TMC MM I 3.3: Development, validation, utilization and / or commercialization of biopesticides, bioinoculants and disease diagnostic tools

INTRODUCTION

The project was mooted to identify potential microbia ls for biocontrol of insect pests and diseases of cotton for eco-friendly agriculture. A mission mode approach was initiated to generate data for their registration to pave way for their commercialization. Potential microbial biopesticides and bioinoculants identified during the first phase of TMC MM I programme were further evaluated for validation of their efficacy through field and lab experiments during 2007-08 and 2008-09. Besides, during last two years, some new microbials which were found promising against insect pests and diseases were also tested in multi location lab as well as field trial.. The scope of the project was further enlarged during 2009-2010 by including diagnostic tools developed (during phase I of TMC) to detect cotton pathogens for further optimization, validation and commercialization for deployment as decision support system for crop protection. The bioformulations, biopesticides and cultures developed under the project can cater to the needs of farming community in practicing eco-friendly agriculture while molecular diagnostic tools would ensure rapid and precise detection of pathogen ensuring need based and timely chemical intervention based on prevalence of disease.

Based on in-vitro screening and limited field experiments conducted earlier, two bioformulations against sucking pests including Mealy Quit and Mealy Kill; four entomopathogenic fungal species viz. *Verticillium lecanii* (NBAII), *Lecanicilfium lecanii* (CICR, Coimbatore), *Fusarium palfidoroseum* (CICR, Sirsa) and *Metarrhizium anisopliae* (NBAII); two microbial pesticides viz. *Photorhabdus* sp. (CICR, Nagpur) and *Xenorhabdus* species (CICR, Coimbatore), four biocontrol species against diseases viz. *Pseudomonas f1uorescens* (CICR H1a), *Bacillus subtilis* (TNAU) and *Trichoderma harzianum* and *viride* strains identified in TNAU, PDBC and Dharwad were shortlisted for further evaluation during 2009-10. Besides,PCR protocols were developed for detection of strains of CLCuV and fungal pathogens of cotton viz. *Alternaria macrospora, Rhizoctonia bataticola,* R. *solani, Ramularia areola* and *Myrothecium roridum* were further taken up for optimization and validation.

OBJECTIVES

- Exploration of potent microbials and their screening against major pests and diseases of Bt cotton
- Evaluation of induced systemic resistance of potential antagonists
- Development of data for registration for potential microbes
- Commercialization of potent bioagentsl formulations / molecular diagnostic kits
- Molecular diagnosis of major pathogens of cotton including optimization of immunodiagnostic protocols.

SALIENT FINDINGS

Commercialization of Molecular Diagnostic tools

Optimization of PCR protocols for efficient detection of pathogen

Five diagnostic primers designed based on specific genetic signatures of *Alternaria macrospora, Rhizoctonia bataticola,* R. *solani, Ramularia areola* and *Myrothecium roridum* were developed during first phase of TMC MM I. The pathogens are detected in polymerase chain reaction (PCR) using genomic DNA as templates. However, for effective development of molecular diagnostic tools in decision support system for sustainable agriculture, the protocols should be robust enough for *in situ* detection of pathogen within the sources viz., infected plant materials, soils etc. The

detection of the pathogen within their sources of perpetuation and perennation is highly complicated due to the presence of large number of inhibitors of PCR within these sources.

Substitution of BSA and glycerol in PCR enhanced the efficacy of amplification and detection of pathogens in the infected plants or in soils. The efficiency of detection of CLCuV within the infected cotton was improved by addition of BSA in the reaction mixture @ 0.2% and glycerol from 0.2 -2.0% (Fig. 3.3.1 a&b).

Fig. 3.3.1 a. : PCR Detection of CLCuV in infected cotton through amelioration of PCR inhibitors with BSA. Lanes 1-4: Plant genomic DNA without BSA; Lanes 6-9, with BSA (0.2 %); C, Control

Fig. 3.3.1b : PCR Detection of CLCuV in infected cotton through amelioration of PCR inhibitors with increasing concentration of Glycerol.. Lane1, Marker; lanes 2-10, 0.2 - 2.0% of Glycerol..

Application of BSA (0.2%) and glycerol (2%) in the PCR reaction also enhanced the efficiency of detection of A. *macrospora* in soil by 40% and 80%, respectively. These experiments showed that the DNA isolated from plants and soil samples possessed some inhibitors of PCR which can be controlled using PCR suppressors and facilitators of PCR.

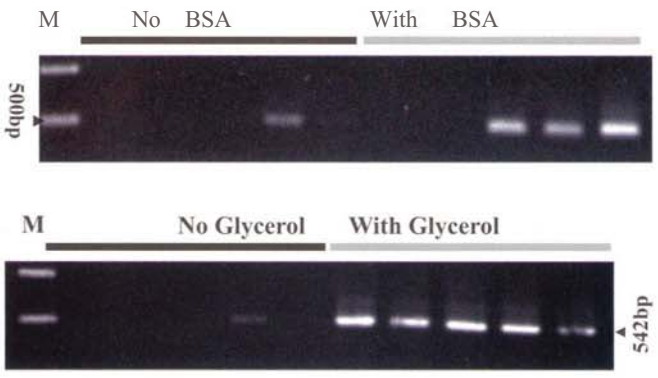

Fig. 3.3.2 a&b : Increase in efficiency of PCR detection of *A. Macrospora* with 0.2% BSA - a & 2% glycerol - b.

Effects of some potential inhibitors on inhibition of PCR

Humic acid in soil is one of the potential inhibitors of PCR.. The humic acid @ 0.02% reduced efficiency of PCR detection of R. *so/ani* by 50% while amplification was completely inhibited @ 0.03%.

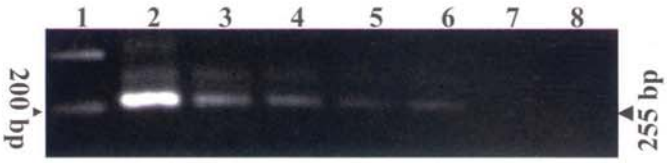

Fig. 3.3.3 a : Effect of increasing Concentrations of *humic* acid in PCR amplification & detection of *R. so/ani.* Lane 1, Marker; Lanes 2-8, humic acid from 0.004%-0.03%.

Phenol, another organic compound frequently used for DNA isolation is another potential inhibitor of PCR.. Contamination of phenol (0.4- 4%) in PCR drastically affected detection of *A.macrospora.*

Fig 3.3.3 b : Effect of Phenol on PCR amplification of A. macrospora Lane 1, Marker; lanes 2-4, 1.2%, 1.6%, 2,; Lane 5 - Control, without Phenol, lanes 5-7, 0.2% BSA+2% Phenol..

Overcoming effects of PCR inhibitors through suppressors and facilitators

Substitution of glycerol @ 1.4% overcame the inhibitory effect of 0.02% humic acid (Fig 3.3.4a). Humic acid concentration beyond the threshold level is difficult to overcome through suppressors. Doubling the concentration of glycerol (2.8%) could not completely overcome the effects of 0.4% humicacid.

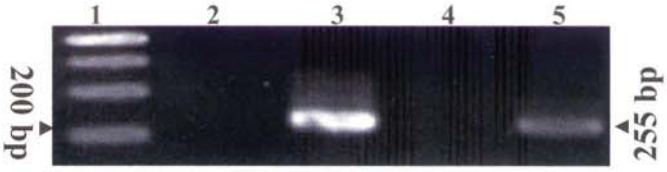

Fig. 3.3.4 a : Effect of Glycerol on PCR inhibition of *R. so/ani* caused by humic acid. Lane 2 & 4, 0.02% & 0.04% of humic acid; Lanes 3 & 5, 1.4% & 2.8% of Glycerol substituted in reaction containing 0.02% & 0.04% humic acid, respectively.

Besides glycerol, BSA in the increasing concentration of 0.2% - 0.4 % effectively mitigated the inhibitory effects of 0.02% humic acid in PCR amplification of strains of *Rhizoctonia so/ani.*

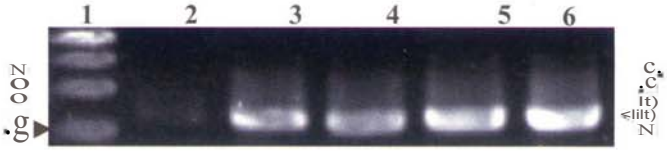

Fig. 3.3.4 b: Effect of increasing concentration of BSA on overcoming the effect of 0.02% humic acid in PCR amplification & detection of *R. So/ani.* Lane 1, Marker; lanes 2-6,0.2%-0.4% of BSA.

Combined substitution of BSA (0.2%) and glycerol (1.2 %) in a reaction containing 0.02% humic acid greatly improved the efficiency of PCR detection *of R. solani* (Fig 3.3.5).

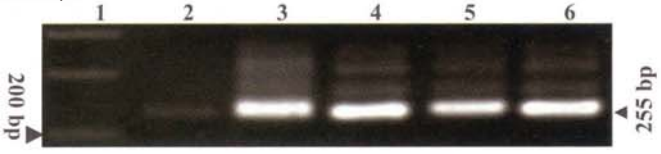

Fig. 3.3.5 : Effect of combined application of BSA (0.2%) & Glycerol (1.2%) on overcoming the effect of humic acid (0.02%) in PCR amplification of *R. so/ani.*. Lane 1, Marker; lane 2, humic acid alone; lanes 2-5, 0.02% humic acid + 0.2% BSA +1.2% Glycerol..

Similarly, Substitution of 0.2% BSAand 2% glycerol in the reaction mixture containing 2% phenol completely reversed the effects of phenol resulting in detection of the pathogen.

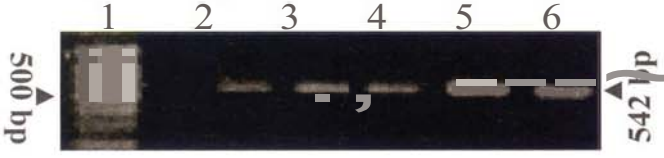

Fig 3.3.6 : Effect of Phenol on PCR inhibition & its mitigation by BSA & Glycerol.. Lane 1, Marker; lanes 2% Phenol; lanes 3-5, 0.2% BSA+2% Phenol; lanes 6-8, 2% Glycerol+2% Phenol..

Evaluation of bioformulation against whitefly

Mealy Quit and Mealy Kill, two formulations were developed for use against sucking pests of cotton

Significantly, high mortality of whitefly was observed with Mealy Quit (71.78%), the effect of which was statistically at par with Neem oil (59.78%), Limonene (56.76%) and Thiomethoxam (51.43%). Effect of Mealy quit was more pronounced upto four days after application. On the contrary, Mealy Kill showed relatively greater efficacy 8 days after application.

Amongst various formulations tested at 4 DAT against jassids, Mealy Quit (59.78%) was also significantly superior to all other bioformulations including neem oil, fish rosin oil, limonene and even insecticide Confidor.

Biocidal formulation developed from Entomo-pathogenic nematodes for use against sucking pests

Biocidal formulation developed from bacteria associated with entomopathogenic nematodes effectively caused mortality of mealy bug crawlers to the extent of 85%. The formulation was compatible with other bio-agent such as *Trichogramma.* Bacterial symbiont grew best at 37°C with an incubation period of 16 hrs on rotary shaker.

Characterization of *Photorhabdus* sp - a bacterial symbiont of *Heterorhabditis indica*

16s ribosomal RNA of *Photorhabdus* sp, the endosymbiont of *H. indica* was PCR amplified. The rDNA sequence of 1.5 kb was cloned and submitted for sequencing.

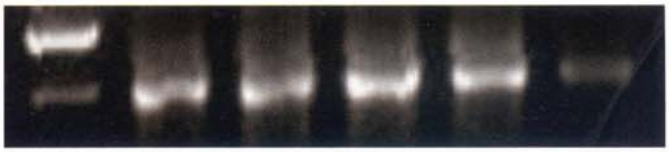

Fig.3.3.7 : PCR amplification of 16s RNA (1.5 kb) of *Photorhabdus sp.*

Isolation and identification of potent entomopathogenic fungi from mealy bug

Out of 43 resident mycoflora isolated from mealy bug, *Lecanicillium lecanii* (Zim,Zare & Gam) was found to be highly virulent against two species of mealy bug viz. *Phenacoccus solenopsis* and *Para coccus marginatus* under laboratory, green house and field conditions (Fig. *3.3.7). Cladosporium cladosporioides* was reported for first time to be associated with mealy bug. Pathogenicity of the fungus was established by artificial inoculation. *L./ecanii* was most effective fungal biopesticide in controlling *Phenacoccus solenopsis* and *Paracoccus marginatus,* two species of mealy bugs affecting cotton, with lowest LC50 values. *B.bassiana* and M *Anisopliae* also provided very effective control of the bug ranking second and third best biopesticide both under laboratory and field evaluations. Besides maintaining effective control, the LT50 (days) of *L. lecanii* was also documented to be the lowest on both the species of mealybugs.

Pathogenesis of entomopathogenic fungi on mealy bug

Pathogenesis of *Manisopliae, B.bassiana, L./ecanii* and *C.cladosporioides* on mealy bug was studied under laboratory conditions. The infection of *Manisopliae* was clearly observed at 24 hours after infection (HAI). The infection of the fungus was associated with wax degradation in patches. At 48 HAI, hyphae were seen ramifying the body. Thick hypha l mat was apparent at 72 HAI which was followed by sporulation at 96 HAI (fig. 3.3.8a). In case of *B.bassiana,* hypha l mat was apparent at 48 HAI and at 72 HAI, 75 per cent of the insect body was covered with mycelium. It took 96 hours to completely ramify the insect (fig. 3.3.8b). In *L. lecanii, 50* % of the insect body was covered with mycelium within 48 hours and ramification was complete by 72 HAI (fig. 3.3.8c). While in case of *C.cladosporioides,* cuticular degradation and oozing out of body fluid was observed after 24 HAI.. At 48 HAI, wax layer was completely removed and the entire body was covered over with spore mass after 96 HAI (fig. 3.3.8d).

Cultural conditions for optimum growth and sporulation of *L. lecanii* were determined. The mycelial growth was highest in SDAY medium followed by that in the sorghum extract and PDA.. Sorghum based medium was found to be significantly superior for spore production followed by SDAY medium. Optimum temperature for virulence and sporulation of three potential entomopathogens were determined. *B.bassiana* and *M.anisopliae* were found to be the most virulent to *Paracoccus marginatus* at 30°C, while differential temperature optima of 25 and 30°C was

documented in case of *L. lecanii* for nymph and adult respectively.

Based on this information, a mass production protocol for *L./ecanii* was standardized.

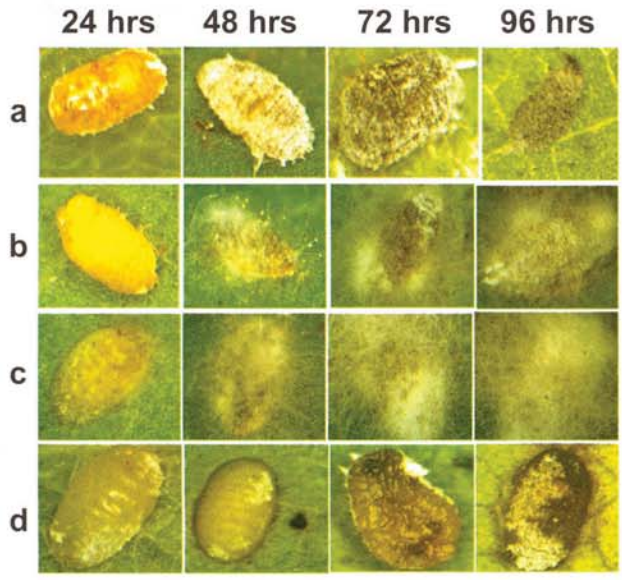

Fig.3.3.8 : Pathogenesis of entomophagous fungi on mealybug. *a) Metarhizium anisopliae b) Beauveria bassiana c) Lecanicillium lecanii d) Cladosporium cladosporoides*

Fusarium pallidoroseum - a potential entomopathogen of mealy bug

During the surveys conducted consecutively for three years from 2007-2008, *Fusarium pallidoroseum* was predominantly found associated with cadavers of mealy bug samples. Efficacy of *Fpallidoroseum* was evaluated under lab and field conditions on cotton mealy bug at various concentrations. Under in-vitro conditions, highest mortality of mealy bug to the extent of 96.67% was documented with two applications of *F. pallidoroseum*@ 5%. Under field conditions, significantly high mortality of 96% was obtained with two sprays of 2.5% talc formulation.

In-vivo comparative evaluation of *F pallidoroseum* with *Vlecanii* (VL 3 and VL 9) strains of NBAII, Actinomycetes, nematode endosymbionts *Xenorhabdus* and *Photorhabdus,* showed all the strains to be highly effective with mealy bug mortality ranging between 82.23% to 92.80%.

In a field trial conducted under AICCIP at three locations including Sirsa, Faridkot and Coimbatore, *F. pallidoroseum* caused mealy bug mortality ranging from 32.5 to 52.99%. The fungus was most effective of the four biopesticides tested including strains of *Vlecanii, M.anisopliae* and *B.bassiana.*

Mass multiplication of *F. pallidoroseum* on agricultural waste

Out of six agricultural wastes like wheat straw, paddy straw, guar straw, rice husk, cotton cake and mustard cake tested to multiply the fungus. Guar straw and cotton cake were found to be the most suitable substrate for growth and mass multiplication of *F pallidoroseum.*

Pathogenicity of *Metarhizium anisopliae* isolates on cotton mealy bugs

Bioassay of 19 isolates of *Metarhizium anisopliae* (107spores/ml) against the nymphs of cotton mealy bugs caused mortality ranging from 11.6 to 63.30 %. Highest mortality (63.30 %.) was observed with Ma-4 isolate which was statistically on par with isolates Ma-2, Ma-7, Ma-14b and Ma-16. Among seven strains of *Vlecanii* (NBAII) tested for their biocontrol efficacy against two different mealy bugs at Coimbatore and Sirsa, strains VL-3, VL-5 and VL-9 showed highest mortality both against nymph and adult mealy bugs.

Management offoliar diseases of cotton

Screening of antagonistic bacteria

Out of 15 isolates of B. *subti/is* assayed, the isolate BsC5 significantly reduced mycelial growth of A. *a/ternata* to an extent of 55.55% in-vitro. Out of 10 isolates of *Pseudomonas fluorescens* tested against A. *alternata,* isolate Pf1 caused highest reduction in mycelial growth of the fungus (48.15 per cent), while among the 12 isolates of *Streptomyces spp.* screened, isolate Sc2 caused highest inhibition of the mycelia growth.

In vitro assay offungal antagonists

Out of ten isolates of *T viride* screened against *A .alternata,* the isolate Tv1 (TNAU) significantly reduced mycelial growth of the test pathogen to the extent of 61.9 percent. .

Effect of rhizobacteria on the induction of defense related enzymes against *Alternaria* leaf blight of cotton

Induction of defense related secondary metabolites in cotton pretreated with Bacillus subtilis strain BsC_s was studied following challenge inoculation with A. *a/ternata.* Significantly, pronounced induction of Peroxidase, Polyphenol oxidase, PAL activity and total phenols was observed as a result of inoculation with the BsC_s showing its probable role in induced resistance.

The antagonistic bacteria, B. *subtilis* (BsC_s) and P *fluorescens* (PfC1) were also found to increase vigour index of cotton seedlings to a significant level. Foliar application of BsC_s on 35 and 45 days old plants protected cotton against *Alternaria* leaf spot disease to the extent of 40%.

Lab evaluation against plant pathogens

Among 26 isolates of *Trichoderma* evaluated against major pathogens of cotton in-vitro, highest inhibition was observed in case of *Trichoderma koningi* (TNAU) was most effective with 58.02% inhibition of mycelial growth of both *Ramularia areola* and *Alternaria macrospora,* followed by *Tharzianum* (ARS Dharwad) with 57.73% inhibition. Besides, two *Trichoderma* species from NBAII viz. *T viride* (TV 97) and *T harzianum* (Th-KSD) were also found to provide effective inhibition of mycelial growth of R. *areola* and A. *macrospora.*

Of the *Psuedomonas* fluorescens isolates tested, two PDBC isolates A3 and A 1 provided significant mycelial

growth inhibition of A. *macrospora* to the extent of 43.23 and 43.19% respectively.

Seed treatment of *T. harzianum* (ARS Dharwad) also improved germination of seeds most significantly in *Ramularia areola* (93.30%) and *Alternaria macrospora* (93.40%) infected seeds.

Field evaluation against foliar pathogens

Among five species and isolates of *Trichoderma* screened, based on in-vitro evaluation of 26 *Trichoderma* isolates, the two PDBC cultures *T. viride* (TV-97) and *T. harzianum* (TH-KSD) were found superior over other bioagents.

Seed treatment with NBAII isolates of *T. harzianum* (TH-KSD) and *T. viride* (TV-97) @ 6 g/kg seed plus foliar spray @ 1% on 60, 75, 90 and 105 DAS was most effective in controlling *Alternaria* leaf spot and grey mildew with consistent improvement in yields.

SPECIFIC TECHNOLOGIES / RECOMMENDATION

Biopesticidal formulation developed against sucking pests including mealy bug

(I) MealyQuit and Mealy Kill were evaluated

(ii) Biocidal formulation from symbionts of EPN

Potent microbials / bioagents with proven efficacy against sucking pest of cotton including mealy bug

(i) *Fusarium pallidoroseum,*

(ii) *Verticillium Isecami* (VL 5),

(iii) *Lecanicillium lecanii* and

(iv) *Photorhabdus* species

Predators effective in management of cotton pest particularly mealy bug

(I)Blaptostethus pallescens,

(ii) *Anthcoris sp* and

(iii) *Brumoides suturalis*

Potent microbials found effective as antagonist of cotton diseases

(i) *Trichoderma harzianum (Th-KSD),*

(ii) *Bacillus subtilis (BsC$_{y}$)*

(iii) *Pseudomonas f1uorescens (CICR H1* a)

Development of disease diagnostic tools

PCR based diagnostic protocols fine-tuned and refined for detection offollowing pathogens:

(i) *Cotton Leaf Curl Virus,*

(ii) *Alternaria macrospora,*

(iii) *Rhizoctonia solani,*

(iv) R. *babaticola,*

(v) R. *areola* and

(vi) *immunodiagnostics ofCLCuV*

Standardization of mass production protocols of bioagents

(i)Fusarium pallidoroseum,

(ii) *Lecanicillium lecanii,*

(iii) *Photorhabdus sp.,*

(iv) *Trichoderma harzianum* and

(v) *Pseudomonas f1uorescens*

Standardization of mass production protocols of predators

(i)Blaptostethus pallescens, (ii) *Anthoris sp.* and (iii) *Brumoides suturalis*

PATENTS FILED

1.E-2/509/2010 MUM: PCR Detection of Xanthomonas axonopodis pv. Malvacearum by P.K.Chakrabarty, Rahul Chavhan and CD.Mayee.

2.1854/MUM/2010 Bacterial biopesticidal formulation derived from entomopathogenic nematodes for management of sucking pests of crop by Nandini Gokte-Narkhedkar, N.V.Lavhe, O.M.Bambawale and CD.Mayee.