Journal of Visualized Experiments

Development of Metarhizium anisopliae as mycoinsecticide: From isolation to commercialization

--Manuscript Draft--

Manuscript Number:	JoVE55272R4
Full Title:	Development of Metarhizium anisopliae as mycoinsecticide: From isolation to commercialization
Article Type:	Methods Article - Author Produced Video
Keywords:	Metarhizium anisopliae, Mycoinsecticide, Solid State Fermentation, Insect Bioassay
Manuscript Classifications:	10.1.40: Agriculture; 10.1.40.226: Biological Control Agents; 10.1.897.120: Biotechnology; 95.51.26: microbiology
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Abstract:	A major concern in developing commercial mycoinsecticides is the speed of kill relative to chemical insecticides. Therefore, isolation and screening for selection of fast acting, highly virulent, entomopathogenic fungus are important steps. Entomopathogenic fungi such as Metarhizium, Beauveria, Nomurea species which act by contact are better suited than Bacillus thuringiensis or nucleopolyhedrosis virus (NPV) which needs to be ingested by the insect pest. In the present work, we isolated 68 Metarhizium strains using soil dilution and bait method from infected insects. The isolates were identified by amplification and sequencing of ITS1-5.8S-ITS2 and 26S rDNA region. The most virulent strain of Metarhizium anisopliae was selected based on median lethal concentrations (LC50) and time (LT50) obtained in insect bioassays against 3rd instar larvae of Helicoverpa armigera. The mass production of spores of the selected strain was carried out by solid state fermentation using rice as a substrate for 14 days. Spores were extracted from the sporulated biomass using 0.1% Tween 80 and different formulations of the spores were prepared. Field trials of the formulations for the control of H. armigera infestation in pigeon pea were carried out by randomized block design. Infestation control obtained with oil and aqueous formulations (78.0% and 70.9%, respectively) was better than 63.4% obtained with chemical pesticide.
Author Comments:	Present article and video describe methodology for the development of mycoinsecticide starting from isolation of indigenous entomopathogens upto its demonstration in the field. It will be useful for researchers as well as entrepreneurs working in the field of agricultural biotechnology. To explain entire concept, it was necessary to include series of experiments.
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a	

certain date, please indicate the date below and explain in your cover letter.	

Cover Letter

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March 11, 2017

Dr. Alisha DSouza,
Review Editor
Journal of Visualized Experiments

Dear Dr. DSouza,

I am submitting herewith our revised manuscript (JoVE55272R 4) entitled: "Development of *Metarhizium anisopliae* as mycoinsecticide: From isolation to commercialization" for your consideration for publication in JoVE. All the editorial and reviewers' comments are addressed and manuscript is revised accordingly.

With best regards,

Yours sincerely,

M. V. Deshpande

TITLE:
Development of *Metarhizium anisopliae* as a Mycoinsecticide: From Isolation to Field performance

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KEYWORDS:
Insect bioassay, *Metarhizium anisopliae*, mycoinsecticide, solid-state fermentation, isolation, insect

SHORT ABSTRACT:
Here, we report the different stages involved in the knowledge-based development of an effective mycoinsecticide, including the isolation, identification, screening, and selection of the "best-fit" entomopathogenic fungus, *Metarhizium anisopliae*, for the control of insect pests in agriculture.

LONG ABSTRACT:
A major concern when developing commercial mycoinsecticides is the kill speed compared to that of chemical insecticides. Therefore, isolation and screening for the selection of a fast-acting, highly virulent entomopathogenic fungus are important steps. Entomopathogenic fungi, such as *Metarhizium, Beauveria,* and *Nomurea*, which act by contact, are better suited than *Bacillus thuringiensis* or nucleopolyhedrosis virus (NPV), which must be ingested by the insect pest. In the present work, we isolated 68 *Metarhizium* strains from infected insects using a soil dilution and bait method. The isolates were identified by the amplification and sequencing of the ITS1-5.8S-ITS2 and 26S rDNA region. The most virulent strain of *Metarhizium anisopliae* was selected based on the median lethal concentration (LC_{50}) and time (LT_{50}) obtained in insect bioassays against 3rd-instar larvae of *Helicoverpa armigera.* The mass production of spores by the selected strain was carried out with solid-state fermentation (SSF) using rice as a substrate for 14 days. Spores were extracted from the sporulated biomass using 0.1% tween-80, and different formulations of the spores were prepared. Field trials of the formulations for the control of an *H. armigera* infestation in pigeon peas were carried out by randomized block design. The infestation control levels obtained with oil and aqueous formulations (78.0% and 70.9%, respectively) were better than

the 63.4% obtained with chemical pesticide.

INTRODUCTION:

From the introduction of organochlorine pesticides in the 1940s in India, the use of pesticides has increased many fold[1], with crop pests still costing billions of rupees[2] annually in terms of yield loss in agricultural production. The widespread and non-judicious use of synthetic pesticides is a continuous threat to the environment and human health[1]. The indiscriminate use of pesticides leads to residues in the soil and the depletion of natural pest predators. It also serves as a powerful selection pressure for altering the genetic makeup of a pest population, leading to the development of resistance[1]. Despite the enormous benefits of the green revolution, which required high inputs, like fertilizers and pesticides, pests continue to be a major biotic constraint. A general estimate of recorded annual crop losses in India and worldwide are USD 12 billion[2] and USD 2,000 billion[3], respectively.

When chemical pesticides have detrimental effects when used to control insect pests, it becomes imperative to search for alternative methods that are ecologically sound, reliable, economical, and sustainable. Biological control offers a suitable alternative and includes the use of parasites, predators, and microbial pathogens[4]. Fungi, for instance, are known to infect a broad range of insect pests, including lepidopterans, hymenopterans, coleopterans, and dipterans, often resulting in natural epizootics. Furthermore, unlike other bacterial and viral insect control agents, the mode of action of insect pathogenic fungi is by contact[,5]. These fungi comprise a heterogenous group of over 100 genera, with approximately 750 species reported among different insects. The important fungal pathogens are: *Metarhizium* sp., *Beauveria* sp., *Nomuraea rileyi*, *Lecanicillium lecanii*, and *Hirsutella* sp., to name a few[6]. *M. anisopliae* (Metchnikoff) Sorokin is the second most widely used entomopathogenic fungus in biocontrol. It is known to attack over 200 species of insects[7].

In this study, different stages involved in the knowledge-based development of a mycopesticide using *M. anisopliae* are presented. This includes: 1) the identification of a source (*i.e.,* either soil or mycosed insects) for virulent entomopathogens, 2) entomopathogen identification and selection, 3) strategies to maintain their virulent nature and effectiveness in the laboratory bioassay and in the field, 4) the cost-effective formulation of infective propagules, 5) the development of unique quality-control parameters for virulent preparation, and 6) bioprospecting and value addition.

PROTOCOLS:

1. Isolation of Entomopathogenic Fungi

1.1) Soil dilution method

1.1.1) Collect the soil samples and mycosed insects from different crop fields (**Table 1**). Isolate the entomopathogenic fungi from soil samples using the soil dilution plating method[8].

Note: In this study, samples were collected from the Pune (18°31'13''N; 73°51'24''E) and Buldhana (19°58'36''N 76°30'30''E) districts, Maharashtra, India.

1.1.2) Weigh 10 g of each soil sample and add them separately to 90 mL of sterile 0.1% (w/v) Tween-80.

1.1.3) Thoroughly mix the samples using a magnetic stirrer for 60 min to release the spores adhered to soil particles.

1.1.4) After mixing, spread 100- to 200-µL aliquots from each sample onto selective medium containing (g/L): peptone, 10; glucose, 20; agar, 18; streptomycin, 0.6; tetracycline, 0.05; cyclohexamide, 0.05; and dodine, 0.1 mL; pH 7.0[9]. Incubate the plates at 28 °C for 3-7 days.

1.1.5) Select and subculture the individual sporulating colonies on the same medium to obtain pure cultures.

1.2) From mycosed insects

1.2.1) Collect the mycosed insects from the field.

Note: A hard larval body is likely to be infected with entomopathogenic fungus. With bacterial or viral infection, the dead insect body is soft.

1.2.2) Collect live insects with abnormal behavior, poor coordination, and jerky movements.

1.2.3) Keep the insects until death and then transfer them to moist chambers for further mycosis and sporulation, if any, at 28 °C and 70-80% RH.

1.2.4) Streak the spores from the sporulating cadavers on the abovementioned selective medium and obtain pure cultures by further subculturing 2-3 times on the same medium.

1.3) Bait method

1.3.1) In a vial (3.85 x 6.0 cm) containing 60 g of soil sample, add 4 rice moth larvae (*Corcyra cephalonica*) and keep the vials at 25 ± 2 °C for a period of 14 days.

1.3.2) Turn the vials upside down every day. After 14 days, screen the soil samples for the presence of mycosed rice moth larvae. Isolate the entomopathogenic fungus by streaking spores from sporulating cadavers on selective medium.

1.3.3) After obtaining pure cultures, transfer the isolates to potato dextrose agar (PDA) slants and incubate at 28 °C and 70-80% RH for 7 days to allow sporulation. Following sporulation, maintain the mother cultures at 8 °C until use.

2. Identification of Entomopathogenic Fungi

2.1) Identify entomopathogenic fungi by observing morphological characteristics, *(i.e.,* asexual spore size and shape and the arrangement of the spores on conidiophores); isolates of 3 main genera, *Metarhizium, Beauveria*, and *Nomuraea*, can be identified.

2.2) For the molecular identification of *Metarhizium* strains, extract the genomic DNA from the mycelial biomass using a DNA isolation kit; follow the manufacturer's instructions (see the **Table of Materials**). Check the quality of genomic DNA by performing electrophoresis on a 0.8% agarose gel.

2.2.1) PCR-amplify the ITS1-5.8S-ITS2 and 26S rDNA region. Use genomic DNA as a template, with ITS1 forward (TCCGTAGGTGAACCTGCGG) and ITS4 reverse (TCCTCCGCTTATTGATATGC) primers[10].

2.2.2) Gel-elute and purify the expected-size amplicons using a gel extraction kit; follow the manufacturer's instructions (see the **Table of Materials**). Quantify the purified amplicon and sequence.

2.2.3) Read and edit the sequences using the software and perform a BLAST search of the nucleotide sequences in the NCBI GenBank data library to analyze the close homology[11].

2.2.4) Deposit the sequences of identified entamopathogenic isolates to the NCBI GenBank database to retrieve the accession numbers.

3. Screening of *Metarhizium* Isolates Against *H. armigera*

3.1) Insect rearing

3.1.1) Establish the initial culture of *H. armigera* by collecting healthy larvae and pupae of the insect from the field.

3.1.2) For rearing, grow the larvae individually in sterile polypropylene vials (3.85 x 6.0 cm, 50-mL capacity) containing pieces of okra disinfected with 0.5% (v/v) sodium hypochlorite for 10 min[12].

3.1.3) Collect the insect eggs laid during rearing and surface-sterilize them with 0.5% (v/v) sodium hypochlorite.

3.1.4) Maintain the larvae at 25 ± 2 °C and 65 ± 5% RH.

3.2) Insect bioassay

Note: For the insect bioassay, the production of spores, and field performance studies, the first

subcultures of *Metarhizium* strains from mycosed *H. armigera* larvae were used, unless otherwise noted.

3.2.1) For the insect bioassays, use 3rd-instar larvae of *H. armigera*.

3.2.2) Dip a set of 30 larvae in triplicate individually into a 10-mL spore suspension of *Metarhizium* isolates (1 x 10^7 spores/mL, unless otherwise mentioned; viability > 90%) for 5 s.

3.2.3) After treatment, transfer each larva individually to a separate, sterile vial to avoid cannibalism. To each vial, add moist Whatmann filter paper No. 1 and a piece of disinfected okra as feed. Change the paper and feed on alternate days.

3.2.4) Keep the larvae at 25 ± 2 °C, 65 ± 5% RH, and 16:8 light: dark for 14 days or until they die.

3.2.5) Transfer the dead larvae to sterile Petri plates containing moist cotton swabs and keep them at 28 °C and 70-80% RH for 3-7 days to allow mycelia and spore formation over the cadavers.

3.2.6) For a control, treat a set of 30 larvae in triplicate with 0.1% (w/v) Tween-80 in sterile distilled water.

3.2.7) Conduct all experiment in triplicate using freshly prepared spore suspensions. Collect and pool the data on percent mortality from three experiments to get average values. Calculate the corrected percent mortality using Abbott's formula[13].

3.2.8) Perform the experiments using a randomized complete block design (RCBD) layout, with each treatment containing a set of 30 larvae in triplicate. Based on percent mortality against *H. armigera*, select *Metarhizium* isolates for further screening of the best isolate for commercial production.

3.2.9) Select the isolates demonstrating >90% mortality against *H. armigera* 3rd-instar larvae.

Note: Here, 12 isolates were selected.

3.2.10) Determine the LT_{50} of these isolates and select the isolates demonstrating the fastest killing (in less time).

Note: Here, 5 isolates were selected from the 12 most potent isolates.

3.2.11) Determine the LC_{50} values of the selected isolates using four different concentrations (*i.e.,* 1×10^3, 1×10^5, 1×10^7, and 1×10^9 spores/mL) of spore suspension.

3.2.12) Determine the LC_{50} of the *Metarhizium* isolates against 3rd-instar larvae of *H. armigera* to increase the possibility of identifying the difference in virulence of isolates with high mortality

values that might go undetected if only a single dose is used.

4. Production of Spores of a *Metarhizium* Isolate for Field Performance Studies

4.1) Production of *Metarhizium* spores by SSF

4.1.1) For SSF, prepare the inoculum by adding 2 x 10^7 spores of the *Metarhizium* isolates to 200 mL of YPG (0.3% yeast extract, 0.5% peptone, and 1.0%, glucose) medium. Incubate the flasks at 28 °C with shaking (180 rpm) for 48 h.

4.1.2) For the mass production of spores by SSF, use rice as a substrate unless otherwise noted.

4.1.3) For SSF, fill autoclave bags (type/14 with a single microvented filter of 0.5 µm; 2 kg capacity; 64 × 36 cm) with 2 kg of rice. Add 1,000 mL of distilled water to the rice in the bags and soak overnight[14]. Autoclave the bags with the soaked rice at 121 °C for 45 min[15].

4.1.4) Inoculate the bags with 48-h-old mycelial inoculum (10% inoculum, 200 mL for 2 kg of rice) and incubate at 28 °C and 70-80% RH for 14 days.

4.1.5) Harvest the spores by liquid extraction using 0.1% Tween-80. For this, add the contents of the bag to 0.1% Tween-80 (3 L per 1 kg of rice), mix thoroughly, separate the spores from the liquid by centrifugation, and dry at 37 °C for 2 days.

4.1.6) Alternatively, dry the bags containing rice with the spores and some mycelia at 37 °C for 2 days to reduce the moisture content (<20%). Harvest the spores using a myco-harvester or vibro-sifter.

4.2) Viability studies

4.2.1) Determine the percent viability of the harvested spores using different methods[15]. For this, prepare the spore suspensions in 0.1% (w/v) Tween-80 and adjust the count to 1 × 10^3 spores/mL.

4.2.2) Spread the spore suspensions (0.1 mL) onto PDA plates in triplicate and incubate at 28 °C and 70-80 % RH for 72 h.

4.2.3) Manually count the isolated colonies and determine the total viable count for the respective sample.

4.3) Spore sedimentation rate

4.3.1) For a uniform dosage, the homogenous spore suspension is required; determine the spore sedimentation rates for *Metarhizium* isolates as described[16]. Check the sedimentation rates of spores in 0.2 M ammonium sulphate and 0.1% Tween-80.

4.3.2) Adjust the count of the spore suspension to ~7 × 10^7 spores/mL to obtain an initial absorbance of 0.6 at 540 nm. Allow the cuvettes to stand undisturbed for 6 h for the spores to settle.

4.3.3) Record the absorbance for up to 6 h. Express the sedimentation rate in percent and calculate the time required for 50% sedimentation (ST_{50}). Repeat the experiment thrice using freshly prepared spore suspensions.

5. Field Performance Studies of the Ability of the Selected *M. anisopliae* Isolate to Control *H. armigera* in Pigeon Peas

5.1) Wettable powder formulation of *M. anisopliae* M 34412 spores

5.1.1) Prepare the 2.5-5% wettable powder formulation by mixing the spores with talc.

5.1.2) Adjust the final viable count (TVC) to 1 x 10^{12} spores per kg of formulation.

5.2) Field performance studies of *M. anisopliae* M 34412 spores

5.2.1) For field performance studies of the ability of the selected *M. anisopliae* isolate to control *H. armigera* infestation in pigeon peas, use an RCBD with four replications.

Note: Here, performed at Mahatma Phule Krushi Vidyapeeth (MPKV), Rahuri (19.3927° N, 74.6488° E).

5.2.2) Use two different spray formulations, an oil formulation of spores (5 x 10^{12} spores/3 L of diesel: sunflower oil, 7:3) and an aqueous formulation in Tween-80 (0.1%). Spray the oil formulation with an ultra-low volume (ULV) sprayer (70 min; 3 L/ha) the aqueous formulation with a knapsack sprayer (5 x 10^{12} spores, 500 L/ha).

Note: Here, the larval populations were recorded one day before the spray and 3 and 7 days after the application of each spray to 5 randomly selected plants. The total population was transformed to the square root of n + 1 for the statistical analysis.

5.2.2.1) According to agricultural practices for the pigeon pea crop, perform the first spraying between 10 and 15 d after egg laying and 2 more times with a 14-day interval. Perform the spraying between 16:00 and 18:00 h IST. Monitor the wind direction and, if necessary, use cloth curtains to avoid the drift of spores to neighboring plots.

5.2.3) For comparison, spray the chemical insecticides with a hand compression knapsack sprayer.

5.2.4) Determine the persistence of the inoculum in the field by collecting *H. armigera* larvae 0, 3, 5, 7, and 14 days after spraying.

5.2.5) Keep these larvae under observation for a period of 14 days and after death, transfer them to a plastic vial containing moist filter paper. Incubate at 25 ± 2 °C and 70 ± 10% RH to observe mycosis.

5.2.6) Determine the persistence of the inoculum on the larval population based upon the percent mortality data of the larvae collected from the field after spraying.

5.2.7) Evaluate the field studies on the basis of percent efficacy[17], percent pod damage, and percent yield[18].

Note: Here, the data for the parameters, such as temperature, humidity, wind velocity (km/h), sunshine (h), rainfall (mm), rainy days, and evaporation (mm), were recorded during a trial at an agriculture university (Mahatma Phule Krushi Vidyapeeth, Rahuri, 19.3927° N, 74.6488° E).

5.3) Farmers' participatory program

5.3.1) Select the number of farmers for the demonstration trials. Supply the pigeon pea seeds (BSMR – 736) along with fertilizer to the farmers.

Note: In this study, 20 farmers were involved. Village: Deolali Pravara, (19.473° N 74.6° E).

5.3.2) Use the same spray formulations and number of sprays as in step 5.2.

6. Effect on Non-target Organisms

6.1) Observe the effect of mycoinsecticide spray, if any, on the pigeon pea leaves.

6.2) Collect the soil dwelling arthropods and leaf-inhabiting insects 1 day after each treatment in the untreated plots and in the plots treated with *M. anisopliae*.

6.3) Collect the soil-dwelling arthropods with pitfall traps within 24 h after treatment and collect the leaf-inhabiting insects with a sweep net on the morning following the treatment (*i.e.,* about 15-18 h after treatment).

6.4) Keep them individually in cylindrical plastic boxes with diameters of 3.5 cm and heights of 4.0 cm. Check the insects daily for infection and feed them with appropriate food.

6.5) Record the presence of *M. anisopliae,* if at all, and isolate the fungus.

7. Identification of Quality-control Parameters

7.1) Check the spore viability by measuring the spore germination on PDA at 28 °C.

7.2) Measure cuticle degrading enzyme activities, such as chitinase, chitin deacetylase, chitosanase, protease, and lipase, produced in the YPG and chitin media, as described earlier[15].

7.3) Determine the percent mortality of *H. armigera* in a laboratory bioassay[15].

7.4) Use molecular markers, such as a PCR-RFLP pattern of Chitinases (Chit 1, 2, and 4) and protease (Pr1A) genes, as virulence attributes for *M. anisopliae*.

7.4.1) Extract the genomic DNA from the mycelia biomass using a DNA isolation kit[15]. PCR-amplify the Chit1 and Chi2 gene fragments using genomic DNA as a template, with primer pairs Chit1F/Chit1R (CTCTGCAGGCCACTCTCGGT/AGCCATCTGCTTCCTCATAT) and Chit2F/Chit2R (GACAAGCACCCGGAGCGC/GCCTTGCTTGACACATTGGTAA). For Chit 4, use the primer pair Chit4F/Chit4R (ATCCGGCAGCACGGCTAC/CTTGGATC CGTCCCAGTTG).

7.4.2) For the amplification of the Pr1A gene, use the METPR2 and METPR5 primer pair (AGGTAGGCAGCCAGACCGGC/TGCCACTATTGGCCGGCGCG).

7.4.3) Perform the restriction digestion[19] of the Chit 1 gene with *BsaJI*, *BstUI*, and *ScrFI*; of the Chit 2 gene with *AluI*, *HpyCH4IV*, and *HpyCH4V*, and of the Chit4 gene with *BstUI*, *HaeIII*, and *MboI*. For the digestion of the Pr1Agene, use *RsaI*, *DdeI*, and *MspI*[20].

7.4.4) Observe the restriction fragment length polymorphism (RFLP) pattern on 1.5% agarose gel by electrophoresis for each gene for most virulent strains (>90% mortality); this can be used as a virulence marker for the selection of *M. anisopliae*.

REPRESENTATIVE RESULTS:

During the investigations, different strains of *Metarhizium, Beauveria*, and *Nomuraea* were isolated by various isolation methods (data not shown)[6,14] As *Metarhizium* strains were found to be more effective at controlling *H. armigera*, a dreadful pest in pulses[6,14], further isolations were targeted to isolate *Metarhizium* strains from different crop fields and insects (**Table 1**). The total of 68 *Metarhizium* isolates obtained were identified by cultural and morphological characteristics and by ITS 1-5.8S-ITS 4 sequencing. Based on the >90% mortality of *H. armigera* in laboratory bioassays, 12 *Metarhizium* isolates were further tested for spore production, viability, LC_{50}, LT_{50}, and ST_{50}. **Table 2** describes the data for 3 potential isolates, M34311, M34412, and M81123; M34412 was found to be the best performing isolate.

Among the tested substrates, such as rice, sorghum, corn, and wheat, rice supported the maximum sporulation in the case of *Metarhizium* isolates (60-75 g of spores/kg; 4-4.4 x 10^{10} spores/g of spore powder).

During the field trial for the control of *H. armigera* in pigeon peas, 78.0% and 70.9% efficacies were obtained with the *M. anisopliae* M34412 oil and aqueous formulations, respectively. The pod damage in *M. anisopliae*-treated plots was found to be less (8.76%) than in the untreated control plots (23.63%) and the chemically treated plots (10.24%). The average yield (q/ha) in the

untreated control was 7.31 q/ha, which was less than that after *M. anisopliae* M34412 treatment (14.04 q/ha). Treatment with chemical gave a yield of 12.78 q/ha (**Table 3**).

The observations recorded for phytotoxicity symptoms revealed that no treatments showed a phytotoxic effect on the pigeon pea crop after 3 sprays of *M. anisopliae* formulation. Out of 57 collected soil-dwelling arthropods (field crickets and spiders), none were infected. Out of 590 collected canopy-inhabiting arthropods, two individuals of the order Heteroptera (= 0.3% of the collected arthropods) were found to be infected with *M. anisopliae* (**Table 4**). Neither spiders nor Coccinellids succumbed to the fungus.

FIGURE AND TABLE LEGENDS:

Table 1. Origin of *Metarhizium* strains. The 68 *Metarhizium* strains were isolated from different crop fields (58 strains) and mycosed insects from the crop fields (10 strains).

Table 2. Selection of three best-performing *Metarhizium* isolates. The isolates were selected based on production parameters and performance in insect bioassays with *H. armigera* 3rd -instar larvae.

Table 3. Field performance of the *M. anosopliae* (M34412) strain against *H. armigera*. The efficacies of different formulations of *M. anisopiae* were compared with chemical pesticide treatments against *H. armigera* infestation in pigeon peas under field conditions.

Table 4. Effects of *M. anisopliae* treatments on non-target arthropods. The observations were recorded in three different fields in two replicates. No effect was seen on any of the non-target insects collected.

DISCUSSION:

During the 1880s, the first attempt was made to use *Metarhizium* to control the scarab beetle, *Anisoplia austriaca,* and the sugar beet curculio, *Cleonis punctiventris*[21]. In this protocol, one of the prerequisites was to isolate a virulent strain, either from the soil or from infected insects. Indeed, other parameters, such as LC_{50}, LT_{50}, and ST_{50}, significantly contributed to the cost-effectiveness of the product[22,23]. For the optimization of the spore production, a delicate balance between number of spores, viability, and virulence was maintainend[24].

As agriculture is a high-volume—low-cost product, the quality perception, acceptability by end users, and shelf life of spores are the major concerns. Host specificity is advantageous to avoiding non-target effects[14]. The avoidance of repeated subculturing on artificial medium and the occasional passage through the insect host maintained the virulence and effectiveness of the *Metarhizium* spores in the field[22]. The presented approach does have limitations: the preparation is more effective when the economic threshold level is ~2-3 larvae per plant, and the spore germination is at a maximum in the presence of high moisture and relatively low temperatures.

Here, the fungal preparation is effective after contact, while bacterial (Bt) and viral preparations (-HaNPV) are only effective when digested. Regarding quality-control parameters, in addition to the viability of the spores, for the first time, it has been suggested that biochemical and molecular markers based on cuticle-degrading enzyme activities and specific restriction digestion patterns of the same enzyme genes can assure effectiveness in the field. The quality-control parameters suggested are: (a) the spore viability, measured as spore germination (should be >90% on PDA after 16 h at 28 °C and 70-80% RH); (b) the percent mortality of *H. armigera* (should be >90%, with 1×10^7 spores in the laboratory bioassay); (c) the chitinase activity in the chitin medium after 72 h (should be $>3.5 \times 10^{-3}$ U/mL); and (d) the PCR-RFLP pattern of chitinase genes. This manuscript has essentially described the protocols, from the isolation of an entomopathogenic fungus to the generation of efficacy data against the target pest in the field. This is one of the prerequisites to register any biopesticide formulation with the Central Insecticide Board of India and, eventually, for commercialization.

The series of experiments detailed here will be useful for the development of a potential mycoinsecticide. Furthermore, after the extraction of the spores, the waste mycelial biomass can be used for plant growth promotion or for the isolation of chitosan or glucosamine polymers for healthcare applications.

ACKNOWLEDGMENTS:
The authors acknowledge the contribution of collaborators from the Indo-Swiss Collaboration in Biotechnology (ISCB) program of the Department of Biotechnology, New Delhi and the Swiss Agency for Development and Cooperation, Berne, Switzerland. The contributions of project students and staff involved in the development of the mycoinsecticide, including Vandana Ghormade, Pallavi Nahar, Priya Yadav, Shuklangi Kulkarni, Manisha Kapoor, Santosh Chavan, Ravindra Vidhate, Shamala Mane, and Abhijeet Lande, are acknowledged. EKP and SGT thank the University Grants Commission, India and the Council of Scientific and Industrial Research (CSIR), India, respectively, for research fellowships. MVD acknowledges the support from the Council of Industrial and Scientific Research, New Delhi for the Emeritus Scientist Scheme. The authors are grateful to the Department of Biotechnology, New Delhi, India for the financial support under the ISCB and SBIRI programs. We are thankful to reviewers for their inputs.

DISCLOSURES:
The authors have nothing to disclose.

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Table 1. Origin of *Metarhizium* strains. The 68 Metarhizium strains were isolated from different crop fields (58 strains) and mycosed insects from the crop fields (10 strains).

Soil (58 isolates)		
Isolate No.	**Crop**	**No. of isolates**
M1311, M1322, M1333, M2104, M2305, M2416, M2427, M2508, M42014, M45115, M45216, M45317, M79120, M79221, M79322	Tomato	15
M3419, M34210, M34311, M34412, M34513, M171264	Custard apple	6
M81123, M91124, M91225, M91326, M91528, M91427, M91629, M91730, M91831, M91932, M111145	Sugarcane	11
M101133, M101234, M101335, M101436, M101537, M101638, M101739, M101840, M101941, M102042, M102143, M102244	Brinjal	12
M51118, M51219	Okra	2
M131150, M141151, M141252, M151153	Pigeon pea	4
M121146, M121247, M121348, M121449	Chickpea	4
M183365	Cotton	1
M193166	Jawar	1
Insect host (10 isolates)		
M16255, M16356, M16457, M16558, M16659	Pigeon pea-greasy cutworm	5
M16154, M16760	Sugarcane-mealy bug	2
M16861	Sugarcane-white grub	1
M16962	Sugarcane-beetle	1
M161063	Sugarcane-*Pyrilla perpussila*	1

Table 2. Selection of three best *Metarhizium* isolates. The isolates were selected based on production parameters and performance in insect bioassays with *H. armigera* 3rd -instar larvae.

Isolate	Yield (g/kg rice) Mean ± SD	Viability (%) Mean ± SD	ST50 in T80 (h) (Fiducial Limit)	LC50 (x 103 spores/mL) (Fiducial Limit)	LT50 (days) (Fiducial Limit)	Mortality (%)
M34311	60.00±2.64a	92.00±2.64a	2.47 (2.26-2.69)	2 (0.4-10.3)	3.5 (3.2-3.7)	96.67
M34412	67.00±3.46b	97.00±1.73a	2.3 (2.11-2.52)	1.4 (0.1-1.9)	3.3 (3.0-3.6)	96.67
M81123	75.00±3.60c	93.00±1.73a	2.65 (2.43-2.90)	5.7 (1.2-26.7)	3.3 (3.1-3.6)	95.56

Numbers followed by the same letter within the column are not statistically different.

ST_{50}, time required for sedimentation of 50% spores in 0.1% (w/v) Tween 80.

Numbers followed by the same letter within the column are not statistically different. SD, Standard Deviation. T80, Tween 80 (0.1%, w/v).

LC_{50}, the median lethal concentration of spores calculated to cause 50% mortality of *H. armigera* after 14 days.

LT_{50}, the median lethal time of spores calculated to cause 50% mortality of *H. armigera* .

Table 3. Field performace of *M. anosopliae* (M34412) strain against *Helicoverpa armigera* .
The efficacies of different formulations of *M. anisopiae* were compared with chemical pesticide treatments against *H. armigera* infestation in pigeon peas under field conditions.

Field Trial[$]		
Treatment	**% Efficacy***	**Yield (q/ha)**
Aqueous *M. anisopliae* M34412 (5x 10^{12} spores/ha) 500L	70.93 ± 4.19	14.04
Oil formulation (*M. anisopliae*) (5x 10^{12} spores/ha) 3 L	78.02 ± 4.61	15.53
Chemical pesticide/Farmers' practice (2ml/L, 500 L/ha)	63.43 ± 0.85	12.78
Untreated Control	-	7.31
Demonstration trial in (Farmers' participatory programme)[$$]		
Treatment	**% Pod damage**	**Yield (q/ha)**
Aqueous formulation (*M. anisopliae*); Area 4.2 ha	15.9 ±1.26	10.75
Oil formulation (*M. anisopliae*); Area 0.4 ha	17.74	12.5
Farmers' practice; Area 11ha	22.72 ± 3.37	7.55

Irrigated crop

[$]Randomised Block Design

*After Henderson and Tilton (1955)

HaNPV, *H. armigera* nucleopolyhedrovirus

[$$] Number of farmers involved in the demonstration trials were 20. The pigeon pea seeds (BSMR – 736) were supplied along with fertilizers to the farmers. Village: Deolali Pravara, Tal. Rahuri. Dist. A'Nagar (MS) (19.473° N 74.6° E)

Table 4. Effects of *Metarhizium anisopliae* (M34412) treatment on non target arthropods. The observations were recorded in three different fields in two replicates. No effect was seen on any of the non-target insects collected.

Parameter	Field 1	Field 2	Field 3
Plot size (m)	12 x 17	10 x 10	10 x 15
Replicates	2	2	2
# Arthropods from pitfall traps tested	20	22	15
% Infected with *M. anisopliae* (pitfall traps)	ND	ND	ND
# Arthropods from sweep net collection tested	193	171	226
% Infected with *M. anisopliae* (sweep net collection)	ND	ND	0.9

ND, Not detected

Field 1, Agriculture college, Pune; Field 2, NGO 1, Tulapur, Pune; Field 3, NGO 2, Aalandi, Pune.

Table of Materials/Equipment

Click here to download Table of Materials/Equipment JoVE_Materials MVDeshpande et al.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Agar	Hi-Media	RM666	Reagent
Ammonium sulphate	Thomas Baker	11645	Reagent
DNA analyzer	Applied biosystem	ABI prism 3730	Instrument
DNA islation kit	Qiagen	69104	Reagent
Dodine	Sigma	45466	Reagent
Gel extraction kit	Qiagen	28604	Reagent
Glucose	Hi-Media	GRM077	Reagent
Knapsac sparyer	Kaypee	HY-16L (1004)	Instrument
Peptone	Hi-Media	RM006-500G	Reagent
Polypropylene vials	Laxbro	SV-50	Plasticware
Potato dextrose agar (PDA)	Hi-Media	M096-500G	Reagent
Tween-80	SRL	28940	Reagent
Ultra low volume sparyer	Matabi	INSECDISK	Instrument
Unicorn-bags	Unicorn	UP-140024-SMB	Autoclavalbe bag for SSF
Yeast extract	Hi-Media	RM027-500G	Reagent
Chromas 2.1			software

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Answer to the reviewer's comment:

Reviewer #2:
This study focused on isolation, bioassay, mass production and field trials of entomopathogenic fungi from various insects and soils. Although a lot of work was done, the data presented in Tables are of some technical problems. For instance, the authors claimed LC50 estimates of 1.4, 2.0 and 5.7 x 103 conidia for their selected isolates against the third instar larvae of Helicoverpa armigera but disclosed insufficient technical information. With my 30-year experience working on fungal insect pathogens, the LC50 estimates are too low to be true for fungal insect pathogens. Their multi-concentration bioassays were carried out by dipping the tested larvae in the 10^3, 10^5, 10^7 and10^9 spores/ml suspensions, which are technically questionable and should be changed to 10^4, 10^5, 10^6 and10^8 spores/ml for fungal infection through cuticular penetration. In another bioassay, the use of a 10^7 spores/ml suspension resulted in an LT50 of 3.3-3.5 days, which is also too good to be true. I guess that the authors could encounter a problem in quantifying precisely the concentration of original spore suspension. The results from field trials are encouraging but no details are disclosed on how the field trials were designed and performed. Nor were standard deviations or errors associated with pod damage percentage and pod yield, which should be statistically analyzed. The manuscript was written in a way to make it far away from a scientific report for a journal. I am not sure of that the writing format is acceptable for the e-journal.

Your Response:
The data presented in the present m/s is by using first subculture from the mycosed larva of H. armigera (Ref.15). However, as rightly pointed out, after repeated subuculturing on artificial medium the LC50 for M34412 was 3 x 104/ml and LT50 was increased to 5.6 days. In Table 2, the fiducial limits for LC50, LT50 and ST50 have been given (Ref. 8). In the revised m/s it has been indicated on line 158 (3.2.1) that for insect bioassay, production of spores and field performance studies, the first subcultures of Metarhizium strains from mycosed H. armigera larvae were used, unless otherwise mentioned.

Appreciating expertise of the reviewers and their concern (which now duly acknowledged in the acknowledgement), I would like to add more details regarding our data along with some observations:

I have checked the values presented in Table 2 and all the values are correct and are from published work. I am also adding one more reference of a PhD thesis which is available online for your perusal. The counting of the conidia is routinely done using haemocytometer and it has been done by different students to cross check the values. In my opinion, it is unlikely that there is an error in counting.

We started this project with isolation of different entomopathogenic fungi and we collected *Beauveria* and *Nomuraea* strains also. As conidia are comparatively dry, the yield of

conidia is high in solid state fermentation for *Metarhizium* isolates we further tested 68 *Metarhizium* isolates from soil samples from different host and non host crops for *H. armigera* as well as from different insects. From 68, we selected 12 best isolates which were highly virulent in laboratory bioassay (Table below from Kulkarni S.A. (2015)):

Isolate No.	Chitinase (U/ml)	Protease (U/ml)	Lipase (U/ml)	CDA* (U/ml)	Chitosanase (U/ml)	Mortality (%)
Cluster 1						
M91427	3.90	2.45	0.498	2.50	25.16	91.11
M101537	3.40	3.14	0.290	2.35	12.26	91.11
M16760	3.59	2.72	0.290	2.49	27.60	91.11
M183365	6.90	2.38	0.332	1.43	23.24	92.59
M2104	3.23	3.24	0.498	2.37	49.60	93.33
M101133	3.30	2.91	0.290	2.56	12.50	93.33
M101335	3.74	3.05	0.290	2.46	33.93	93.33
M81123	3.76	3.32	0.747	2.48	35.80	95.56
M91629	5.10	3.18	0.581	2.36	26.23	95.56
M171264	5.70	2.10	0.415	0.45	25.64	96.29
M34311	3.53	3.28	0.747	3.21	35.70	96.67
M34412	3.96	3.38	0.996	1.30	32.40	96.67

The table is from: Kulkarni S.A. (2015) Biochemical and molecular studies of chitin deacetylase from *Metarhizium* species. A PhD thesis submitted to Savitribai Phule Pune University, Pune, India. Available on: http://ncl.csircentral.net/1696/1/Kulkarni%20Shuklangi_Ph.D.%20thesis.pdf

From this, based on LC_{50}, LT_{50}, ST_{50}, yield and other parameters we selected 3 best performers. We can give in detail technical information for all the experiments. As the number of references is restricted we have not given all the references of our published work. I am attaching the list of references published on this work for your ready reference.

It has been published that the LC_{50} value of the 40th sub-culture increased as compared to that of the first sub-culture (Nahar et al, 2008). This will affect the field performance of the 40th sub-culture. The LC_{50} can be decreased by passaging *M. anisopliae* through *H. armigera*. Alternatively, the same level of efficacy can be obtained by applying > 20% conidial inoculum under field conditions. However, the selection of either of the above strategies would greatly depend on the cost factor. Similarly it has been suggested that LT_{50} can also be useful to reduce cost as this will not allow larvae to enter in to further in star which could become more resistant. For example, the virulence of *Paecilomyces farinosus* towards the English grain aphid, *Sitobion avenae* (F.) increased after host passage (Hayden et al. 1992). After three selective sub-cultures of *P. farinosus*, LT50 decreased from 11 to 5 days. The changes in the LT50 of the present strain

M. anisopliae after passage through *H. armigera* were in accordance with the earlier reports (Nahar et al, 2008).

The significance of these parameters, especially LC_{50} and LT_{50} was highlighted in the co-development programme by industry partner for cost reduction. In the solid state fermentation, 70 g (4 x 10^{10} conidia/ g) conidia/ kg of rice are produced. For 1 hectre field to control *H. armigera* in pulses, usually 1 x 10^{12} conidia are sprayed 3 times with 15 days interval, which comes down to 120 g conidia in formulation. The production of conidia cannot be increased beyond certain limit as there is a delicate balance between number and virulence. Under the circumstances, it is possible that strain which has lower LC_{50} and LT_{50} can be used in lower number in the field, which in turn be useful to reduce the cost, which is one of the challenges ahead (Kapoor and Deshpande, 2013). At present, we are working in this direction too.

Insect bioassay (LC_{50})

The insect bioassays were carried out using 3rd instar larvae of *H. armigera*. The set of 30 larvae in triplicate were dipped individually in 10 ml conidial suspension of *Metarhizium* isolates for 5 sec. After treatment, each larva was individually transferred to a separate sterile vial containing moist Whatmann filter paper No. 1 and a piece of disinfected okra that was changed on alternate days. The larvae were kept at 25±2°C, 65±5% RH and 16:8 (L: D) for 14 d or until they died. To allow mycelia and conidia formation over the cadavers, the dead larvae were transferred to sterile petri plates containing moist cotton swabs and kept at 28°C and 70-80% RH for at least 3-7 d. A set of 30 larvae in triplicate treated with 0.1% (w/v) Tween 80 in sterile distilled water served as a control. The experiment was conducted in triplicate using freshly prepared conidial suspensions. The data on percent mortality from three experiments were pooled to get average values, which were corrected by Abbott's formula (Abbott, 1925).

The median lethal concentration (LC_{50}) of *Metarhizium* isolates against 3rd instar larvae of *H. armigera* was determined to increase the possibility of identifying the difference in virulence of isolates with high mortality values that might go undetected if only a single dose was used. The LC_{50} values were determined using four concentrations (1×10^3, 1×10^5, 1×10^7 and 1×10^9 conidia/ml) of conidial suspension. The experimental layout was a RCBD with each treatment containing a set of 30 larvae repeated 3 times.

The field trials were performed with Agriculture University and the results were obtained from them officially, which is the requirement for Central Insecticide Board, India registration. The farmers' participatory trial was for demonstration organized by the Agriculture University. Though we participated in the trials the data was authenticated by Director of Research, (DOR) Agriculture University (MPKV, Rahuri). The values of standard deviation for percent pod damage as per report are included in Table-3.

List of publications on this topic:
Research papers

1. Vidhate R., Singh J., Ghormade V., Chavan S.B., Patil A. and **Deshpande M.V.** (2015) Use of Hydrolytic enzymes of *Myrothecium verrucaria* and conidia of *Metarhizium anisopliae*, singly and sequentially to control pest and pathogens in grapes and their compatibility with pesticides used in the field. Biopestic. Int **11**: 48-60.

2. Kapoor M., Pawar P.V., Joseph M., Sen A. and Deshpande M.V. (2013) Evaluation of biocontrol potential of *Metarhizium anisopliae* strains against larvae and adults of *Aedes aegypti* (L.). J. Biol. Control **2**7: 194-203

3. Yadav P and **Deshpande M.V**. (2012) Control of beet armyworm, *Spodoptera litura* (Fabricius) by entomopathogenic fungi, *Nomuraea rileyi* N812, *Beauveria bassiana* B3301 and *Metarhizium anisopliae* M34412. Biopestic. Int. **8**: 107-114

4. Kulkarni S.A., Ghormade V., Kulkarni G., Kapoor M, Chavan S.B., Rajendran A., Patil S.K., Shouche Y., and **Deshpande M.V**. (2008)Comparison of *Metarhizium* isolates for biocontrol of *Helicoverpa armigera* (Lepidoptera: Noctuidae) in chickpea. Biocontrol Sci. Technol. **18**: 809-828. (IF 1.087; 2)

5. Nahar P.B., Kulkarni S.A., Kulye M.S., Chavan S.B.,Kulkarni G., Rajendran A.,Yadav P.D., Shouche Y. and **Deshpande M.V.** (2008) Effect of repeated *in vitro* sub-culturing on the virulence of *Metarhizium anisopliae* against *Helicoverpa armigera* (Lepidoptera: Noctuidae). Biocontrol Sci. Technol.**18**:337-355 (IF 1.087; 5)

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7. Nahar P.B., Kulye M., Yadav P., Hassani M., Tuor U., Keller S. and **Deshpande M.V.** (2003) Comparative evaluation of indigenous fungal isolates, *Metarhizium anisopliae* M34412, *Beauveria bassiana* B3301 and *Nomuraea rileyi* N812 for the control of *Helicoverpa armigera* (Hüb.) on chickpea. Indian J. Mycol. Plant Pathol. **33**: 372-377

8. Nahar P.B., Yadav P., Kulye M., Hadapad A., Hassani M., Tuor U., Keller S. Chandele A., Thomas B. and **Deshpande M.V.** (2004) Evaluation of indigenous fungal isolates, *Metarhizium anisopliae* M34412, *Beauveria bassiana* B3301 and *Nomuraea rileyi* N812 for the control of *Helicoverpa armigera* (Hübner) in pigeon pea field. J. Biol. Control **18**:1-7

Reviews

1. Vidhate R., Ghormade V., Kulkarni S., Mane S., Chavan P. and **Deshpande M.V.** (2013) Mission Mode Collections of Fungi with Special Reference to Entomopathogens and Mycopathogens. Kavaka 41:33-42

2. Kapoor M. and **Deshpande M.V.** (2013) Development of mycoinsecticide for the control of insect pests: Issues and challenges in transfer of technology from laboratory to field. Kavaka 40: 45-56

3. Yadav P and **Deshpande M.V.** (2010) Fungus- fungus and fungus–insect interactions. Biopestic. Int. **6**:21-35

4. **Deshpande M.V**. (1999) Mycopesticide production by fermentation: Potential and challenges. Critical Reviews in Microbiology **25**: 229-243. (IF 1.917; 26)

Chapters

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2. Chavan S, Ghormade V, Nahar P, and **Deshpande MV.** (2006) Entomopathogenic fungi: A valuable tool to fight against insect pests. In: Plant Protection for the New Millennium.Vol.II eds. AV Gadewar, and BP Singh, pp. 227-243 Delhi: Satish Serial Publishing House.

3. **Deshpande M.V.** (2005) Formulations and applications of mycopathogens. In: Microbial biopesticide formulations and application. Tech.Document No. 55. Eds. R.J.Rabindra, S.S.Hussaini and B.Ramanujam. pp.150-158, PDBC, Bangalore.

4. **Deshpande M.V**. (2005) Mycopesticides: Their potential and challenges In : Fungi:Diversity and Biotechnology. Ed. M.K.Rai and S.K.Deshmukh, pp.375-390. Scientific Publishers, Rajasthan.

5. Hassani M., Tuor U., **Deshpande M**., Hadapad A., Nahar P., Bucher T., Kulye M., Yadav P., Enkerli J. and Keller S. (2004) Mass production of entomogenous fungus Metarhizium anisopliae (Deuteromycotina: Hyphomycetes) for biological control of helicoverpa armigera (Lepidoptera: Noctuidae)on pulses. In: Proceeding of the international workshop on entomopathogenic fungi - a valuable alternative to fight against insect pests (September 16-18, 2002). Ed. M.V.Deshpande, pp. 37-49, National Chemical Laboratory, Pune, India.

6. **Deshpande M.V**., Keller S., Hassani M., Chandele A., Nahar P., Hadapad A., Kulye M., Yadav P. Bucher T. and Tuor U. (2004) Comparative evaluation of indigenous fungal isolates , *Metarhizium anisopliae* M34412, *Beauveria bassiana* B3301 and *Nomuraea rileyi* N812 for the control of *Helicoverpa armigera* (Hüb.) on pulses. In: Proceeding of the international workshop on entomopathogenic fungi - a valuable alternative to fight against insect pests (September 16-18, 2002). Ed. M.V.Deshpande, pp. 51-59, National Chemical Laboratory, Pune, India.

7. **Deshpande M.V.,** Chandele A.G., Nahar P. , Hadapad A. , Patil, G., Ghormade V., Keller S. , and Tuor U. (2003) Entomopathogenic fungi: Mycoinsecticides useful against lepidopteran pest in pulses. IOBC/WPRS Bull. **26**: 27-30.

8. **Deshpande M.V.** and Tuor U. (2001) Microbial control of pests: Entomopathogenic fungi as mycoinsecticides. Proceedings of the III Asia Pacific Crop Protection Conference-2001. Pesticides Manufacturers & Formulators Association of India, Mumbai. pp. 56-59.

9. **Deshpande M.V.** (2000) Mycopesticide production: Contribution of fungal morphologies in the biopesticide formulations. Nat. Bot. Soc. 55:11-17 [Peer Reviewed].

10. **Deshpande M.V.** (1999) Mycopesticide production: Potential and challenges. In: Proc. National Seminar on Advances in Plant Pathology, University of Pune, Pune. pp. 21-30.

11. **Deshpande M.V**. (1998) Biopesticide production by fermentation: Scope and limitations. In: Microbial Pesticides & Insect Pest Management (H.D.Rananavare, S.R.Naik and T.K.Dongre, eds) 75-80, BARC, Mumbai and HAL, Pune.

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