

### TMC-MM 3.3: Commercialization of bioagent mass production technologies in intensive cotton districts

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#### Targets & Achievements

Targets	Achievements
<b>Project Directorate of Biological Control, Bangalore</b>	
Screening of antagonistic organisms against cotton pathogens, viz., <i>Fusarium solani</i> , <i>Ramularia aereola</i> and <i>Alternaria macrospora</i>	Twenty-four isolates of <i>Trichoderma</i> belonging to <i>T. viride</i> , <i>T. harzianum</i> , <i>T. virens</i> and <i>T. hamatum</i> were tested by Dual culture test. <i>T. viride</i> (PDBC-32, 115), <i>T. harzianum</i> (PDBC-15, PDBC-21, IISR-P26) (62.2-62.8% inhibition) were identified as promising isolates.
Effect of promising isolates of <i>Trichoderma</i> sp. on seed germination and seedling growth of cotton.	Fourteen promising isolates of <i>Trichoderma</i> sp. ( <i>T. viride</i> (PDBC-12, 32, 97, 115 and TV-CICR), <i>T. harzianum</i> ((PDBC-10, PDBC-15, PDBC-21, PDBC-M, CPCRI-KD, CPCRI-KS, IISR-GTH7, IISR-P26) and <i>T. hamatum</i> (PDBC-138) were tested for their effect on cotton seed germination (Annaigiri) and seedling growth. All fourteen isolates of <i>Trichoderma</i> tested showed higher seed germination (82-88%) and with good vigour index (1626.5-2273.8).
Growth and sporulation of <i>Beauveria bassiana</i> and <i>Metarhizium anisopliae</i> in different liquid media in stationary and shaker cultures	Ten liquid media were evaluated for mass production of <i>B. bassiana</i> and <i>M. anisopliae</i> in stationary and shaker cultures. Maximum biomass production of <i>B. bassiana</i> was observed with Jaggery-soy broth in stationary and shaker cultures (12.5 and 20.0g/100ml wet wt., respectively) and highest cfu counts in stationary culture were observed with Yeast peptone soybean oil broth ( $5.1 \times 10^8$ cfu/ml). Maximum biomass production of <i>M. anisopliae</i> (20 and 48.8g/100 ml wet wt.) and cfu counts ( $9.8 \times 10^8$ and $4.3 \times 10^8$ cfu/ml) was observed with yeast peptone dextrose broth in both shaker and stationary cultures.
Development of pilot scale production system for <i>B. bassiana</i> in fermentor	Among the three media tested (Jaggery-soy broth, Jaggery-soy yeast broth and Corn meal broth), Corn meal broth supported maximum biomass after 7 days (17.3g/100ml wet wt.) and highest cfu counts ( $240 \times 10^{10}$ cfu/ml).
Development of pilot scale production system for <i>M. anisopliae</i> in fermentor	Jaggery-soy yeast broth gave highest biomass production of <i>M. anisopliae</i> (20.7g/100ml wet wt.). Regarding cfu counts, Sabouroud dextrose yeast broth yielded highest cfu count of $2.7 \times 10^{10}$ / ml.

Targets	Achievements
Shelf-life studies of talc formulations of <i>B. bassiana</i> and <i>M. anisopliae</i> prepared from the fermentor biomass	Talc formulations of <i>B. bassiana</i> prepared from Corn meal broth remained viable in sufficient numbers in the samples stored at room temperature as well as refrigerated temperature for seven months period ( $5.4 \times 10^8$ and $30.0 \times 10^8$ cfu/g, respectively). Shelf-life studies of talc formulations of <i>M. anisopliae</i> (Jaggery-soy yeast broth, Jaggery-soy broth, Sabouroud dextrose yeast broth and Potato dextrose broth) were completed for the period of four months and the cfu counts are as per the CIB standard in all the samples
<b>Central Institute for Cotton Research, Nagpur</b>	
EPN bacterial symbionts as a new pest management option for sucking pests	Preliminary field trials indicated that the bacterial symbiont, <i>Photorhabdus</i> sp. broth when sprayed was found to cause 24 to 80% mortality of sucking pests.
EPN against <i>H. armigera</i> and semilooper on cotton	Single field application of <i>H. indica</i> at 1 billion/m <sup>2</sup> was found to reduce the population of the pests.
Evaluation of shelf life of EPN	<i>H. indica</i> having origin from hot dry cotton ecosystem stored better at high temperature of 28°C. Anti-desiccant AV Gel 1 and 10% enhanced the storage viability.
Standardization of mass production protocol for <i>H. indica</i>	Nutrient agar based medium was the best as maximum number of <i>H. indica</i> were produced at 28-30°C with humidity ranging between 80-90%.
Media for <i>Metarhizium</i> and <i>Nomuraea</i>	Soaked grains of rice and sorghum with 1% yeast granules at 28°C with above 70% humidity was the best for <i>Nomuraea</i> . For <i>Metarhizium</i> the same conditions was found suitable with slightly reduced humidity (65%)
Selection of heat tolerant isolate of <i>H. indica</i>	One isolate of <i>H. indica</i> isolated from cotton fields of CICR, Nagpur.

## Progress of work

### PDBC, Bangalore

#### Develop pilot-scale production system for *Trichoderma*, *Beauveria*, *Metarhizium* and *Nomuraea*

#### ❖ Screening of antagonistic organisms against cotton pathogens, viz., *Fusarium solani*, *Ramularia aerea* and *Alternaria macrospora*

Twenty four isolates of *Trichoderma* belonging to *T. viride* (8 isolates), *T. harzianum* (11 isolates), *T. virens* (5 isolates) and *T. hamatum* (1 isolate) and an isolate each of *Chaetomium globosum* and *Bacillus subtilis* and three isolates of *Pseudomonas fluorescens* were tested against *Alternaria macrospora*, *Fusarium solani* and *Ramularia*

*aereola* (cotton isolates) by Dual culture test. The percent inhibition of the pathogen and over growth of antagonists on the pathogen are presented in Table 3.3.1. For the cotton pathogen - *A. macrospora*, *T.viride* (PDBC-12, 32, 97), *T.harzianum* (PDBC-21, CPCRI-KD, CPCRI-KS, IISR-GTH7) and *T. hamatum* (PDBC-138) were identified as promising isolates (Figs 1 & 2) causing 67.4-70.7% inhibition with 32.6-36.0 mm overgrowth. Against *F. solani*, *T.harzianum* (PDBC-M, IISR-GTH7 and IISR-P26) showed 55.9-56.7% inhibition with 30-36.5 mm over growth. Against *R. aereola*, *T.viride* (PDBC-32, 115), *T.harzianum* (PDBC-15, PDBC-21, IISR-P26) showed 62.2-62.8% inhibition with 41-43 mm over growth.

❖ **Effect of promising isolates of *Trichoderma* sp. on seed germination and seedling growth of cotton**

Fourteen promising isolates of *Trichoderma* sp. belonging to *T. viride* (PDBC-12, 32, 97, 115 and TV-CICR), *T. harzianum* ((PDBC-10, PDBC-15, PDBC-21, PDBC-M, CPCRI-KD, CPCRI-KS, IISR-GTH7, IISR-P26) and *T. hamatum* (PDBC-138) were tested for their effect on cotton seed germination (Annaigiri) and seedling growth. Hundred seeds were treated with the spore suspension of each isolate at the spore dose of  $2 \times 10^6$  spores and dried and planted in the pots. The percentage seed germination, the root length and shoot length were recorded and based on these observations, vigour index was worked out. All fourteen isolates of *Trichoderma* tested showed higher seed germination (82-88%) and vigour index (1626.5-2273.8) compared to the untreated seed which showed 72% seed germination with seedling vigour index of 1531.9. Among the fourteen isolates of *Trichoderma* tested, *T.viride* (CICR isolate) showed highest seedling vigour index of 2273.8 followed by *T.harzianum* (IISR-P26 isolate) showing vigour index of 2010.3.

**Growth and sporulation of *Beauveria bassiana* and *Metarhizium anisopliae* in different liquid media in stationary and shaker cultures**

Ten liquid media like, Potato dextrose broth(PDB), Sabouoroud dextrose broth with yeast extract (SDYB), Sabouoroud maltose broth with yeast extract (SMYB), Malt extract broth (MEB), Corn meal broth (CMB), Jaggery soya broth (JSB), Yeast peptone dextrose broth (YPDB), Yeast peptone soluble starch broth (YPSS), Czapek dox broth (CDB) and Yeast peptone soybean oil broth (YPSB) in stationary and shaker culture conditions were evaluated for mass production of *B.bassiana* and *M.anisopliae*. Biomass production and cfu counts were taken after 10 days of incubation.

**Table 3.3.1. Efficacy of fungal and bacterial antagonists against *Fusarium solani*, *Alternaria acrospora* and *Ramularia aereola* (Dual culture Test)**

Antagonist	<i>A. macrospora</i>		<i>F. solani</i>		<i>R. aereola</i>	
	Inhibition (%)	Over growth antagonist (mm)	Inhibition (%)	Over growth antagonist (mm)	Inhibition (%)	Over growth antagonist (mm)
<i>T. viride</i> PDBC12	68.5	35.5	48.9	6.5	57.8	38.0
<i>T. viride</i> PDBC23	61.7	12.5	47.0	3.5	55.6	35.0
<i>T. viride</i> PDBC31	49.4	0.0	47.8	0.0	58.9	37.0
<i>T. viride</i> PDBC32	68.5	35.5	47.8	0.0	62.3	43.0
<i>T. viride</i> PDBC97	68.5	36.0	43.4	4.0	56.7	39.0
<i>T. viride</i> PDBC115	59.6	23.0	51.2	7.0	62.2	41.0
<i>T. viride</i> CICR	65.1	31.0	47.8	11.5	58.9	30.5
<i>T. viride</i> TNAU	65.1	31.0	48.9	10.5	55.6	40.0
<i>T. harzianum</i> PDBC10	64.0	27.0	48.9	21.5	60.0	36.0
<i>T. harzianum</i> PDBC15	61.7	12.5	52.3	4.0	62.8	41.0
<i>T. harzianum</i> PDBC21	67.8	33.0	47.8	17.5	62.4	41.0
<i>T. harzianum</i> PDBC-M	63.0	7.5	55.9	36.0	58.9	37.0
<i>T. harzianum</i> ITCC	52.8	0.0	47.8	0.0	55.6	40.0
<i>T. harzianum</i> CPCRI-KD	67.8	33.0	47.8	32.0	58.9	37.0
<i>T. harzianum</i> CPCRI-KL	52.8	0.0	42.3	2.0	57.8	38.0
<i>T. harzianum</i> CPCRI-KS	67.4	32.8	47.8	9.0	56.7	39.0
<i>T. harzianum</i> IISR-GTH7	69.7	32.6	56.7	36.5	58.9	37.0
<i>T. harzianum</i> IISR-P26	64.2	30.0	56.4	36.5	62.2	41.0
<i>T. harzianum</i> CICR	63.2	19.5	47.8	7.0	55.6	15.0
<i>T. virens</i> PDBC12	53.9	0.0	46.7	12.5	55.0	41.0
<i>T. virens</i> IISR-P12	52.8	0.0	46.7	11.5	55.6	40.0
<i>T. virens</i> ITCC	52.8	0.0	44.5	6.5	55.6	35.0
<i>T. virens</i> CICR	65.1	22.5	53.4	9.0	55.0	40.0
<i>T. hamatum</i> PDBC138	70.7	33.0	50.0	10.0	55.6	0.0
<i>C. globosum</i> - PDBC	50.5	0.0	22.3	0.0	34.0	0.0
<i>P. fluorescens</i> PDBC	43.8	0.0	20.0	0.0	44.5	0.0
<i>P. fluorescens</i> PDBC-1	49.4	0.0	22.3	0.0	44.5	0.0
<i>P. fluorescens</i> PDBC-2	48.3	0.0	17.8	0.0	45.6	0.0
<i>B. subtilis</i> -PDBC	46.0	0.0	13.0	0.0	32.3	0.0
CD at 5%	4.92	3.32	2.18	4.62	1.96	1.21

Maximum biomass production of *B.bassiana* in stationary and shake cultures was observed with Jaggery soya broth (12.5 and 20.0g/100ml wet wt. respectively), followed by and Corn meal broth (11.6 and 18.6g/100ml wet wt. respectively), Yeast peptone soybean oil broth (YPSB) provided maximum cfu counts ( $5.1 \times 10^8$ cfu/ml) in stationary culture. In shake culture corn meal broth provided maximum cfu counts ( $38 \times 10^8$ cfu/ml) followed by Yeast peptone soybean oil broth ( $25 \times 10^8$ cfu/ml).

Maximum biomass production of *M.anisopliae* was observed with yeast peptone dextrose broth (YPDB) in both shaker and stationary cultures (20 and 48.8g/100 ml wet wt. respectively) followed by Sabouroud maltose with yeast extract broth (17.9 and 37.3g/100 ml wet wt. respectively). Similarly maximum sporulation was observed with YPDB broth in both shaker and stationary cultures ( $9.8 \times 10^8$  and  $4.3 \times 10^8$  cfu/ml respectively).

#### **Development of pilot scale production system for *B. bassiana* and *M. anisopliae* in fermentor**

Studies on biomass production and cfu counts of *B.bassiana* and *M. anisopliae* in Jaggery-soya broth (JSB), Jaggery-soya yeast broth (JSYB) and Corn meal broth (CMB) in ten-liter fermentor has been studied for seven days at 24 intervals. Peak biomass production and cfu counts were observed after 7 days in all the media tested. Corn meal broth (CMB) supported maximum biomass after 7 days (17.3g/100ml wet wt.) followed by JSYB (12.79g/100ml wet wt.) and JSB (12.0g/100ml wet wt.). Highest cfu counts were observed with corn meal broth ( $240 \times 10^{10}$  cfu/ml).

Among the four media tested for *M. anisopliae* in fermentor for the specified period and time as mentioned earlier (Jaggery Soya yeast broth (JSYB), Jaggery Soya broth (JSB), Sabouroud dextrose yeast broth (SDYB) and Potato dextrose broth (PDB)), Jaggery-Soy yeast broth (JSYB) gave highest biomass production of *M. anisopliae* (20.7g/100ml wet wt.) followed by Jaggery Soya broth (16.5g/100ml wet wt.). Sabouroud dextrose yeast broth (SDYB) yielded highest cfu count of  $2.7 \times 10^{10}$ / ml.

#### **Shelf-life studies of talc formulations of *B. bassiana* and *M. anisopliae* prepared from the fermentor biomass**

Talc formulations of *B. bassiana* were prepared from the fermentor biomass (7days) obtained from JSB, JSYB and CMB by mixing with sterilized talc at 1:1 ratio and then drying to 8% moisture. Similarly talc formulations of *M. anisopliae* were prepared from fermentor biomass (7days) obtained from JSYB, JSB, SDYB and PDB. After drying, the

samples were packed in polypropelene bags and stored at room temperature (17-36°C) and refrigerated temperature (6-8°C) for shelf life studies. The samples were drawn at monthly intervals and cfu counts were estimated on PDA plates.

The talc formulations of *B. bassiana* prepared from Corn meal broth (CMB) remained viable in sufficient numbers in the samples stored at room temperature as well as refrigerated temperature for seven months period (5.4X10<sup>8</sup> and 30.0X10<sup>8</sup> cfu/g respectively) and were as per the CIB standard. Further shelf life studies are in progress. The talc formulations of *B. bassiana* prepared from Jaggery-soya broth and stored at room temperature remained viable in sufficient numbers 1.3 X10<sup>8</sup> cfu/g (as per the CIB standard) up to six months period. The cfu counts fell below the CIB standard (<10<sup>8</sup> cfu/g) after 7 months of storage.

After four months of storage, talc formulations of *M. anisopliae* prepared from Jaggery Soya yeast broth (JSYB) showed highest cfu counts at room temperature (264 X10<sup>8</sup> cfu/g) as well as refrigerated temperature (287 X10<sup>8</sup> cfu/g) followed by the talc formulations prepared from Sabouroud dextrose yeast broth (50X10<sup>8</sup> and 136 X10<sup>8</sup> cfu/g respectively). Further shelf life studies are in progress.

### **Central Institute for Cotton Research, Nagpur**

#### **Entomopathogenic nematode bacterial symbionts recorded as new management option for management of sucking pests.**

Preliminary field trials of the two *Photorhabdus* (symbiont of entomopathogenic nematode) isolates recorded to have antifeedant properties against insect pests of cotton were field tested at Sirsa, Nagpur and Nanded for control of sucking pests of cotton. The bacterial symbiont *Photorhabdus* sp. Broth (Fig. 3) when sprayed was found to cause population reduction of sucking pests, viz., aphids, *Aphis gossypii* (80%), leafhopper, *Amrasca devastans* (56%), whitefly *Bemisia tabaci* (45%) and thrips, *Thrips tabaci* (24%). Repeating the spray after three days was found to substantially enhance mortality of sucking pests.

#### **EPN Against *Helicoverpa armigera* and semilooper on cotton**

Single field application of *H.indica* at 1 billion/m<sup>2</sup> was found to reduce populations of cotton bollworm and semilooper by 58 and 50%, respectively .

### **Evaluation of shelf life of EPN**

Parameters influencing storage viability of *H. indica* isolates and evaluation of anti-desiccants in enhancing the viability of EPN under storage conditions was carried out. Four temperature conditions viz. 5, 13, 25, 28 and 30°C were evaluated. *H.indica* having origin from hot dry cotton ecosystem can be stored better at higher temperature of 28°C. Of the various anti-desiccants tested, A.V.gel at 1 and 10% was found to enhance storage viability (Table 3.3.2).

**Table 3.3.2 Evaluation of different anti desiccants to improve shelf life of *H.indica***

Antidesiccants used	%Viability after 1 month	%Viability after 2 months
A.V. 1%	80	45
A.V.10%	90	56
CMC 1%	78	48
Glycerine 1%	67	40
G.A. 1%	70	45
Control	56	15

Slurry formulation of EPN had higher survival (88-98 days) when stored for 40 days as compared to suspension and granular formulation.

### **Modification and standardization of mass production protocol for Entomopathogenic nematode *H. indica***

Large quantity of inoculum of EPN *H.indica* is required for field application against cotton bollworm *H. armigera*. Among the various non-synthetic, synthetic and semi-synthetic culture media evaluated in the laboratory, nutrient agar based medium was the best as maximum numbers of *H. indica* were produced on it. However, the infectivity of nematode population produced was found to be adversely affected, therefore, a new culture substrate for higher EPN population with increased efficacy against *H.armigera* was aimed at.

The corn oil constituent of the nutrient agar based culture was substituted with other vegetable oils, viz., soybean, sunflower and groundnut at 1% as well as animal fat at 1, 5 and 10%. All the plates with 20 ml medium and 50±5 numbers of *H. indica* juveniles were incubated for a period of 21 days. Three weeks later the number of nematodes produced was estimated.

Besides these, intensity of infectivity of nematodes thus cultured was also evaluated against third instar larva of Cotton bollworm *H. armigera*. 10-15 infective juveniles per insect larva was standardized as effective dose for evaluation of EPN isolates for their insect larvicidal efficacy.

Media containing vegetable oils had a population harvest that ranged between 150 –190 X10<sup>3</sup>. Animal fat at 1 and 5% was an excellent producer of nematodes and nematode juveniles produced were highly effective against cotton bollworm larvae (Table 3.3.3). This new media can thus be used for mass multiplication (Fig. 4) and application of *H.indica* on cotton crop for management of Cotton bollworm, *H.armigera*.

**Table 3.3.3. Evaluation of different fat/oil constituents of Nutrient agar based media**

Fat constituent of Nutrient agar based media	Production of infective juveniles (in 000) <i>Yield Range</i>	Per cent larval mortality of <i>H. armigera</i>
Soybean oil 1%	150-185 ( 160)	40
Sunflower oil 1%	168-190 (175)	55
Groundnut oil 1%	130-156 (148)	36
1% Corn oil (Original Wout's media )	180-205 (170)	35
Animal Fat 1%	90-115 (110)	85
Animal Fat 5%	145-180 (156)	97
Control (Insect reared)	6-8 (7.3)	98

Uninoculated Control- 1.6%

Figures in parenthesis denote average values

C.Difference for Production of infective juveniles-5.19

C.Difference for Larval Mortality-0.98

Maximum production of nematode juveniles was obtained at temperature of 28-30°C with humidity ranging between 80-90%. Nematodes can be cultured on nutrient broth based medium consecutively for four generations without any loss of infectivity.

#### **Modification of media for *Metarhizium* and *Nomuraea* culture**

*Nomurea rileyi* was isolated from infected larvae of *Helicoverpa* and Semilooper collected from cotton fields. It can be easily multiplied on insect larvae such as *Heliocoverpa*, Semilooper and Spodoptera. However, humidity need to be maintained above 80% for development of fungus. Mycelial growth on the mummified larvae occurs within 24 h and spores are formed 2-3 days later. *Nomuraea rileyi* can also be easily cultured on cereals such as rice and sorghum.

Protocol was developed using soaked grains of rice and sorghum fortified with 1 % yeast granules for development of *N. rileyi* mycelia and sporulation. Temperature of 28°C and humidity above 70% was found optimum for sporulation. No sporulation was observed at humidity below 60% RH. On sorghum the sporulation was delayed by one day as compared to that on rice.

Protocols for culturing of *Metarhizium* were standardized using soaked grains of rice, sorghum and small millets fortified with 1 and 5% yeast granules. Temperature of 28°C was found congenial for sporulation. Minimum humidity of 65 % was found necessary for sporulation.

### **Selection of heat tolerant isolate of *Heterorhabditis indica***

Sixteen isolates of EPN belonging to *Heterorhabditis bacteriophora* and *H. indica* and *Steinernema glaseri* isolated from cotton growing ecosystems and found effective against cotton insects particularly cotton bollworms were quantified for variation in tolerance to temperature, moisture stress and host finding ability. Sufficient variability existed in temperature tolerance of different isolates of EPN. One isolate of *H. indica* isolated from cotton field of CICR, Nagpur was made tolerate to high temperatures by periodic exposure to high temperatures and selection of individuals. Besides *H. armigera*, this isolate was found to be effective against other cotton insect pests.

### **Technologies developed**

1. Liquid fermentation technology of *Beauveria bassiana*, *Metarhizium anisopliae* and *Trichoderma harzianum* and *T. viride*.
2. Solid state mass production of *Nomuraea rileyi*