Title: Development, validation, utilization and commercialization of bio-pesticides and bio inoculants

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INTRODUCTION

Biological control of pests is a component of IPM. Several pathogenic strains which are antagonistic to diseases as well as entomophagous nematodes have been known to exist. The project aims to develop protocols for mass multiplication of such bio control agents.

OBJECTIVES

1. To explore potent microbials and their screening against major pests of Bt-cotton
2. To evaluate induced systemic resistance of potential antagonists
3. To develop of data for registration of potential microbes for commercialization
4. To commercialize the new bio-pesticide strains

ACTIVITIES

- Isolation/collection of antagonists and insect pathogens from agro ecosystems of three cotton growing zones/national and international institutions
- Exchange of isolates and their maintenance at cooperating centres
- Development of insectary of mealy bugs, thrips, spodoptera and cultures of virulent isolates of insect pathogens
- Lab evaluation of isolates against target insects and pathogens including dose response relationships, glass house/small pots testing
- Field evaluation and generation of bioefficacy data
- Characterization of isolates/molecular aspects for registration/patent application
- Development of mass production protocols
- Generation of toxicological data
- Registration of potent isolates and their commercialization

EXECUTIVE SUMMARY

Procedure standardized under TMC MM 3.1 has been used for rearing of mealy bug, *Phenococcus solenopsis,* the dominant species in the North and Central zones. The culture is now being maintained at PDBC, NCIPM, CICR, Nagpur and Sirsa for evaluation of entomopathogens.

Field survey carried out in North Zone led to collection of mealy bug cadavers from cotton stalks and isolation of *Verticillium lecaniifrom* these cadavers.

PDBC has provided fifteen isolates (VI-1 to VI-5, VI-2a, VI-2c, VI-2d, VI-3a, VI-3d, VI-3g, VI-7, VI-8, VI-9 & VI-10) of *Verticillium lecaniifor* evaluation against mealybugs to NCIPM, CICR, Nagpur and Sirsa.

Field testing of different formulations of *V lecanii* along with important insecticides against *Phenacoccus solenopsis* carried out at Sirsa indicated maximum reduction in the population of crawlers in profenophos followed by *V. lacanii* formulations. Quinalphos, profenophos, carbaryl and chloropyriphos were found to be non compatible with *V.lecanii* cultures as they caused more than 80% inhibition.

Fifteen isolates of *Trichodema harzianum, T viride, and T virens* have been isolated and are now being maintained for evaluation against the foliar diseases of cotton at UAS, Dharwad. Twenty five isolates of *Ramularia areola* causing grey mildew in Karnataka, Coimbatore and Maharastra and isolates of *Rhizoctonia bataticola, Alternaria macrospora, Xanthomonas axonopodis* pv *malvacearum* and *Vertici//ium albo-atrum* have been collected and maintained for future research with antagonistic fungi and bacteria.

Spraying the debris with nematode suspension (5000 nematodes / ml) of *Heterorhabditis indica* and *Steinernema riobrave* was found to cause mortality of red cotton bug by 78-75% and S. *riobrave* recorded higher mortality of mealy bug females.

Out of 100 soil samples collected from the rhizosphere of cotton from different cotton growing regions of Tamil Nadu five samples were positive for *Heterorhabditis sp.* and 11 for *Streinernema sp.* Bacterial symbionts *Xenorhabdus sp* and *Photorhabdus sp.* (based on biochemical characterization were isolated from entomopathogenic nematodes. Out 16 bacterial symbionts isolated, Xeno-1, Xeno-12 and Photo -3 were found to be highly pathogenic against mealy bug. Primary phase alone was found to be pathogenic to mealy bug. Method for mass multiplication of bacterial symbiont was standardized.

Screening of *Metarhizium anisop/iae, Beauveria besiana* and *B. brongniartii* against mealy bugs under laboratory condition indicated only 10 -20 % mortality.

SALIENT FINDINGS

Entomopathogens For Mealy Bug Management

Mass Production of mealy bugs

Procedure standardized under TMC MM 3.1 has been used for rearing of mealy bug, *Phenococcus solenopsis,* the dominant species in the North and Central zones. The culture is now being maintained at POBC, NCIPM, CICR, Nagpur and Sirsa for evaluation of entomopathogens. A method was standardized to multiply mealy bugs on sprouted potato. These mealy bugs do not multiply well on any other known natural sources. Procurement and mUltiplication of the predator, *Cryptolaemus montrouzeri* was carried out with mealybugs multiplied in the lab at CICR, Nagpur.

Pathogenicity of Il. *leeanii* (VI-8 isolate) on cotton mealy bugs under laboratory

At POBC, Bangalore, nymphs of cotton mealy bugs reared on potato sprouts were used for pathogenicity tests. Two concentrations of spores viz., 10^7 and 10^6 spores/ml of VI-8 isolate of *V. lecanii* were sprayed on nymphs in three replications. Nymphs treated with sterile water served as control.. Observations on mortality were recorded at 24 hours intervals for period of thirteen days. The dead ones as and when noticed were transferred to sterile wet filters for development of external fungal growth. Mycosis of the dead ones were confirmed by microscopy and plating on SOYA medium.

The results indicated that VI-8 isolate is pathogenic and caused 21.43% of mycosis on nymphs atthe spore dose of 10^7 spores/ml. The mycosed nymphs showed external fungal growth (Fig.1) and the fungus was re-isolated from the mycosed nymphs.

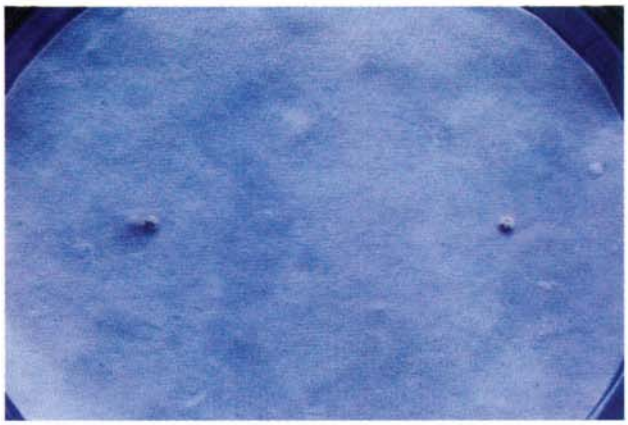

Fig. 1. Mycosed nymphs with external fungal growth of *V. lecanii*

Efficacy of *Vertici//ium lecanii,* for the control of mealy bug in cotton under field conditions

At CICR RS Sirsa, two *V. lecanii* preparations of POBC were tested along with Thiodicarb (625 g/ha) and Profenophos (1250 ml/ha) under field conditions. A plot size of 6.0 m x 2.7 m (60 plants) with 1m x 30 cm spacing on variety H-1117 in three replications were tested. Two sprays were given in the experiment and data on mealy bug severity using the recommended rating scale.

Significant reduction in severity after first and second spray was noted in all the treatments. Maximum reduction was noted in profenophos followed by thiodicarb and *V. lecanii* preparations.

In second experiment, Ecocil, Bioprahar and Indian oil product were tested along with Acephate (2000 g/ha) under field conditions. A plot size of 6 m x 2.7 m (60 plants) with 1m x 30cm spacing on variety H-1117 in three replications was kept. Three sprays were given in the experiment at weekly interval and data on mealy bug severity was recorded using a rating scale of 0 to 4. Data on severity was recorded from all plants in the plot.

Bioprahar and Indian oil did not show any reduction in percent

severity even after three sprays. In Ecocil treatment slight reduction was observed. Acephate showed more than 50% reduction after first spray and almost complete reduction of severity after second spray.

The third experiment was conducted on *RCH-134* Bt hybrid with plot size $5.4mX$ 6.0m anda spacing of 1 metre X 60cms at CICR, RS, Sirsa. The preparations of *V.lecanii* were sprayed at 2 g/l and other insecticides were used at recommended concentration. The trial was conducted in three replications using RBD. Five plants were tagged from each plot and observations on marked 5cm area from each plant were recorded. Maximum reduction of mealy bug population was noted in thiodicarb followed by profenophos.

Dose-response relationship for fungal pathogen on mealy bugs

The experiment was conducted with the following treatments

1. *V.lecanii* -108cfu/g (talc powder)
2. *V.lecanii* -109cfu/g(talc powder)
3. *Ecoci/*
4. lab culture
5. *V.lecanii-PDBC*
6. *Photorhabdus*
7. *Profenophos-check*
8. Control

Five concentrations of each treatment were used. Five mealy bugs with three repetitions were taken for each treatment.. The mealy bugs were dipped in solutions for 5-10 seconds and put on leaves in petri dishes. The leaves were keptturgid by putting water soaked cotton swabs on petioles and were kept wet subsequently also. Observations on mortality were taken at 24 hour intervals up to 10 days. The experiment was repeated using same treatments but sticker (Helper of Tropical Agro @1 mill) was added.

Without sticker, slight mortality (6.6%) at concentration of 7.5 &10g/l was observed in *V./ecanii* preparations after 24 hours. In other bio preparations the mortality was noted at 24 h in lower concentrarions. Complete mortality was observed in profenophos treatment after 24 hrs. After ten days, an increased mortality was observed in all treatments. A clear response of increased dosages was also seen in all treatments. Maximum mortality of 93.2% was observed in case of *V./ecanii* culture received from PDBC at 7.5 & 10 g/l concentrations. Almost similar trend of results was observed in case of different treatments tested in combination with sticker but the mortality started early and was more as compared to treatments tested without sticker.

Three species of entomopathogenic fungi viz., *Metarhizium anisopliae, Beauveria besiana* and *B. brongniartii* were screened against mealy bugs under laboratory condition at CICR RS Coimbatore. The per cent mortality ranged from 10-20 % for all the three species.

Compatibility of entomopathogens with insecticides

Two cultures 1. lab culture and 2. *V lecanii* culture isolated from mealy bug cadavers were taken for compatibility studies with seven insecticides at CICR RS Sirsa. Recommended doses of insecticides were tested using poison food inhibition technique with three Petri dishes for each treatment..

The results revealed that in lab culture, more than 80% inhibition was noted in case of quinalphos, profenophos, carbaryl and chloropyriphos indicating their incompatibility with this culture (Fig 2). However, imidacloprid followed by thiodicarb and acephate appeared to be compatible.

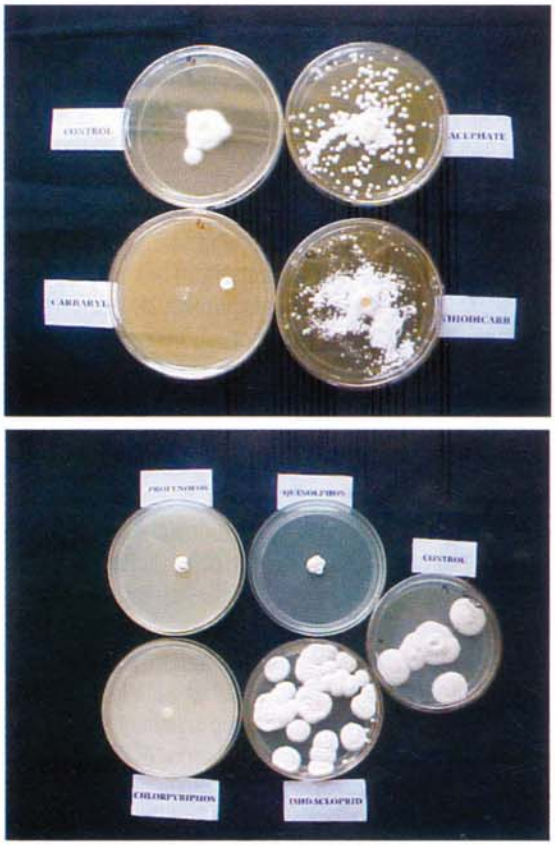

Fig 2 : *V.lecanii* - Insecticide interactions

In case of *V /ecanii* also the same four insecticides showed more than 60% inhibition whereas no inhibition was noted in imidacloprid and acephate while thiodicarb showed some stimulation of growth indicating the possibility of its integration with *V. /ecanii* for effective and successfull management of mealy bugs in cotton ecosystem.

ENTOMOPATHOGENIC NEMATODES AGAINST COTTON INSECTS

A total of 100 soil samples were collected from the rhizosphere of cotton at CICR RS Coimbatore from different cotton growing regions of Tamil Nadu and baited with *Corcyra cephalonica* and *Galleria mellonella* to isolate entomopathogenic nematodes. Dead larvae collected were placed in White trap to extract nematodes. They were stored in sterile water at room temperature. Out of 100 samples 5 samples were positive for *Heterorhabditis sp.* and 11 samples were positive for *Streinernema sp.* At CICR, Nagpur heat tolerant isolate of *Hetrorhabditis indica* and *Steinernema riobrave* was evaluated against mealy bug and red cotton bug of cotton. Results indicate that inoculum levels of 20-25 infective juveniles per insect was the optimum dose for both EPNs against mealy bug females. Among the two EPN tested, S. *riobrave* recorded higher mortality of mealy bug female. It is possible that smaller body size of juveniles may result in better penetration of the nematode.

Experiments with species of *Steinernema* and *Heterorahbditis* using 1-25 *Corcyra cephalonica* larvae at a time as host did not support this hypothesis as most dauers could be recovered in first baiting round. However, very small percentage (2-3%) could be recovered in subsequent baiting rounds. This indicates that while this may not qualify to be called as phased infectivity, a very small percentage infects later. This has significance that in case of invading juveniles not completing their life cycle, a small percentage of population always remains to ensure survival and perpetuation of species.

Bacterial symbionts of entomopathogenic nematodes against mealy bugs

Bacterial symbionts of entomopathogenic nematodes were isolated at CICR RS Coimbatore from dead cadavers or directly from the infective juveniles (IJ). Nematodes were inoculated @ 10 IJ/ larva and 24 hours after death they were surface sterilized in alcohol, dissected and heamolymph collected was inoculated onto NBTA medium. Bacterial isolation from IJ was carried out by sterilizing nematode and crushing them in sterile water. The suspension was inoculated onto NBTA. After several subculturing individual colonies were isolated and stored at low temperature. Based on biochemical characterization they were identified as *Xenorhabdus sp* and *Photorhabdus sp.*

Sixteen bacterial symbionts along with a bacterial symbiont, *Xenorhabdus poinari* (isolated from *Steinernema glaseri)* was screened against mealy bug under laboratory condition at CICR RS Coimbatore. Three isolated viz., Xeno-1, Xeno-12 and Photo -3 were found to be highly pathogenic against mealy bug. This experiment was repeated twice and the same trend of results was obtained. Primary and secondary phase of three bacterial isolates viz., Xeno-1, Xeno-12 and Photo -3 were screened against mealy bug. Primary phase alone was found to be pathogenic to mealy bug. Different temperatures viz., 15,20,25,30 and 35°C were tested for multiplication of bacterial symbiont in Nutrient broth. Maximum multiplication was observed at 25 and 30°C. Different pH viz., 5,6,7,8 and 9 were tested for multiplication of bacterial symbiont (Fig.1) in Nutnent broth. They were able to multiply on all pH tried. But maximum multiplication was reported in pH 6-8. Based on !abora~ory screening, 0.5 % Sucrose, Liquid paraffin (2%) were Identified as the phagostimulants and additive respectively to Increase the effectiveness of bacterial formulation. They were also not harmful to the symbionts.

EPN bacterial biocide developed at CICR, Nagpur was evaluated against cotton mealy bug *Phenoccocus solenopsis.* In a replicated trial sprouting potato was placed in plastic container with provision for sufficient aeration. The formulation was sprayed on the sprouting potatoes and allowed to air dry for an hour. Thirty crawlers were placed on each potato. **In** another treatment the crawlers were placed on the potato sprouts and formulation sprayed. Formulation carrying all the constituents except for the bacteria served as one of the control treatments. The other control treatments were: water spray and no spray. There were five replications. The experiment was repeated on one month old cotton seedlings .The observations on insect mortality were recorded daily for four days. Final observation was taken after one week and number of crawlers developing into adults was recorded. Mortality recorded after both prerelease and post release sprays ranged between 78-88 with only 8-11 % crawlers developing into adults.

Biosafety of EPN against egg parasitoid Trichogramma

Strips containing *Corcyra* eggs freshly parsitized by Trichogramma were used. Strips were cut into 1 cm^2 size were placed on to moist filter paper placed in petriplate. The strips were subjected to following treatments

1. Bacterial suspension 50 III
2. Water 50 III
3. Suspension minus bacterium
4. No treatment

The plates were sealed with parafilm and observations on emergence was taken daily up to five days. The percent emergence in all treatments ranged between 80-92%. The results indicated thatthere was no significant difference among the treatments and all were at par with control..

RHIZOBACTERIA AS POTENTIAL BIOAGENT

Evaluation of *rhizobacteria* isolated from cotton rhizosphere against mealy bug

At CICR, Nagpur, rhizobacteria were isolated from cotton rhizosphere and evaluated against plant parasitic nematodes of

cotton. A total of 40 isolates were screened for efficacy against root-knot and reniform nematodes. Out of these five isolates were found to have antagonistic properties against nematodes. These isolates were taken up for their possible efficacy against mealy bug of cotton. The methodology was the same as adopted for evaluation of EPN bacterial biocide against mealy bug. The results indicated that isolate 4 is effective against mealy bug crawlers. Initial identification indicates this isolate as belonging to *Bacillus sp.* Further identification as well as screening of more isolates is in progress.

ISOLATION OF ANTAGONISTIC MICROORGANISMS

At CICR RS Sirsa, antagonistic microorganism were isolated from mealy-bug cadavers. From cadavers, isolation was carried out by two methods i.e. (1) by directly keeping mealy-bug cadavers onto solidified PDA plates and (2) by keeping surface sterilized mealy-bug cadavers onto solidified PDA plates with the objective to get all possible fungal flora. Fungi obtained thus, further sub-cultured to purify the fungal culture, by hyphal-tip and single spore technique and incubated at 25±2°C. In both methods yellowish white /pinkish white colony appeared in all seven samples.

Microscopic examination showed the presence of microconidia, macroconidia and chlamydospores in some of the cultures. Fungi isolated from mealy-bug cadavers of different locations will be identified from NBAIM / ITCC, IARI, New Delhi.. Several antagonistic microorganisms and biocontrol agents were isolated and maintained at TNAU, Coimbatore.

Antagonistic activity on soil borne pathogens

At TNAU, Coimbatore, out of six *Trichoderma* isolates, Is01 was most effective in inhibiting the growth of the major soilborne pathogens viz., *Rhizoctonia solani* (65.55 % inhibition), *Fusarium oxysporum pv.vasintectum (70.00%* inhibition) and *Macrophomina phaseolina* (68.88% inhibition) where as the existing *Trichoderma viride* (check TNAU culture) recorded 62.22, 66.66 and 65.66 percent inhibition respectively. Among the ten isolates of *Bacillus spp* screened at TNAU, Coimbatore against *R. solani, F o.pv. vasintectum and M. phaseolina,* in the lab by dual plate technique, the isolates Iso 5 was highly inhibitory in reducing the growth of the pathogens by 72.22, 71.10 and 74.96 per cent respectively comparing to the existing check of TNAU culture which recorded 71.32, 68.33 and 73.44 per cent inhibition respectively.

Out of six isolates of *Pseudomonas* spp screened against *R. solani, F o.pv. vasintectum* and *M. phaseolina,* the isolate Iso 16 was most effective in reducing the growth of the pathogens by 52.22, 53.33 and 54.44 per cent respectively where as the existing check TNAU culture recorded 50.11,49.55 and 46.60 per cent inhibition respectively.

Preliminary screening of ten isolates of *Streptomyces* spp against *R. solani, F o.pv.vasintectum* and *M. phaseolina,* the isolate Iso 2 was highly inhibitory in reducing the growth of the pathogens by 55.33,55.55 and 54.44 per cent respectively.

It was observed that the *Trichoderma* isolates of ARS, Dharwad farm, TNAU, Coimbatore, Sriganganagar, Pantnagar, MARS, Dharwad were found superior in inhibiting the growth of Sclerotium rolfsii..

Maintenance of cultures

Twenty two isolates of *Verticillium lecanii* collected from different insect hosts from different locations are being maintained at PDBC, Bangalore for screening against mealy bugs of cotton. Eleven isolates of *Verticillium* lecanii (VI-1, VI-2a, VI-2c, VI-2d, VI-3a, VI-3d, VI-3g, VI-7, VI-8, VI-VI-9 & VI-1 0) were supplied to NCIPM, New Delhi for testing against cotton mealy bugs. Two hundred grams of talc formulation of *V lecanii* was sent to CICR regional station, Sirsa (Haryana) for field evaluation against cotton mealy bug.

STUDIES ON VARIABILITY IN FUNGAL PATHOGENS

At UAS, Dharwad, nine isolates of *Fusarium solani* collected from different parts of Karnataka are being maintained for future research. Seventeen isolates of *Sclerotium roltsii* and one isolate each of soil borne pathogens viz. *Verticillium alba atrum and Rhizoctonia bataticola* and *Alternaria macrospora* have been collected from different parts of Karnataka .Forty isolates of *Ramularia areola* causing grey mildew have been collected from Karnataka, Coimbatore and Maharashtra. Morphological studies on *Sclerotium roltsii* indicated that there were differences among the isolates with respecttheir mycelial growth ranging from 6.05 em (Garag isolate) to 7.78 em (Chandanamatti - 2). For mycelial colony characters all were having similar white cottony growth except UAS Dharwad isolate 2 where it was slightly compact.. All sclerotial bodies were dark brown except the Amminabhavi isolate which was slightly reddish brown. The size of sclerotia also relatively differ from small to big.

Out of the three genotypes used under study (two Bt and one Non Bt) for virulence of 17 isolates of *Sclerotium* rolfsii, the Lokur isolate was found more virulent followed by Tadkod isolate and hence the Lokur isolate was considered for its interaction with 17 isolates of *Trichoderma sp.* At 30 DAS it was observed that there was 89.17 % mortality of seedlings in Lokur and Marewad isolates followed by Tadkod isolate (87.50) indicating that they are more virulent isolates. Lokur isolate appeared a better candidate for its reaction with Trichoderma isolates