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Mass multiplication and formulation of *Metarhizium anisopliae* (Metsch.) Sorokin

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ABSTRACT: ***Metarhizium anisopliae*** **is a potent microbial insecticide against** ***Helicoverpa armigera*** **(Hübner), a potential threat to successful cultivation of many economically important crops. Pathogenicity study conducted with 10^8 spores/ml concentration of fungal suspention revealed larval mortality of 100.00, 90.00, 76.67, and 56.67 per cent against Ist, IInd, IIIrd and IVth instar larvae of** ***H. armigera*****, respectively. Five media were compared at four temperature levels. Emersons's Yeast Phosphate Soluble Starch (YPSS) medium recorded highest conidial count of 22.00 x 10^7 spores/ml at 27° C followed by 25° C, Sabouraud's Dextrose Broth + Yeast (SDB+Y) and Potato Dextrose Broth (PDB) being on par with YPSS at 27° C. Poor cultural growth was recorded in Barners medium at all temperature levels. Amongst the five media used for the mass multiplication studies, Potato Dextrose Broth was found to be better in terms of spore production, viability of conidia and its production cost. Bioassay data analyzed by log dose Probit method revealed LC_{50} values of $6.0x10^4$, $1.2x10^6$, $4.8x10^6$ and $5.0x10^7$ spores/ml for Ist, IInd, IIIrd and IVth instar** ***H. armigera*** **larvae, respectively, with very shallow slopes.**

KEY WORDS: *Helicoverpa armigera*, media, *Metarhizium anisopliae*, pathogenicity, temperature

INTRODUCTION

Helicoverpa armigera (Hübner) is posing a potential threat for successful cultivation of several economically important crops in India. For instance, more than 55 per cent of total insecticide used in the country is being utilized in cotton pest management with a special emphasis on *H. armigera*, wherein, the crop occupies just about 5 per cent of total cultivable area in India (Puri, 1995)

Even though pesticides form the first line of defense against the pest preventing the economic damage due to its rapid curative action, wide spread report of insecticide resistance in *H. armigera* to almost all popular insecticides has forced the farmers to think about better alternatives. Taking into consideration the encouraging results obtained by using microbial insecticides, studies were initiated on *Metarhizium anisopliae* (Metsch.) Sorokin, a green muscardine soil inhabiting fungus to assess the effect of temperature and media on growth and sporulation of this fungus with a special emphasis to track down a cheaper formulation along with its pathogenicity against *H. armigera* larvae.

MATERIALS AND METHODS

Isolation of *M. anisopliae*

For the isolation of the entomopathogen, a number of diseased *H. armigera* larvae with dense

coating of dark green fungal growth were collected from cotton fields at the College of Agriculture, Nagpur in August 2000.

Isolation was done by the standard method on potato dextrose agar. Out of 19 samples only one showed the mycelial growth, which was later, identified as *M. anisopliae*. Identification was done on the basis of external symptoms, morphology of spores and sporulating structures (Radha *et al.*, 1955; Gopalakrishnan and Narayanan, 1988).

Pathogenicity

The sample identified as *M. anisopliae* was used for proving Koch's postulate on IIIrd instar larvae of *H. armigera*. A set of 10 larvae was sprayed with 2 ml of fungal suspension of 10^7 spores/ml grade with sticker (Hamam toilet soap) using hand atomizer. The characteristic green fungal growth was reisolated, purified, identified and was used for further study. (Table 1).

Cultural media

Sporulation of *M. anisopliae* was subjected to assessment in five liquid cultural media, *viz.*, Sabouraud's Dextrose Broth + Yeast medium (SDB+Y), Emerson's Yeast Phosphate Soluble Starch (YPSS), Potato Dextrose Broth (PDB), Czpeck's and Barner's medium at four temperature regime, *viz.*, 20, 25, 27 and 30°C. One ml pure fungal suspension from 7-day-old culture (10^8 spores/ml) was inoculated in 100 ml of each autoclaved medium in 250 ml conical flasks in four replications. Cultures incubated for 10 days in BOD incubator at predecided temperature levels were subjected to spore count studies with haemocytometer (Table 2).

Mass Multiplication

Emerson's Yeast Phosphate Soluble Starch (YPSS), Potato Dextrose Broth (PDB), Rice Hull (RH) + Sawdust (SD) + Rice Bran (RB) (75: 25:100) and Rice Husk supplemented with 2 per cent dextrose were inoculated with *M. anisopliae* pure colony suspension (10^8 spores/ml) for the biomass and spore count study after 15 days (Table 3).

Formulation

Fifteen days after inoculation, the spent medium with fungal biomass of *M. anisopliae* was blended in electric mixer for 1-2 minutes to homogenize the slurry. It was filtered through muslin cloth to remove debris under aseptic condition with spore count maintained at 10^7 spores / ml. The slurry of known microbial strength along with sticker Hamam toilet soap @ 0.23 g / litre (Puzari *et al.*, 1991) was packed in already sterilized polypropylene bags with proper label and instructions. Bags were stored in the refrigerator and were subjected to the counting of colony forming units after 45 days.

***H. armigera* culture**

Rearing of *H. armigera* culture was done at the Insectary unit, Department of Entomology, College of Agriculture, Nagpur under standard conditions (25±2°C temperature, 75 per cent relative humidity, 12:12h light and dark period). Stage wise larval culture was procured as per the experimental requirement.

Bioassay

For ascertaining efficacy of *M. anisopliae*, 2-ml fungal suspension was sprayed on a set of 10 larvae in a Petri-plate with the hand atomizer and replicated thrice. Ist- IVth instars of *H. armigera* larvae were surface sterilized with sodium hypochloride solution (1%) followed by three changes of sterilized distilled water. Five concentrations, *viz.*, 10^4, 10^5, 10^6, 10^7 and 10^8 spores/ml of fungal suspension were used to obtain the Probit kill. *H. armigera* larvae were maintained at ambient condition individually in 12 well plastic trays with soaked Kabuli gram seeds as diet. Corresponding control without spray of fungal suspension was also maintained to record the natural mortality. Cumulative larval mortality was recorded from fourth day after the application of treatment up to eighth day (Table 4).

Statistical analysis

Completely randomized design (CRD) was used to analyze the effect of temperature and media on sporulation of *M. anisopliae* for the test of

significance. Data on test of pathogenicity were analysed under CRD with per cent larval mortality subjected to arcsine (X+1) transformation.

Log dose Probit assay

Bioassay data from 3-replications of five concentrations were pooled and subjected to Probit analysis using software POLO-PC for computing dose mortality regression. The control mortality was corrected as per Abbot's formula (Abbott, 1925).

RESULTS AND DISCUSSION

Studies on pathogenicity of *M. anisopliae* against Ist- IVth instar *H. armigera* larvae revealed larval mortality to the extent of 100.00, 90.00, 76.67 and 56.67 per cent, respectively, on 8th day with significant differences between them when sprayed with fungal suspension of 10^8 spores/ml (Table 1). Cent per cent larval mortality up to IVth instar of *H. armigera* with 1.8 x 10^9 spores/ ml suspension was reported by Gopalakrishnan and Narayanan (1988).

Amongst the various media tested, YPSS media proved to be better at all the temperatures recording highest spore count of 22 x 10^7 spores/ml at 27°C followed by 17.12 x 10^7spores/ml at 25°C (Table 2). The findings of Barnes *et al.* (1975) that yeast containing media are the best in terms of growth and sporulation of *M. anisopliae* are in corroboration with the present finding. SDB and PDB medium also registered high spore count of 18.00 x 10^7 and 17.25 x 10^7 spores/ml, respectively at 27°C, being on par with the best medium, YPSS. The high sporulation responses of the above medium has similarity with the observation of Mietkiewski *et al.* (1994) and Sharma *et al.* (1998) reporting 27°C and 28°C to be the best temperature for optimum germination, growth and sporulation of *M. anisopliae*. Czapek's and Barner's medium recorded significantly poor culture growth at all the temperatures tested when compared to the other media.

Mass multiplication studies revealed superiority of Emerson's YPSS medium in terms of highest spore count of 1.71 x 10^7 spores and bio mass production of 480 mg/ 100 ml followed by PDB with 1.03 x 10^7 spores/ml spore count and 450 mg/ 100 ml bio mass production (Table 3). Khan *et al.* (1993) reported highest conidial count of 4.5 x 10^7 spores/ml on Emerson's YPSS medium. RH: SD: RB takes a long time of 24 days for spore production (Puzari *et al.*, 1997) and rice husk supplemented with 2 per cent dextrose recorded poor sporulation reflecting its unsuitability for the mass production of *M. anisopliae*. PDB was selected for mass multiplication of *M. anisopliae*, being more

Table 1. Pathogenicity test for *M. anisopliae* at (10^8 spores/ml) on various instars of *H. armigera* larvae

Instar larvae	No. of treated	Per cent larval mortality at				
		4th Day	5th Day	6th Day	7th Day	8th Day
Ist	30	36.67 (37.88)	70.00 (57.42)	83.33 (66.66)	96.67 (81.24)	100.00(90.00)
2nd	30	33.33 (35.85)	66.67 (55.37)	80.80 (64.16)	83.33 (66.66)	90.00(72.54)
3rd	30	33.33 (35.85)	53.33 (47.47)	66,67 (55.37)	76.67 (61.82)	76.67(61.82)
4th	30	26.67 (31.76)	43.33 (41.73)	56.67 (49.43)	56.67 (49.43)	56.67(49.43)
Control	30	0.00 (5.47)	0.00 (5.47)	0.00 (5.47)	0.00 (5.47)	0.00(5.47)
SEM±		2.59	3.00	4.31	4.92	4.87
CD (P=0.05)		5.76	6.69	9.62	9.20	4.97

* Figures in parentheses are arcsine (X+1) transformations.

Table 2. Effect of media and temperature on sporulation of *M. anisopliae*

Media	x10^{7}spores/ml			
	20°C	25°C	27°C	30°C
Potato Dextrose Broth (PDB)	6.37	10.25	17.25	7.00
Sabouraud's Dextrose Broth +Yeast (SDB+Y)	6.87	11.87	18.00	7.80
Yeast Phosphate Soluble Starch Broth (YPSS)	8.00	17.12	22.00	10.50
Czapek's medium	2.50	4.12	6.50	3.00
Barner's medium	1.50	3.00	5.50	1.75
SEM ±	1.48	1.31	2.28	2.62
CD (P=0.05)	3.79	3.49	4.87	5.75

Table 3. Mass multiplication of *M. anisopliae* on different media

Medium	Mycelial dry wt. (mg/100ml)	Spore count ml^{-1}
Yeast Phosphate Soluble Starch (YPSS)	480	1.71x10^7
Potato Dextrose Broth (PDB)	450	1.03x10^7
Rice hull + Saw dust + Rice bran (RH+SD+RB)	-	1.32X10^7
Rice husk supplemented with 2% dextrose	-	7.90X10^5

Table 4. Log dose Probit response of *H. armigera* larval instars to fungal suspension of *M. anisopliae*

Instar	No.	LC_{50}	95% FL	LC_{90}	95% FL	Slope ± SE	Het.	X^2
Ist	150	6.0 x 10^4	1x10^4-1.8 x 10^5	6.4 x 10^6	1.9x10^6-4.9x10^7	0.64± 0.12	0.54	1.60
IInd	150	1.2 x 10^6	3.1x10^5-3.7 x 10^6	2.8 x 10^8	5.4x10^7-6.1x10^9	0.54 ± 0.1	0.38	1.12
IIIrd	150	4.8 x 10^6	1.5 x 10^6-2.2x 10^7	3.8 x 10^9	3.7x10^8-4.7x10^{11}	0.44± 0.08	0.27	0.80
IVth	150	5.0 x 10^7	1.2 x 10^7-7.6x 10^8	6.1x 10^{10}	2.5x10^9-1.1x10^{14}	0.42± 0.69	0.03	0.10

FL = Fiducial limit

Het = Heterogeneity

economical in comparison to the Emerson's YPSS medium (Zade, 2000).

PDB along with fungal growth was mixed with sticker (Hamam toilet soap @ 0.23 g/litre) and was packed in already sterilized polypropylene bags with proper labeling and instructions and stored at 8°C in refrigerator (Walstad *et al.*, 1970). Colony forming units (CFU) recorded after 45 days were to the extent of 3.4 x 10^6 spores/ml.

M. anisopliae exhibited promising toxicity levels against Ist–IVth instar larvae of *H. armigera*, reflecting LC_{50} values as 6.0×10^4, 1.2×10^6, 4.8×10^6 and 5.0×10^7 spores/ml, respectively later instars being more susceptible (Table 4). The slopes indicating heterogeneity of *H. armigera* strain used.

Kencharaddi and Jayaramaiah (1997) reported LC_{50} value of 6.07×10^4 (1st), 6.15×10^5 (3rd) and 1.1×10^8 spores/ml against 5th instar, which strengthen the reports of Gopalakrishnan and Narayanan (1988) and they are in line with the present finding.

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