ORIGINAL RESEARCH ARTICLE

INTERNATIONAL JOURNAL OF BIOASSAYS
ISSN: 2278-778X
CODEN: IJBNHY
OPEN ACCESS

DEVELOPMENT AND TESTING OF MYCOPESTICIDE FORMULATIONS OF *METARHIZIUM ANISOPLIAE* (METSCHNIKOFF) FOR SHELF LIFE AND FIELD APPLICATION AGAINST *SPODOPTERA LITURA* (FEB) LARVAE

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Received for publication: June 11, 2015; **Revised**: June 28, 2015; **Accepted**: August 5, 2015

Abstract: Conidiospores of the entomopathogenic fungus *Metarhizium anisopliae* were produced by solid state fermentation using sorghum/barley/rice grains. Shelf life and viability of oil formulatations as well as unformulated infective propagules was tested by germination bioassay and pathogenicity studies on *Spodoptera litura* larvae. Viability of unformulated conidiospores stored at three temperatures (4°C, -30°C and room temperature) was understood using germination bioassay. Studies on seven oil formulations and two powdered formulations for shelf life at 4°C, revealed 60% viability of conidiospores formulation with gingelly oil, stored for six months. Virulence of the infective propagules studied against third instar *Spodoptera litura* larvae during six months storage demonstrated insect mortality ranging from 40 to 88.33% with LT50 values of 4.63 to 13.07 days. Field application of infective propagules on *Vigna sinensis* crop heavily infested with *S. litura* suggested insect mortality ranging from 51.35% to 88.65% at $1X10^{12}$ conidia/ ml of gingelly oil formulation.

Key words: *Metarhizium anisopliae, Spodoptera litura,* shelf life, viability, field applications, formulations.

INTRODUCTION

Use of entomopathogenic fungi for biological control of insect pests is assuming increasing attention in recent times. It is believed that either fungal cells themselves or cell-free components would be equally effective. The first attempt to control a pest with a fungal agent was carried out in Russia in 1888, when the fungus now known as *Metarhizium anisopliae* (Metchn.) Sorokın was mass produced on beer mash and sprayed in the field for control of the beet weevil *Cleonus punctiventris* (Germar) (Lord, 2005). Deshpande (1999) studied the production of mycopesticides by Solid State Fermentation (SSF) and Submerged Fermentation (SMF), while Soccol *et al.,* (1997) developed a SSF based process and investigated several agro-industrial substrates to produce spores from *Beauveria bassiana* for use in the biological control of pests of banana, sugarcane, soybean and coffee. Desgranges *et al.,* (1993) produced *B. bassiana* spores by SSF for use against European corn borer. Statistical optimization of process variables has been reported for large scale production of *M. anisopliae* spores by SSF (Bhanu Prakash *et al.,* 2008). The development and optimization of a simple production procedure will not only be valuable for the supply of reliable material for laboratory and field efficacy trails, but will also help to ensure the compatibility of the product with the formulation and application system.

Formulation technology is widely viewed as having great potential to improve the efficacy of microbial biocontrol agents (Burges 1998). The broad range of contemporary mycoinsecticide formulations, based on aqueous or oil carriers and incorporating surfactants, emulsifiers, and other adjuvants, have the potential to influence secondary dose acquisition. A number of laboratory and field studies indicate that oil formulation improves the efficacy of fungal pathogens (Inglis *et al.,* 2002).

Oils and wetting agents have been extensively investigated and adopted as means of enhancing the delivery, persistence, and efficacy of mycoinsecticides. Oil based formulations of *Beauveria bassiana* (Balsamo) Vuillemin were introduced by Prior *et al.,*(1988), who reported efficacy of coconut oil for *B. bassiana* conidia rather than 0.01% aqueous Tween 80 for the weevil *Pantorhytes plutus* (Oberthur). Oil-based formulations of mycopesticides have been tested against various insect pests with positive results (Bateman *et al.,* 1993; Filho *et al.,* 1995; Inglis *et al.,* 1996; Hidalgo *et al.,* 1998; Legaspi *et al.,* 2000; Malsam *et al.,* 2002). Because of the lipophilic nature of phialoconidia that do not bear a mucus coating, they can be easily suspended in oils to achieve greater efficacies than when used in water (Bateman *et al.,*1993; David-Henriet *et al.,*1998). Cotton seed oil, soybean oil, and mineral oils reportedly do not adversely affect the viability of *B. bassiana* (Smart and Wright 1992, Grimm, 2001). Objectives of the present study were to test the unformulated and formulated conidiospores of *M. anisopliae* conidiospores during six months storage and to assess their viability as well as efficacy against *S. litura* in laboratory and field conditions.

MATERIALS AND METHODS

Preparation of infective propagules of M. anisopliae

Metarhizium anisopliae isolate M25 obtained from *Spodoptera litura* cadavers collected from the agricultural fields of Andhra pradesh, India was used for mass multiplication and formulation. Aerial conidia were produced using a standard two-phase production system (Jenkins *et al.,* 1998; Bhanu Prakash *et al.,* 2008), which consists of a submerged liquid culture followed by conidiation on a solid substrate. The submerged liquid culture comprise of simple liquid medium containing Dextrose-2g and peptone- 0.5g and 100 ml of distilled water to generate hyphal bodies and mycelium which forms inoculum for the second phase. Autoclaved grains of Barley/Sorghum/ Rice in polypropylene bags were used as solid substrates for the second phase. The bags were incubated for 15 days at 25±1ºC for fungal development and subsequent sporulation. The substrate with conidia was transferred to plastic trays and kept for drying in the cabinet at 30±1ºC. After two days when the moisture content was around 20%, conidia were separated by sieving through laboratory sieves of 1 mm mesh and further dried in an

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auto-desiccator cabinet for four days to reduce the moisture content to 5%, because suitable optimal moisture content for long term dried conidia storage was found to be 4-5% (Moore *et al.,* 1996).

Conidial shelf life and viability at different temperatures

The unformulated conidia were stored at different temperatures for a period of 6 months (4°C, -30°C and room temperature (29±1°C)). Conidial germination was tested at constant control temperature of 25±1°C. The germination assay was done with freshly isolated spores as control. An aqueous conidial suspension (100 µl containing 2.5 X 10^2 conidia/ ml) was spread on SDAY medium and the inoculated Petri dishes in triplicate were incubated at 25±1°C in an environmental chamber. Plates were observed at 24 hourly intervals to test for germination. The count of germinated conidia was taken from 24h-96h after inoculation. Viability of conidia was tested at one month intervals for a period of 6month.

Testing viability by laboratory bioassay against larvae of S. litura.

Larvae of *Spodoptera litura* were obtained from the infested cauliflower fields of Visakhapatnam, Andhra Pradesh, India. A laboratory colony of *S. litura* originated from insect eggs was maintained at 25°C (8/16 h photoperiod) in plastic tubs. Nymphs and adults were reared on castor leaves.

Conidiospores harvested after drying at 30°C for 24 to 48h and stored at 4°C, -30°C and room temperatures for 15days, 2 months and 6months were used for pathogenicity studies on 3rd instar *S. litura* larvae. Conidial suspension in 0.02% Tween-80 (Sigma–Aldrich, India) was placed in a 50 ml glass vial with approximately 50 glass beads (3 mm diam.) and agitated for 5 min on a vortex mixture. Propagule concentrations were estimated using a hemocytometer and adjusted to 10^8 propagules ml^{-1}. Conidial viability was determined by plating preparations onto SDAY medium and examining colonies/ plate for each of the three replicates. An aliquot of the suspension was taken to check the viability of conidia as described by Varela and Morales (1996). The remaining suspension was stored in a refrigerator (4°C) for the treatments the next day. In a few samples, the viability was also tested after storing in the refrigerator. No significant variation in viability was observed in the aqueous conidial suspension tested before and after storing at 4°C.

Third instar *S. litura* larvae were chosen at random for each treatment batch, to test virulence of the fungal propagules stored at different temperatures. The larvae obtained from a single egg patch were used for each experiment. Larvae were reselected on the basis of size of the insect for homogeneity of the sample. Three replicates were maintained throughout the experiment. The insects were treated singly with 100µl of inoculum, dispensing with a micropipette (Gilson®) on the surface of the insect all over its body and head as per the method 0f Butt and Goettel (2000). *S. litura* larvae were placed in perforated plastic boxes (15 cm). Fresh castor leaves were provided as feed every day to the larvae, and the boxes were cleaned of insect litter daily. The insects were treated for 48h and the fatally injured larvae during inoculation were removed during treatment period. Mortality was recorded at 24h intervals till pupation. Controls were treated with an equal volume of 0.02% Tween 80® in sterile distilled water. Dead insects were immediately surface sterilized with 1% sodium hypochlorite followed by three rinses with sterile distilled water transferring to moist chambers (autoclaved Petri dishes with a moist filter paper lining) to facilitate mycosis. Pathogenicity experiments of *S. litura* against *M. anisopliae* were repeated twice. The pupae obtained from the remaining infected insects were kept in sterile moist sand for post pupation monitoring. The cumulative insect mortality in each treatment was corrected for control mortality (Abbott 1925). Median lethal time (LT_{50}) was calculated from the cumulative mortality data on each day post treatment, using probit analysis.

Formulations

Seven oil formulations and two powdered formulations were prepared with *M. anisopliae* conidiospores. One gram of Conidia from 2-week-old cultures grown on complete medium (SDAY) were collected aseptically and suspended in 2 ml of autoclaved and adequately cooled Peanut oil/ Gingelly oil/ Palm oil/ castor oil/ Sunflower oil/ Kerosene/ or Turpentine. For powder formulations, 1gm of dry conidial powder was mixed with 2gms of chalk powder/ talk powder in plastic vials. All the 7 formulations were stored at 4°C in refrigerator for 6 months.

Laboratory bioassay of formulations against S. litura larvae.

Laboratory evaluations of formulated *M. anisopliae* spores seven oil formulations and two powdered formulations were evaluated on 3rd instar *S. litura* larvae at 1st, 3rd and 6 months on gingelly oil, sunflower oil and peanut oil.

Field application of unformulated and formulated conidiospores against S. litura larvae.

Aerial conidia of an *S. litura* derived isolate of *Metarhizium anisopliae*, were mass multiplied on rice grains and harvested using a 1mm sieve (Bhanu prakash *et al.,* 2008). The harvested conidia were dried to a water content of 5% at ambient temperature and suspended in a 0.02% of liquid detergent solution.

The formulated spores of *M. anisopliae* showing 86% of germination after storage for three months at 4°C in gingelly oil were used for field evaluations. The emulsifiable formulation (EF) was prepared by mixing the oil formulation with 0.02% liquid detergent so as to get $1x10^{12}$ conidia/ml.

Vigna sinensis crop was raised in field plots of 2 sq. m. by maintaining 2 feet space within rows and 2 feet between rows. *M. anisopliae* conidiospores were used for spray application in the evenings between 16:00 h and 18:30 h. Field trials were conducted on bean crop (*Vigna sinensis*) infested with *Spodoptera litura*. All the larval stages of the pest were present on the crop at the time of application. Pest

load was noted before application of spores from randomly selected areas in the field, which were covered and protected using nylon net to avoid escape of pest from the plants. Control plots were sprayed with liquid detergent mixed water. First trial of mycopesticide application was conducted during winters of two successive years.

RESULTS

Shelf life of infective propagules

Shelf life of the unformulated spores stored at 4°C, -30°C and room temperature for six months revealed stability at -30°C after six months storage. Spores collected from sorghum and barley substrates revealed better viability after six months at -30°C (80%) in terms of germination (Fig. 1). Samples stored at 4°C and 30°C showed better viability than those stored at room temperature. Conidia stored at room temperature displayed gradual decrease in germination starting from 85% in the first month to 38% at the six month. Conidia harvested from rice substrate appeared to be drastically effected during storage based on the drop in germination percent from 75% to 28% during six months storage period.

Viability testing by laboratory bioassay against 3rd instar S. litura larvae

Effect of storage temperature and time on the virulence of *M. anisopliae* towards *S. litura* indicated decrease in insect mortality with increase in storage time (15day's to 6months). Pathogenicity studies of the mass multiplied conidia of *M. anisopliae* stored at the three temperatures on the third instar larvae of *Spodoptera litura* at different time intervals revealed drastic decrease in time of kill and LT_{50} values with respect to spores stored at room temperature compared to the corresponding values of 15th day sample.

Lt_{50} values of fresh spores on 15th day of harvesting ranged between 4.63 to 5.14 days (Table 1). Low LT_{50} values were recorded for propagules produced on rice (4.95). Lt_{50} values of the spores stored for 6 months at different temperatures was more compared to the value of 15th day sample. High LT_{50} values were recorded for propagules obtained from rice substrate and stored at -30°C (6.25) and on SDAY at 4°C (6.39). While the corresponding values for spores produced on barley was 5.39 and 5.06 for 4°C and -30°C storage respectively. At room temperature spores produced on sorghum showed lower LT_{50} values than spores produced on barley. LT_{50} values of propagules stored for six months at the same temperature revealed low LT_{50} values for barley followed by sorghum at 4°C and -30C°. At room temperature sorghum showed low LT_{50} value of 9.80 days and high LT_{50} value of 13.07 days on rice substrate (Table 1).

Table 1: Laboratory bioassay data of mass multiplied dry spores of the entomopathogenic fungus *Metarhizium anisopliae* post 15th day, 2nd month and 6th month against 3rd instar *Spodoptera litura* larvae.

Time	Temperature	Substrate	%Mortality	%Mycosis	LT_{50} (days)	Fiducial limits	*RVI*
15 days	Room Temperature	SDAY	86.67	57.69	5.14	4.76 – 5.54	0.181
		Barley	88.33	54.72	4.63	4.69 – 5.42	-1.378
		Sorghum	86.67	59.62	5.08	4.72 – 5.45	1.017
		Rice	86.67	57.69	4.95	4.58 – 5.31	0.181
2 Months	4°C	SDAY	71.67	44.19	6.91	6.35 – 7.57	0.730
		Barley	83.33	40.00	5.39	4.96 – 5.84	-1.202
		Sorghum	78.33	40.43	5.62	5.13 – 6.15	-0.440
		Rice	70.00	42.86	6.39	5.88 – 6.97	0.911
	-30°C	SDAY	81.67	46.94	5.51	5.06 – 5.99	-0106
		Barley	88.33	45.28	5.06	4.24 – 5.01	-1.207
		Sorghum	80.00	43.75	5.20	4.75 – 5.65	0.077
		Rice	73.33	47.73	6.25	5.73 – 6.84	1.236
6 Months	Room Temperature	SDAY	70.00	40.48	7.43	6.79 – 8.24	0.320
		Barley	73.33	38.64	7.09	6.49 – 7.82	0.664
		Sorghum	71.67	39.53	6.80	6.28 – 7.42	0.500
		Rice	58.33	34.29	9.16	8.26 – 10.47	-1.485
	At 4°C	SDAY	55.00	30.30	9.52	8.55 – 10.94	-0.557
		Barley	73.33	36.36	6.84	6.29 – 7.48	1.435
		Sorghum	58.33	34.29	8.71	7.91 – 9.82	-0.090
		Rice	53.33	28.13	9.27	8.40 – 10.51	-0.789
	At -30°C	SDAY	71.67	34.88	6.65	6.11 – 7.27	-0.050
		Barley	76.67	39.13	5.49	5.03 – 5.97	1.052
		Sorghum	73.33	36.36	6.20	5.70 – 6.77	0.330
		Rice	75.00	28.89	5.97	5.50 – 6.48	-1.334
	Room Temperature	SDAY	46.67	28.57	10.89	9.59 – 13.02	-0.156
		Barley	50.00	33.33	9.86	8.92 – 11.26	0.699
		Sorghum	51.67	32.26	9.80	8.78 – 11.34	0.810
		Rice	40.00	25.00	13.07	11.17 – 6.63	-1.353

Viability testing by germination assay for formulated spores of M. anisopliae

Viability of conidia (expressed as percent germination after 24h) in the formulations displayed reduction over a period of six months (Fig. 2). Gingelly oil and sunflower oil formulations showed better viability compared to other formulations. Analysis of variance indicated significant effect of isolate ($P<0.001$) and a significant interaction between time and formulation (<0.01). The overall decline of viable conidia was 13.33% per month (calculated from the mean slope of regression). Differences between the oil as well as powder formulations were moderately less, though significant in some cases. Difference in the mean values among the storage schedules

is greater than would be expected by chance after allowing for effects of differences in oil/powder and the values are statistically significant ($P \leq 0.05$).

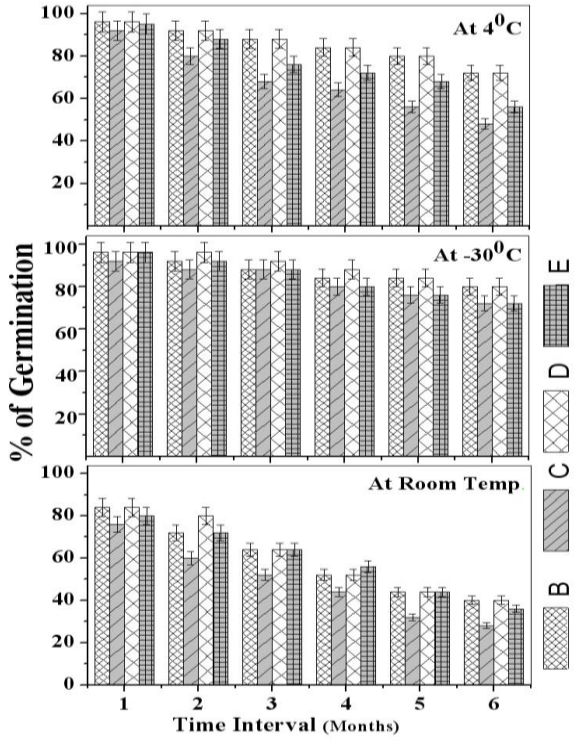

Fig. 1: Germination percentage of *Metarhizium anisopliae* dry spores over a period of six months storage at 4°C, -30°C and room temperature.

Viability testing of three of the oil formulations by bioassay against 3rd instar Spodoptera litura larvae.

Gingelly, sunflower and peanut oil formulations were tested at 1st, 3rd and 6th months of storage for viability by bioassay against 3rd instar larvae of *Spodoptera litura*. Gingelly oil formulation displayed maximum mortality of 86.33% of *S. liturai* after one month storage and 73.33% after six months. The corresponding LT50 values were 4.33 and 6.89 days respectively (Table 2). On the other hand peanut oil formulation displaced least RVI value of -0.924 at 1st month compared to the value of 1.86 for gingelly oil formulation.

Table 2: Laboratory bioassay data of formulated spores of the entomopathogenic fungus *Metarhizium anisopliae* post 1st, 3rd and 6th month against 3rd instar *Spodoptera litura*

Time	Substrate	% Mortality	%Mycosis	LT_{50} (days)	Fiducial limits	*RVI*
1st Month	Gingelly	88.33	84.91	4.33	3.91-4.73	*1.062*
	Sunflower	81.67	81.63	5.12	4.63-5.61	*-0.137*
	Peanut	76.66	76.08	5.21	4.70-5.73	*-0.924*
3rd Month	Gingelly	86.67	78.85	4.76	4.31-5.20	*0.962*
	Sunflower	78.33	78.23	5.34	4.80-5.90	*0.072*
	Peanut	73.33	77.27	6.56	5.97-7.24	*-1.034*
6th Month	Gingelly	73.33	79.55	6.89	6.26-7.65	*0.963*
	Sunflower	71.67	74.41	6.98	6.31-7.81	*0.071*
	Peanut	*70*	*71.42*	*7.36*	*6.74-8.15*	*-1.034*

Table 3: Mortality of *S. litura* on *Vigna sinensis* crop after application of formulated/unformulated spores of *M. anisopliae.*

Instar	unformulated spores		with gingelly oil	
	4th day	8th day	4th day	8th day
2nd	34.65	74.65	45.56	84.1
3rd	21.64	55.86	34.54	64.68
4th	8.65	45.64	22.61	68.3
5th	-	42.36	3.12	55.63

Field application

Field performance of fungal propagules was tested on *Vigna sinensis* crop heavily infested with *S. litura*. Data was taken at four random places in the field. Mortality of the pest was observed between fourth and eighth day after spraying. No mortality was detected in plots treated with distilled water + 0.02% liquid detergent which served as control. The mean percent larval mortality with respect to 2nd, 3rd, 4th and 5th instar larvae of *S. litura* was high in the application of formulation compared to application of unformulated spores (Fig. 2). Though maximum mortality (Fig. 3) of larvae at 2nd instar stage was recorded in the gingelly oil formulation as well as unformulated spore application, the values were superior in the gingelly oil (84.10%) compared to unformulated spores (74.65%).

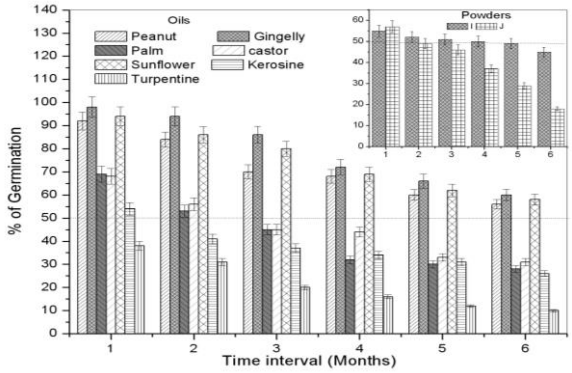

Fig. 2: Effect of different formulating agents on conidial viability (percent control values) of *Metarhizium anisopliae* over a six months period.

Fig. 3: Dead larvae of *Spodoptera litura* showing mycosis prior to sporulation on the eighth day of application using *M. anisopliae* formulation (infected larva of *S. litura* showing mycosis).

DISCUSSION

The experiment was conducted to assess the impact of storage temperatures on *M. anisopliae* mass multiplied spores and its infectivity against *S. litura*. The viability of conidia recoded a decline at room temperature and relative stability at -30°C. On the other hand Consolo *et al.,* (2003) reported no difference in viability of the formulation of hyphomycetous fungi with vegetable oils (corn, sunflower and canola) at different temperatures (4, 17 and 26°C). Goettel *et al.,* (1995) reported uniform germination of conidia of FHD13 isolate formulated in different vegetable oils and kerosene. On the other hand, Hidalgo *et al.,* (1998) observed well maintained conidial viability in the fat pellet formulations and in the dustable powder (DP) but not so good in the oil suspension (OS) formulation. Luz and Batagin (2005) reported the influence of oils on the settling behavior of *Triatoma infestans* nymphs and the activity of an oil–water formulation of the fungus against this vector under laboratory and simulated field conditions. The response of conidial longevity to temperature and humidity is predictable under consistent and fluctuating conditions (Hong *et al.,* 1997 and 1999). Conidia can withstand high temperatures when dry and high humidity when cool, and different isolates can exhibit greatly different responses (Hong *et al.,* 2001). Consequently, isolates could be selected on characteristics that include longevity under warm and humid conditions.

Shelf-life as well as biological and physical properties of the formulation are important issues in commercial formulations. Couch and Ignoffo (1981), suggested that formulations must remain stable for at least 1 year, but preferably for greater than 18 months for commercialization to occur. We found viability of around 40% of the conidia of *M. anisopliae* in the formulation after 6 months at room temperature. This decline in conidial viability of the fungus is acceptable in practical use or in the development of inert emulsions for crop protection purposes. Prior *et al.,* (1988) suggested that oil-based formulations of *B. bassiana* have the advantage of allowing better adhesion of conidia to the hydrophobic cuticle of insects and reported better conidial survival in coconut oil than in water. Daoust *et al.,* (1983) found that oils of different types, in a concentrated or pure form, have detrimental effect on *M. anisopliae* conidial viability during storage at certain temperatures, and relative humidity. Batta (2003) reported *M. anisopliae* formulation with coconut and soybean oils used in preparation of selected inert emulsion towards nymphs of the tobacco whitefly, *Bemisia tabaci* and the changes which occurs in the physical properties of these oils during rapid mechanical homogenization of emulsion ingredients.

In the field trials, gingelly oil formulation of *M. anisopliae* applied at 10^{12} spores/ml showed effective control of 84.10% to 55.63% against the larval stages of *S. litura* completed to that of unformulated sample. Kaaya and Hassan (2000) found that mortality of unfed nymphal *R. appendiculatus* was greater when treated with oil-based than with aqueous formulations. Hornbostel *et al.,* (2005) reported that in a field study, 10^{9} spores/ml *M. anisopliae* did not effectively control questing *I. scapularis* nymphs, and significant differences were not detected in pre- and post-treatment densities. The enhanced efficacy of formulation is generally attributed to the fact that oils are excellent stickers, promoting contact between the formulated active ingredient and the lipophilic insect cuticle and increasing rain-fastness on the waxy leaf cuticle of treated host plants. The good pest control achieved in the field trial is a positive indication for inclusion of this fungus in the integrated pest management programmes.

The simple technology involved in the mass production of this easily cultured fungus makes it feasible for adoption by entrepreneurs interested in cottage industry to the supply of formulation at a lower price as compared to the chemical pesticides. Further the method of broadcasting the fungus along with the culture substrate is also feasible for promoting its further multiplication in the field, generating the high spore inoculum required for controlling the major leaf eating lepidopteran pests of economically important crops. Environmental safety and ecosystem stability considerations lead to the conclusion that the use of native isolates in a microbial control program is more convenient (Lockwood 1993). The practice of developing indigenous fungal strain is preferable for development of mycopesticide formulation for application against local pests in view of better ability to adapt to the local conditions.

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CITE THIS ARTICLE AS:
GVS Bhanu Prakash, UV Ravi Sankar and V Padmaja. Development And Testing Of Mycopesticide Formulations Of *Metarhizium Anisopliae* (Metschnikoff) For Shelf Life And Field Application Against *Spodoptera Litura* (Feb) Larvae. *International Journal of Bioassays* 4.9 (2015): 4284-4289.

Source of support: Nil
Conflict of interest: None Declared