used as differentials for determining physiological races.

The weed (*Euphorbia heterophylla*) has been identified as an alternate host for *R. areola*. The isolate from this host was able to infect the susceptible lines of all four cultivated *Gossypium* spp. and *vice versa* producing typical areolate spots. The hosts exhibited different reaction to different isolates. The *G. barbadense* isolate was the most virulent followed by the isolate from *G. herbaceum*. Contrastingly, *G. barbadense* lines could not be infected by artificial inoculation in Nagpur. Also arboreum isolates were found to be more virulent compared to others. The variation in observations may be due to difference in inoculum preparation at two places. In Coimbatore fungal spores harvested from the infected plants were used for inoculation while in Nagpur the spore mass raised on synthetic medium was used for inoculation.

Cotton LRA 5166 (*G. hirsutum*), Cernuum, Chandrolla and G. 27 (*G. arboreum*), Jayadhar (*G. herbaceum*) and the weed host have been identified as the universal suscepts. All *G. arboreum* lines except AC 36 and *G. herbaceum* lines have been found highly susceptible to all isolates of *R. areola*. The isolate from the weed host showed only hypersensitive reaction or few spots on IC 629 (*G. hirsutum*), AC 36 (*G. arboreum*), GB 119 and ERB 3758 (*G. barbadense*). Similarly the *G. barbadense* host GB 119 expressed hypersensitive reaction to all isolates. GB 124 (*G. barbadense*) was resistant (no symptoms) to both *hirsutum* and *arboreum* isolates. ERB 3758 (*G. barbadense*) resistant to the *arboreum* isolate and expressed hypersensitive or few spots to *hirsutum*, *herbaceum* and weed isolates. The *G. barbadense* – Suvin was resistant to the *arboreum* isolate. Inclusion of new *G. barbadense* lines might help in differentiation of races/strains of *R. areola* of the isolates.

Biochemical basis of variation

Even though there were minor variations among the cotton leaf constituents (phenol, protein, proline and gossypol contents), these might not serve as a tool for differentiation of *R. areola* isolates .

Ability of different isolates to evoke differential defense enzyme activity within same host points towards physiologic variability in pathogen isolates.

- The *barbadense* isolate when inoculated on the four *Gossypium* spp. exhibited higher catalase activity and low super oxide dismutase (SOD) and Ascorbic acid oxidase (AAO) activities except on LRA 5166 (*G. hirsutum*).

- The *herbaceum* isolate when inoculated showed lower catalase activity (except on Cernuum – *G. arboreum*) and very high AAO activity (except on Suvin – *G. barbadense*)
- The *hirsutum* isolate led to high SOD activity upon interaction with all hosts.
- The *arboreum* isolate exhibited high polyphenol oxidase (PPO) activity on RAHS 14 (*G. herbaceum*) and LRA 5166 and low PPO activity on Cernuum and Suvin.

The above experiments were repeated several times and the results clearly indicated that the induction of differential enzyme activity in same host is possible only if there is a physiological and genetic variability among the *R. areola* isolates.

At Dharwad center twenty-five *areola* isolates were collected from different cotton growing areas of Maharastra and Karnataka. These isolates were cultured from three cultivated species of cotton including *G. herbaceum, G. arboreum* and *G. hirustum* varieties and hybrids. The isolates have been maintained on Potato Sucrose Agar medium. Detailed investigations on pathological, morphological and cultural variability of isolates *of R. areola* exhibited wide diversity with regard to growth, colony characteristics, pigmentation and formation of reproductive propagules on the culture medium. Based on these characters the 25 isolates were categorised in four major groups viz., Nagpur, Coimbatore, Dharwad and Kumbhapur. An attempt was made to differentiate representatives of each group based on their peroxidase isozyme pattern. The variability among members of four groups is presented in the zymogram.

All isolates possessed a common band of Rm value 0.41. The Coimbatore isolate exhibited two additional bands of 0.84 and 0.91, while Nagpur group possessed the band of 0.84 and differed from former in lacking the band at Rm 0.91. There was no variability between Dharwad and Kumbapur isolate based on the peroxidase isozyme pattern both of which possessed a single band that is

common to all isolates. Biochemical groups in *areola* isolates corroborate with the groupings done earlier on the basis of pathological and morphological groups.

Cotton Wilt *Fusarium oxysporum* fsp. *Vasinfectum*

During crop season 2004-05, thirteen wilt affected cotton plants were collected from in different cotton growing areas of Maharashtra and Punjab.

A. Isolation, identification and maintenance of cultures

Fusarium wilt affected samples of cotton plants were obtained from different areas of Maharashtra and Punjab to study the genetic diversity and development of diagnostic methods. Thin slices of affected roots after surface sterilization were inoculated on petridishes containing potato dextrose agar medium After 2-3 days of incubation at 28°C$\pm$1°C in BOD incubator initial growth of *Fusarium oxysporum* fsp. *vasinfectum* was observed. Pure cultures of the isolates of pathogen were maintained by hypal tip isolation method. Preliminary identification was done on the basis of cytomorphological features. Temporary slides of the isolated culture were made for microscopic observations of the micro conidia and macro conidia of the pathogen *F. o.* fsp. *vasinfectum*. Pigmentation of mycelial mat etc. was also studied for the purpose of identification of the pathogen. Pure cultures of *F. o.* fsp. *vasinfectum* were maintained on potato dextrose agar medium. Subcultures were made every 1-2 month to ensure viability of the cultures. Cultures were stored at 4 °C in refrigerator for use in future study.

Differentiation of biotypes/strains of pathogen:

Mycelial growth of different isolates

During the year 2004-05, thirteen new isolates were made for various methods of differentiation of strains of the pathogen. The growth pattern of these isolates was studied to know the visual differences in growth rate on potato dextrose agar medium.

Visual differences in growth rate and colony morphology were observed on PDA. Minimum growth of 46.66 mm and 0.518-mm/h growth rate was observed in culture FOV-18. However, the maximum diametric growth of 60.33 mm with 0.704 mm/h growth rate was recorded in case of FOV-19 .

It appeared that the isolates of *F. o.* fsp. *vasinfectum* of diploid cottons had some variations in the mycelial growth pattern and growth rate. Some isolates of *F. o.* fsp. *vasinfectum* showed slow to rapid growth with raised to smooth surface and regular to irregular margins. Highly variable pigmentation i.e. dark violet, violet, pink, pinkish white and white was produced by different isolates on PSB in medium'Wide variability in cultural and morphological characters of the isolates of *F. o.* fsp. *vasinfectum* indicated existence of physiological strains.

Salt tolerance capacity of different isolates of *F. o.* fsp. *vasinfectum*:

Thirteen new isolates were studied for their salt tolerance capacity. An experiment was conducted to differentiate different isolates of *F. o.* fsp. *vasinfectum* on the basis of salt tolerance capacity. Minimum salt tolerance was observed in isolate FOV- 23.

Pathogenicity tests:

Twenty-three isolates of f. o. fsp. *vasinfectum* including 13 new isolated collected during 2004-05 were tested for pathogenicity on a highly susceptible cultivar G-27 (*G. arboreum)*. The inoculum was multiplied on cottonseed meal having 2.0 per cent sugar solution. Experiment was conducted under glass house conditions by providing optimum environmental conditions for the initiation and development of the disease. . Pathogenic variability was observed in initiation and further development of the disease. The wide variability in pathogenic ability of isolates was observed among the different isolates in infecting the cotton plants indicated the variation in the pathogen. Isolates FOV-12, FOV-15, FOV-16 and FOV-30 predominantly caused pre-emergence wilting compared to rest of the isolates, which caused post emergence wilting. Post emergence wilting of 90.67 per cent wilting was observed within 30 days of germination in case of isolates FOV-19 and FOV-23. Lowest mortality was observed with isolates FOV-13, FOV-14, FOV-22, FOV-27 and FOV-28. Observations on incidence and nature of cotton wilt in twenty-three isolates of *F. o.* fsp. *vasinfectum* revealed wide variability in their pathogenicity .

Fungal DNA Isolation:

Genomic DNA was isolated from the fresh mycelium of *F. o.* fsp. *vasinfectum* by using a miniprep protocol. The DNA was resolved by electrophoresis on 0.7% agarose.

Polymerase chain reaction

A polymerase chain reaction assay for detection of isolates of *F. o.* fsp. *vasinfectum* was previously standardized. Using the conserved primers for amplification of internal transcribed spacer regions between rDNA, a 400-bp DNA fragment is unambiguously amplified from Fusarium isolates (Fig.3.2.19)

RAPD Analysis of *F. o.* fsp. *vasinfectum* isolates

Twenty arbitrary primers OPF (Operon Technologies) were screened for their RAPD pattern. Primer OPF-5 successfully amplified most of the isolates generating polymorphic banding pattern and variability among *F. o.* fsp. *vasinfectum* isolates (Fig 3.2.20).

The primer OPF-5 generated amplicons between 0.1 - 0.6 kb. Cluster analysis based on the RAPD generated fragments clearly assigned *F. o.* fsp. *vasinfectum* isolates in two distinct groups as shown in dendrogram.

Preliminary investigations showed that molecular approaches could detect the polymorphism between the *F. o.* fsp. *vasinfectum* isolates and as such can aid in development of diagnostic tools for differentiation of fusarium strains.

Variability in cultural characteristics, such as growth rate, irregular appearance and pigmentation, salt tolerance ability, pathogenic variability and RAPD pattern provides evidence of genetic variability between the isolates of *F. o.* fsp. *vasinfectum* collected from different cotton growing areas.

Root Rot *(Rhizoctonia* species)

Isolation and multiplication of the pathogens

Different isolates of *R. solani* and of *R. bataticola* used in this study were isolated from cotton root rot affected plants collected mainly from different places in North India (Haryana, Punjab and Rajasthan). Pure cultures of isolates were maintained on Czapek-dox agar slants and stored at 4 ^{0} C till further use.

Chlorate phenotype

Recent efforts to classify isolates of *M. phaseolina* have considered colony morphology on media amended with chlorate. Pearson *et al.* showed that isolates from corn stalks were chlorate-resistant and had dense phenotype,

whereas isolates from soybean root tissue and field soil were chlorate-sensitive and had feathery or restricted phenotypes. In another report, isolates from sorgum had a dense phenotype, whereas soybean isolates had a restricted phenotype. It was suggested that this apparent relationship between phenotype and host could serve as a marker for identifying host specific isolates.

Keeping the above facts in view the isolates of *R. bataticola* were examined for chlorate phenotype on PDA medium amended with 100mM, 120 mM and 150 mM potassium chlorate . Cultures were incubated at 30 ^{0} C in darkness and phenotypes were recorded after 4 days. It was however noted that the phenotype of tested isolates did not change with addition of chlorate to the medium. These isolates mostly belonged to cotton crop only. Further studies using isolates from other crops will be undertaken to find out host specific reaction of isolates to chlorate if any.

RAPD analysis of isolates of *R. bataticola* and *R. solani* were done using Operon primers from the series OPM and OPN. The cluster analysis was done using the RAPD fingerprints. Based on similarity coefficients isolates of these two species of *Rhizoctonia* were grouped. One representative isolate from each group was further selected for rDNA sequence analysis for mapping the variability and development of group specific SCAR markers.

Grouping of isolates *Rhizoctonia* based on RAPD Analysis

Groups	***R. solani* isolates**	**Groups**	***R. bataticola* isolates**
Group-I	2, 3, 5, 6, 7, 8, 9, 10, 12, 13, 14, 15 & 16	Group-I	16
Group-II	19, 20, 22 & 23	Group-II Group- IIa	1 & 11
Group-III	17, 18 & 21	Group- IIb	2, 3, 4, 5, 6, 7, 8, 9 & 19
Group-IV	1, 4 & 11	Group- IIc	10, 13, 17, 18, 20, 21, 23, 24 & 25
		Group- IId	14 & 15
		Group- IIe	12 & 22

It is possible that isolates with similar morphology may not be genetically identical. Ribosomal DNA has been used as an important genetic marker to differentiate species and strains of many organisms. Mitochondrial rDNA and nuclear rDNA have been useful in identifying fungal genera and species and in

recognizing intraspecific variation in ectomycorrhizal fungi. Fungi can be differentiated on the basis of variability in the internal transcribed spacer regions of rDNA .

The strategy was utilized to test genetic difference among isolates of *R. solani* and *R. bataticola*. The sequences of the primers used for above study are:

ITS-1 5'- TCCGTAGGTGAACCTGCGG

ITS-2 5' – TCCTCCGCTTATTGATATGC

ITS- 5 5' - GGAACTAAAAGTCGTAACAAGG

The PCR amplified DNA was resolved by electrophoresis on 1.5 % agarose gel using 100 bp ladder as marker. ITS1 and ITS2 regions of ribosomal DNA were successfully amplified from Isolates of *R. solani* and *R. bataticola*. The ITS region in case *R. solani* was of ~700 bp fragment while that in case of isolates of *R. bataticola* was of 550 bp fragment. Howcver, all isolates within two species possessed amplicons indistinguishable from each other based on the sizes of amplified fragments.

Restiction enzyme analysis (REA) of amplified ITS region of *R. bataticola and R. solani.*

To find variability among different isolates of ***R. bataticola and R. solani*** on the basis of amplified region DNA restriction analysis PCR amplified rDNA fragment of each isolate was digested with different restriction enzymes viz., *Alu* I, *Bam*H I, *Eco*R I, *Hae* III, *Hind* III, *Hpa* II, *Mbo* I, *Pst* I, *Sal* I and *Xba* I. The restriction fragments were size fractionated on 2.0 % agarose gels. Fragmentation pattern were analysed after staining gels with ethidium bromide and viewing under the under U.V. light. Variability between some isolates of *R solani* and *R bataticola* could be delineated on the basis of REA (Fig.3.2.22 a & b). To determine precise variability between the isolates, variability in nucleotide sequences of the rDNA and its ITS regions were analysed.

Cloning and sequencing of rDNA

It was planned to clone the ITS amplified fragments from one isolate each in four groups of *R solani* and also the two main and five sub groups of *R bataticola* . The recombinant plasmids were digested with *Pst*1 and *Sac*II in the MCS in the vector, flanking the cloned insert.

After ascertaining that the rDNA fragment has been cloned at appropriate sites, the sequencing was done using vector based promoter primers (Banglore Genei Pvt Ltd)

The cloned sequences were analysed after deleting the vector sequences. The sequence was subjected to blast analysis at www.ncbi.nlm.nih.gov and got hits from rDNA sequences. The 503 bp nucleotide sequence of the rDNA and ITS region of one of the *R. solani* isolates is given below:

TAGTTCGCGGGTATCCCTACCTGATCCGAGGTCTACCTTGAGAAAAGTTCAGAAG
GTTCGTCCGGCGGGCGACGCCAACCGCTCCAAAGCGAGGTGTATTCTACTACGC
TTGAGGGCTGAACAGCCACCGCCGAGGTCTTTGAGGCGCGTCCGCAGTGAGGA
CGGTGCCCAATTCCAAGCAGAGCTTGAGGGTTGTAATGACGCTCGAACAGGCAT
GCCCCCCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACT
GAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAG
AACCAAGAGATCCGTTGTTGAAAGTTTTAGTTTATTAACTTGTTTATCAGACGTCTG
CGTTTACTGACTGGAGTTTGAAGGTCCTTTGGCGGCCGGAGCCGCCAAAGCAAC
AGAGGTACGTTCACAAAGGGTGGGAGAGTCGAGCCGGAGCTCGAAAACTCGGTA
ATGATCCAATCA

The cloning and sequencing of other amplified rDNA fragments to understand variability among isolates of *R solani* and *R bataticola* is in progress

Helicoverpa armigera

PCR-RFLP to differentiate two species of *Helicoverpa*

A PCR-RFLP based technique to distinguish two species of *Helicoverpa.* was developed. *H. assulta* larvae were collected from *Datura* in and around cotton fields of Nagpur. *H. armigera* were collected as eggs from cotton. Larvae were reared till pupation on semi synthetic diet. The emerging moths were identified using the taxonomic key described. Genomic DNA was isolated from the thorax of female moths using the protocol described by Zhang and Hewitt.The mid CO-1 (Cytochrome Oxidase) region possesses high functional significance and was therefore chosen for the study. The primers amplified 598 bp fragment corresponding to mid to near terminal region of COI.

Rsa I approximately cut the 598 bp-amplified fragment in the center yielding two fragments of approximately 333 bp and 265 bp each (Fig.3.2.25). *Rsa*I

recognition site is masked by a mutation in *H. assulta* and as such the amplified PCR product remain uncut. PCR-RFLP thus can serve as a tool for differentiation of the two species of *Helicoverpa*, which in turn could significantly influence the adoption of pest management strategies. This tool can offer support to the conventional taxonomic differentiation based on morphological features.

During 2004-05, genomic DNA from 4 cotton strains and 4 non- cotton strains were subjected to post PCR sequencing of the COI, COII, SrRNA and LrRNA regions. This resulted in 16 nucleotide reads for each region. The sequences corresponded to the observed sizes of the PCR.

Cytochrome oxidase I (COI): Primers were designed to amplify a fragment of approximately 600 bp. The 16 sequences obtained were aligned using the program Clustal X. Two distinct mutations were obtained between the cotton and non-cotton strain as indicated in the Table 3.2.2.

Table 3.2.2. Mutations in partial nucleotide sequences and the deduced amino acid sequences of Cytochrome Oxidase I (COI) gene of Cotton and Non-cotton strains of *Helicoverpa armigera*.

	Nucleotide sequence*	Corresponding amino acid seq
Ha Cotton	(2206)5'-ATTTTACC***G***GGA-3' (2217)	(245)ILPG (248)
Ha Non-Cotton	(2206)5'-ATTTTACC***A***GGA-3' (2217)	(245)ILPG (248)
Ha Cotton	(2377)5'-TATTT***T***ACATCAGCT-3' (2392)	(302)YFTSA (306)
Ha Non-Cotton	(2377)5'-TATTT***C***ACATCAGCT-3' (2392)	(302)YFTSA (306)

*Values in brackets indicate position of nucleotides with reference to *Drosophila yakuba* mitochondrial genome and that of amino acids in the deduced protein sequence. Letters in bold and italics indicate base substitutions.

Both the mutations involved transversions where A in the non- cotton strain was replaced with G in the cotton strain at the 2214 bp position while the second mutation involved replacement of T in the cotton strain with C in the non- cotton strain (2383 bp). These mutations were consistent between 4 cotton and 3 non- cotton individual sequences in both the forward and reverse reads. The CO-I amino acid sequence is divided into twenty-five regions comprising of five structural classes (twelve transmembrane helices- M1- M12, six external

loops E1-E6, five internal loops, carboxy and amino terminals, Lunt *et al.* 1996). The nucleotide sequence in the present study corresponds to the two external loops (E3 and E5), five trans membrane helices (M6, M7, M8, M9, M10) and two internal loops (I3 and I4), described in other insects. One mutation has been found in the M 6 region (at the 155 bp position with the sequence obtained with specific primers) while the second mutation is found in the E4 (external loop) region (323bp position).

Partial CO1 sequences were subjected to the Restriction mapper version 3.0, online software for the identification of restriction sites unique to the cotton or non-cotton strain. *Nci* 1(*Bcn*1) was identified as the unique restriction enzyme that could differentiate, at the point of the first mutation, between the cotton and non- cotton strain. The enzyme cuts at CC↓SGG where S= C/G. A virtual digest demonstrated that the RE could selectively cut the amplified CO1 fragment of non-cotton strain into two bands approximately 127 bp and 458 bp in size. (Fig. 3.2.26) *Bst* 2UI was the second restriction enzyme (ACC↓AGGA) that could selectively cut the PCR amplified product of cotton strains into 2 band of sizes of approximately 150 bp and 450 bp, respectively (Fig. 3.2.27). A unique restriction enzyme for the second mutation was not found. PCR – RFLP using enzymes *Nci*1 and *Bst*2 UI is being proposed for the identification of the two haplotypes of *H. armigera*.

SrRNA: This region corresponds to 14197bp and 14622bp of *D. yakuba*. It was amplified from three cotton and non- cotton strains each, using primers specific to the region (oligos SR-J-14199 and SR-N- 14594). PCR amplification resulted in a fragment of 395bp that corresponded to the region of the full sequence. A distinct mutation was observed between the cotton and non- cotton strains consistent in all the 12 sequence reads. Mutation was in the form of transversion with G in the cotton strain being replaced with A in the non-cotton strain. A PCR RFLP tool with *Mae*III (restriction site of ↓GTNAC where N=A/T/C/G) was found to distinguish clearly the cotton and non cotton strains.

It is important to mention here that in a parallel study where we studied the geographical variability of *Helicoverpa*, 18 haplotypes were identified with

respect to mutations in the partial CO-I region. Studying the tree it was observed that a large group was formed in which individuals had 'G substituted with A' mutation and this mutation did not necessarily go hand in hand with the second mutation, 'T substituted with C'. Incidentally the cotton and non-cotton strains differed in these mutations. This could just be coincidental and our original populations may have been the two distinct haplotypes. However we now have a molecular tool, PCR- RFLP that can readily identify these haplotypes. The question we now need to answer is whether these haplotypes differ in their feeding behavior, insecticides susceptibility etc.

Separation of strains from field collected populations of *H. armigera* mating preferences

Field populations, as large full-grown larvae (cotton strain), were collected from the first brood occurring on cotton in Nagpur. Single pair crosses were set up when these larvae turned into adult moths. Larvae obtained from these crosses were separated into cotton and non- cotton strains based on the weight gained in a 7- day feeding period on cotton squares of LRA 5166. The non- cotton larvae were discarded. Cotton larvae were reared till the F2 generation when selections were made for the third time on cotton squares. Non- cotton strains were obtained from releasing neonates obtained from field collected eggs on cotton squares and those larvae that did not gain more than 20mg weight on cotton squares were continued through further selections. Crosses were made between cotton and non- cotton strains as single pairs. Both inter- strain and intra strain crosses were made. Reciprocal crosses were made in the two categories of crosses. This experiment was designed to understand whether the two strains interbreed freely under laboratory conditions and to understand the feeding preference of their progeny. The crosses were made and observations were recorded on oviposition and incubation periods.

Of the 109 single pair crosses made 48 crosses were inter-strain crosses and 61 were intra strain crosse. It was observed that the two strains mate and inter breed freely under laboratory conditions. Of the 109 crosses, fertile crosses ranged between 22-38%. In fact, the percentage of fertile crosses is similar to that occurring in any set of single pair crosses of *H. armigera* in the lab. There

were no statistically significant differences between the oviposition and incubation periods of inter and intra-strain crosses.

The progeny of those crosses that hatched were selected by releasing 2-day-old larvae on cotton squares of LRA 5166, chickpea and red gram pods. Larvae from each set were weighed at the end of 7 days and categorized as cotton and non- cotton based on the weight gain.

Feeding preferences of cotton and non cotton strains

It was amply clear from this study that progeny of cotton strain feed on both cotton squares and Tur pods while progeny of non- cotton strains prefer to feed more on Tur pods than on cotton squares. In inter strain crosses where the mother was a cotton strain female, the progeny preferred to feed on both Tur pods and cotton squares. In inter strain crosses where the mother was non-cotton female the progeny preferred to feed on Tur pods when compared to cotton squares. Progeny obtained from 2 crosses each, representing each of the 4 groups indicated that the feeding preference patterns of the progeny on chickpea pods were similar to that of Tur. A similar response of the feeding preference of progeny was also observed with chickpea. Thus the non-cotton strain prefers to feed on legumes to cotton even under no choice condition.

Cornutal spines as a taxonomic tool

It was described 2003-04 that those male moths with cornutal spine numbers between 13-15 belonged to the cotton strain while those that belonged to the non- cotton strain demonstrated the presence of cornutal spines between 9-11. Male moths with cornutal spine number of 12 behaved as both cotton and non-cotton strains in terms of their feeding behavior. The temporal variation in the cornutal spine numbers of pheromone trapped male moths was studied at Wardha and Nagpur from July to April.

Utilisation of cornutal spine numbers as a taxonomic tool has its limitation on account of the grey area in our understanding of the feeding behavior of larvae that develop into moths with 12 cornutal spines that incidentally were in large numbers this season.

Studies at Coimbatore center showed that the cornutal spines on male moths of *Helicoverpa* collected from different hosts were variable in number. Cornutal spine numbers recorded from the individuals collected from cotton varied between 9-14 while the population collected from red gram recorded 10-12

number and those from pigeon pea showed spine numbers between 12-13. Though *Helicoverpa* strains exhibited variability in their cornutal spine numbers they possessed, no correlation seemed to exist between the spine numbers and host specificity. Thus the applicability of cornutal spines as morphological marker, if any, needs further investigation.

Whitefly (*Bemisia tabaci*)

Documentation of various biotypes and variants of whitefly in cotton growing areas of India:

Whitefly types in different parts of India had remained uncharacterized with respect to its biotype until B-biotype was first reported to exist in tomato fields in Kolar region of Karnataka State (Bank et al., 2000). Since the start of this project, an exhaustive work has been in progress at PAU Ludhiana to identify and map the prevalence B- biotype of *B. tabaci* in different regions of India, which were grouped as North Indian states (Punjab, Haryana, Rajasthan), Central Indian states (Gujarat, MP) and South Indian States (Karnatka, TN, AP). RAPD-PCR with OPH-16 and three B-biotype specific SCARs (Bta $B7_{F-R}$, Bta $B15_{F-R}$, Bta $B29_{F-R}$) were effectively used to map the prevalence of B-biotype in whitefly populations from different regions of South India and is summarized below:

- All whitefly populations in the South Indian region (Karnataka, Tamil Nadu and Andhra Pradesh) are constituted by B-biotype of whitefly. This type is different from the B-biotype from Australia and Israel but is a closely related to it genetically (80 %).
- Besides the predominant Indian B- biotype-1 (89 %), the populations from south India are found mixed with other variants of this biotype (Indian B-biotype-2 & 3) to the extent of 5.5 % each.

Since the developed SCAR markers specific to B-biotype do not differentiate non B-biotype whitefly populations, the analysis of whitefly populations from other areas of India was performed through RAPD-PCR. Using RAPD-PCR analysis (OPH-16), 11 whitefly populations collected from cotton fields in different areas (Punjab-7, Haryana- 2, Rajasthan- 2) of north India were found to be non B-biotype (Fig 28A). Subsequently comparative molecular analysis of whitefly populations collected from cotton fields from five different locations in Punjab,

one location in Rajasthan and one location in Pakistan (Faislabad) was conducted using two primer (OPH-16 and OPB-10). The uniform banding pattern with both the primers clearly established that a single whitefly type (non B-biotype) prevailed in cotton growing areas of neighboring Pakistan and Indian Punjab as well as Rajasthan. This whitefly type was also different from the Q- biotype, which is prevalent in European countries.

Similar analysis of whitefly populations from 15 fields of different crops (5 cotton fields) located in the Nagpur and Wardha Districts of Maharashtra State (Central Indian States) established the complete absence of B-biotype in this State.

All populations were constituted of a non B-biotype whitefly of a single major type although some minor genetic variants existed in most populations from this region. Compared to above, though a non B-biotype whitefly (identical to that prevalent in Maharashtra) predominated in all the 36 populations collected from fields of different crops in 3 different districts of Gujarat State (Anand, Vadodhara, Bharuch), 1/3rd of the populations were found to be contaminated with a B-biotype.

Table 3.2.3: Prevalence of B-biotype in different Districts of Gujarat State.

Parameter	**District**			*Total*
	Anand	**Vadodara**	**Bharuch**	
WF-Populations studied	14	17	5	36
% Population with B-biotype	43.0	29.4	20.0	33.3
No. of WFs studied	103	136	37	276
% B-biotype in all Populations	27.2	4.4	2.7	12.7
% B- biotype in B-biotype populations	60.9	15	12.5	37.2

Distribution of B-biotype in population from this state showed that this biotype was most prevalent in Anand District with heaviest contamination of populations from GAU campus located at Anand City. In this district, this type existed as major whitefly accounting for 28 out of 46 whitefly (60.9 %) individuals in six populations. In Vadodara district, B-biotype was present at low level (4.4 %). In Bharuch District as well out of five populations (37

whiteflies) studied, only single population of eight individuals from Sarsod was found to contain a single B-biotype individual.

Genetic relatedness amongst whitefly types predominant in different regions of India:

Using 20 different RAPD primers, comparative RAPD-PCR with eight previously identified whitefly individuals representing major whitefly types (only major types) identified from different states of India and two standard B-biotypes (Australia & Israel) was performed for evaluating genetic relatedness. These whitefly types from different areas of India included the following:

- Indian B-biotype-1, -2 & -3 from Kolar- Karnataka
- Major non B-biotype prevalent in Gujarat & Maharashtra
- Non B-biotype of Punjab, Haryana and Rajasthan

The comparative RAPD- PCR profile of different whitefly types with most of the primers generated number of marker bands that brought out genetic dissimilarity as well as similarity amongst different whitefly types occurring in India (Fig 3.2.31).

In total 20 primers generated 235 marker bands out of which 179 (76.1 %) bands were polymorphic (Table 3.2.4).

Table 3.2.4: Details on different parameters for deriving similarity coefficient amongst different whitefly types identified from India.

The data on these 235 marker bands vis-à-vis respective whitefly types was analyzed for genetic similarity using the software NTSYSpc and the genetic similarity values developed into a dendrogram .

Analysis of the genetic similarity data in the dendrogram led to following inferences:

- B-biotypes from Australia and Israel are closely related to each other by 86.0 % genetic similarity.
- Indian B-biotypes-1 & -2 from Kolar are similarly related to each other by 86.5 % genetic similarity. However, Type 3 is genetically similar to the above types to a lower extent of only 62 %.

- Indian B-biotypes-1 & -2 are related to two standard B-biotypes (76%) and thus are distinct forms of B-biotypes types. As Indian B-biotypes-3 is genetic similar to both the B-biotypes and Indian B-biotypes (though to a lower extent), it appears that all three Indian B-biotypes identified from Kolar (Karnataka) have evolved possibly due to interbreeding of whitefly types native to South India with lately introduced B-biotype in recent past.
- Prevalence of single whitefly type in Punjab & Haryana (97% genetic similarity) as well as Rajasthan is established by very high genetic similarities (94 %) amongst the whitefly types representing these states.
- The major whitefly type (non B-biotype) prevalent in States of Gujarat & Maharashtra forms a separate identity/ type, which is different from whitefly type prevalent in Punjab, Haryana & Rajasthan (74 % genetic similarity).

Identification of molecular markers specific to different whitefly types detected in India

The comparative RAPD- banding profile of different whitefly types found in India (with 20 RAPD- primers) generated 179 polymorphic bands (Table 3.2.20). RAPD analysis resulted in identification of 71 polymorphic RAPD- bands, which appear to hold high specificity for one or the other whitefly type .

The specificity of these marker bands is described below:

i) Standard B-biotype: 13 markers are specific for standard B-biotypes (Australia and Israel).

ii) North and Central India: 9 markers each have specificity for the non B-biotype whitefly presently native to north (Punjab, Haryana and Rajasthan) and Central (Gujarat and Maharashtra). Besides six other markers could be useful in detecting whiteflies (without any discrimination) native to areas in these States.

iii) South India: All the three Indian B-biotypes (Kolar region) have shown considerable genetic relatedness to both of the standard B-biotypes (Australian and Israel). In spite of such a high genetic similarities observed amongst the above variants of B-biotypes, considerable number of molecular markers could be identified for internal differentiation within this group. Whereas 11 markers were found specific to both the standard B-biotypes as well as all the three Indian B-biotypes, another set of 3 markers were identified for detection of all the three Indian B-biotypes in South India. These markers hold immense

practical significance both in the monitoring the presence of specific whitefly type(s) in different regions of India as well as their spread to other newer areas and regions for their effective management.

Validation of SCAR primers for identification of B-biotype of whitefly

In the year 2003-04, three B-biotype specific SCAR primers (primers Bta $B7_{F-R}$, Bta $B15_{F-R}$ and $B29_{F-R}$) were developed. This year, in order to validate these SCARs, the Gujarat populations in which B-biotype was earlier identified through RAPD marker ($OPH16_{850}$) were analyzed with two SCAR primers (Bta-$B7_{F-R}$, Bta-$B29_{F-R}$). These two SCARs effectively identified B-biotype individuals in such populations. This whitefly type was not detected in populations from Punjab, Rajasthan and Maharashtra.

Thus due to the specific ability to identify B-biotype whitefly individuals in whitefly populations, the three SCAR primers B-7_{F-R}, B-15_{F-R} & B-29_{F-R} developed in this study hold immense practical significance for monitoring of B-biotype and related whitefly types in populations.

Different whitefly types identified by use of RAPD-PCR and SCAR markers that exists in India are given in Table 3.2.5.

Table 3.2.5. Documentation of whitefly types in areas of India

Geographical Area	**State**	**Prevalent biotype of Whitefly**
North India	Punjab	Non B-biotype*
	Haryana	
	Rajasthan	
	Pakistan*	
Central India	Maharashtra	Non B-biotype**
	Gujarat	Non B-biotype** mixed withB-biotype
South India	Karnataka	B-biotype
	Tamil Nadu	B-biotype
	Andhra Pradesh	B-biotype

*This type is prevalent in Faisalabad (Pakistan) as well

**This type is different from the type prevalent in North India and Pakistan

- **Identification of polymorphic DNA fragments in host specific whitefly population for conversion into specific primers and SCARs**

In the year 2003-04, RAPD-PCR analysis of DNA from whitefly populations collected from five different host crops (cotton, brinjal, soybean, tomato and potato) and two weed species (*Abutilon* sp and *Sida* sp) with 17 different RAPD-primers, established high genetic relatedness (80 percent and above) amongst whitefly types. Although the above polymorphic markers could have been processed for developing specific markers, it was thought appropriate to develop SCAR markers only from homogeneous whitefly populations reared on specific hosts under lab conditions. In view of above the whitefly populations were reared on the respective host plants in isolated screen houses for three months (4 generations). Homogeneity in the populations was confirmed by uniform RAPD banding pattern of 15 individuals from a particular population using three different RAPD primers (OPB-2, OPC-07, OPC-01) (Fig. 3.2.32).

Continuous rearing of whitefly populations from different hosts on respective host plants made all the individual whitefly populations homogenous. Such populations are ideal for development of host-specific molecular markers. RAPD-PCR amplifications were performed with DNA of 10 whitefly individuals from different host specific populations with six different primers.

Comparative banding pattern resulted in identification of 26 polymorphic bands (markers) some of which were uniquely specific for specific host crops. Thirteen of these bands were specific to single hosts while others showed specificity to whitefly from two or more host crops. Based on the nucleotide sequences of the SCAR markers, specific primers will be developed which will be evaluated for identification of host-specific whitefly type(s).

- **Efficiency of whitefly populations from different hosts for CLCuV acquisition and transmission in cotton**

Studies were carried out to explore the efficiency of whitefly from different plant hosts for acquisition of CLCuV from infected cotton plants and subsequent transmission to healthy cotton plants. The CLCuV specific primers ($P_{1800\text{-}500}$) were custom synthesized. The sequences of the primers are given below:

F1800 5' CCT CCT TTA ATT TGA ACC GG 3'

R500 5' GGC TTT CTG TAC ATG GGC CTG T 3'

Primers amplified a 1.5 kb DNA fragment from CLCuV infected plants and whitefly vectors.

Transmission of CLCuV by host specific whitefly populations

Whitefly populations collected from fields of five different crop plants (cotton, brinjal, potato, tomato, soybean) and one weed plant (*Sida* species) were maintained in isolation on respective host plant in screen cages for three generations to introduce homogeneity. For detection of infection in whitefly and in cotton plants, PCR amplifications were performed with $P_{1800\text{-}500}$. The results showed that whitefly populations from different host plants greatly differed with respect to virus acquisition from the diseased cotton plants, being highest (100 %) in cotton whitefly and lowest (20%) in tomato whitefly (Table 3.2.6). Similarly the transmission efficiency of acquired virus to healthy cotton plants was also highest (100%) in case of cotton whitefly and lowest in tomato whitefly.

Table 3.2.6: Efficiency of CLCuV acquisition by different host specific whiteflies from diseased cotton and its transmission.

Host plant	Virus acquisition (%)	Virus Transmission (%)	CLCuV infection in cotton* (%)
Cotton	100	100	100
Potato	40	60	60
Tomato	20	20	20
Soybean	80	70	70
Brinjal	40	40	40
Sida species*	60	50	50

* Thickening of veins and upward curling of leaves along the margins

Cotton whitefly exhibited 100% efficiency both in acquiring as well as transmitting CLCuV. Whitefly from soybean showed high efficiency for virus acquisition and transmission (80 and 70 %, respectively). Such efficiency was comparatively lower in case of whiteflies from other host crops. Tomato whitefly was poorest in acquiring as well as transmitting CLCuV (20% each) to cotton. It was inferred that probably the specific host crop induced some definite genetic changes in the whitefly, which influences their role as vector of CLCuV. The disease symptoms developed in those plants only in which CLCuV DNA could be amplified/ detected through use of CLCuV- specific primers. These observations clearly suggest that whitefly from different hosts greatly differ with respect to their role as vectors of the virus and in causing CLCuV disease in cotton.