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# Metarhizium anisopliae as an Entomopathogenic fungi: optimization of mass production with diverse grain substrates

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**ABSTRACT :** To overcome the harmful impacts of toxic chemical insecticides on the environment, biological control has been developed. Biological control is the use of natural fungi-extracted products to kill insects and pests. Entomopathogenic fungi are a promising alternative for insect pest control in agriculture. Entomopathogenic fungus also colonizes plant root endophytically, thus showing potential as a plant symbiont. The objectives of this work were to isolate and identify Entomopathogenic fungi, *Metarhizium anisopliae*, from agricultural soil based on their characteristics for the development of bio insecticides. Mass production of *Metarhizium anisopliae* was carried out in the laboratory by using different grains as substrates. The spore count and biomass were maximum when *Metarhizium anisopliae* were cultured in rice, which is cost-effective and economically viable. *Metarhizium anisopliae* is typically used as a liquid formulation that contains $2\times10^6$ CFU, which directly infect insects.

**KEY WORDS**: *Metarhizium anisopliae*, Entomopathogenic, Bio insecticides, mass production, grain

## I. INTRODUCTION

The excessive use of chemical products in agricultural crops is a problem, since some insects have developed resistance to them [1]. In order to reduce the use of toxic pesticides, it has become vital to explore for alternate method for the biological management of pests [2]. More than 750 species of Entomopathogenic fungi are widely distributed in the environment, which can control up to 80% of an insect's population by infecting it with fungi [3]. *Metarhizium*, *Beauveria*, *Aschersonia*, *Trichoderma*, *Entomophthora*, *Zoophathora*, *Hirsutella*, *Fusarium*, and *Verticillium* are a few of the most significant genera [4]. An approach for integrated pest management includes biological control. It is characterized as the control of pest populations by natural enemies and typically involves an active human role. Remember that without human intervention, all insect species are also suppressed by naturally occurring organisms and environmental circumstances. Oftentimes, this is referred to as natural control. Although the biological control of insects is the focus of this manual. Predators, parasitoids, and diseases are examples of natural enemies of insect pests, commonly referred to as biological control agents. Biological antagonists are the most popular term used to describe plant disease management agents [5]. Now a days, High concentrations of chemical pesticides are used to control pests and insects that can attack and injure crops. Chemical pesticides benefit crop, but they also have an influence on the ecosystem. The destruction of biodiversity, aquatic species, animals, birds, and natural adversaries of agricultural pests may result from the overuse of pesticides [6], [7], [8], [9].

Agriculture, horticulture, and forestry all use fungi for biological control. It is possible to prevent organisms from harming the plant by including a specialised fungus. The fungal genera *Gliocladium*, *Coniothyrium*, *Penicillium*, *Myrothecium*, *Chaetomium*, and *Laerisaria* are frequently employed for biological control [10].
Due to the negative effects of chemical pesticides, cost-effective and environmentally friendly pesticide management is currently required for sustainable crop production. To solve this problem, we must create bio pesticides, or biological control agents, which are an environmentally friendly substitute for chemical pesticides [11]. Biopesticides provide a number of benefits, including safety, friendliness towards non-target species, targeted activity against the desired pests, cost effectiveness, effectiveness in lower quantities, hence offering lower exposure, and fast decomposition, which largely avoids pollution issues [12].Among the examined microorganisms, Entomopathogenic fungi have a lot of potential as a plant pesticide [13]. The development of Entomopathogenic fungi as a biological control agent is centered on the fact that fungi in the genus Metarhizium produce deadly mycoses in a range of significant insect pest species (*Lepidoptera*, *Helicoverpa*, *Coleoptera*, etc.). The one of these fungus, *Metarhizium anisopliae* that has been studied the most thoroughly as an insecticide for crop pest management [14].

*Metarhizium anisopliae* is a species of Entomopathogenic fungus that belongs to the order Hypocreales. *Metarhizium anisopliae* is found in various habitats worldwide, including soil, decaying organic matter, and insect cadavers.As facultative fungal parasites, *Metarhizium anisopliae* can infect and thrive on insect hosts and

can be mass manufactured (in a solid condition). The presence of *Metarhizium anisopliae*, which has the potential to develop into bio pesticides, and occurs in soils of different agro ecosystem. As an Entomopathogenic fungus, *Metarhizium anisopliae* has the ability to infect and kill a wide range of insect pests. It has been extensively studied for its potential use as a biological control agent in agricultural and forestry systems. The fungus has a unique mode of action, wherein it attaches to the cuticle (outer covering) of the insect and penetrates it using specialized structures called appressoria. Once inside the insect's body, it multiplies and releases enzymes that break down the host tissues, eventually causing death [15]. The fungus has gained interest because of its capacity to manage insect pests in forestry, horticulture, and agriculture. It is available in a variety of forms, including sprays, granules, and dusts, and can be used as a bio pesticide. The use of *Metarhizium anisopliae* as a bio control agent has a number of benefits, including the capacity for long-term pest suppression, compatibility with integrated pest management techniques, and ability to remain in the environment [16].

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. The avoidance of repeated sub culturing on artificial medium and the occasional passage through the insect host maintained the virulence and effectiveness of the *Metarhizium* spores in the field [17]. The objective of this research was to determine mass production of *Metarhizium anisopliae* in different grain and check viability of spore.

## II. MATERIALS AND METHODS

**RHIZOSPHERIC SOIL COLLECTION :** Agricultural soil samples were collected randomly from the rhizospheric plant. Soil sample were collected from middle of the field in May 2023. Rhizospheric soil collection was done by using standard procedure.Soil were pooled from in depth 5-20 cm by avoiding external parts and debris in sample. About 100gm of sample were taken using cylindrical sampler and stored in sterile polythene bags sealed with a rubber band followed the method of T. Castro et al.,2016 [18] and brought to the laboratory for further test [19].

**ISOLATION OF *METARHIZIUM ANISOPLIAE* BY SERIAL DILUTION METHOD :**

1. Isolation of different fungi from collected soil samples was carried out by using the serial dilution method.
2. Weigh 10 g of soil samples and add them to 90 mL of sterile distilled water.
3. Thoroughly mix the samples using a magnetic stirrer for 60 minutes to release the spores adhered to soil particles.
4. After mixing, spread 0.1 ml of the sample from the aliquot into the selective medium. To avoid lown growth, dilute the sample by the serial dilution method, and each dilution was then spread on the respective plates. Selective agar media was prepared by using Potato Dextrose Agar (PDA) (composition g/L: Infusion from potatoes: 200, dextrose: 20, agar: 15). Add antibiotics for inhibition of bacterial growth.
5. Incubate the plates at 28 °C for 5-7 days.
6. Select and subculture the individual sporulating colonies on the same medium to obtain pure cultures [20] [21].

**IDENTIFICATION OF FUNGAL ISOLATES *METARHIZIUM ANISOPLIAE* :** During the seven-day incubation period, Petri dishes were evaluated daily for the existence of colonies of different fungi. Identification of *Metarhizium anisopliae* was made by observing morphological characteristics and microscopic examination [20].

**Morphological Identification of Fungal Isolates :** In the morphological identification of the fungal strains, the macroscopic traits of the colonies were observed first. For each colony, characteristics such as the color on the upper and lower sides of the plates, size, and pigmentation were observed [22] [23].Colonies were identified by morphological characteristics as described by M Tulloch 1976 & R. A. Humber 1997 [24] [25].

**Microscopic Examination :** When fungal colonies sporulated on PDA, small plaques appeared from the edge and the center of each growing colony were transferred onto glass slides containing drops of lacto phenol mounting medium and then crushed properly for better examination. A microscopic examination was made at 400 and 1000 magnifications using a compound light microscope. Which represents their vegetative and

**PURIFICATION OF DESIRED FUNGI BY USING SELECTIVE MEDIUM :** Serves as an additional carbon and energy source for the fungi and agar as well as solidifying the fungal colonies selected from the previous examination were further spread on potato dextrose agar and sabouraud dextrose agar for purification and further examination. Prepare suspension from selected colonies and spread it on selective medium

separately [20]. PDA and SDA are Selective mediums for *Metarhizium anisopliae.* In PDA medium, Potatoes are a source of essential nutrients such as carbohydrates, vitamins, and minerals, which support fungal growth. In addition, dextrose provides a stable surface for fungal colonies to grow. SDA medium contains a combination of ingredients such as dextrose, peptone, and agar that provide essential nutrients for fungal growth. Addition of antibiotics suppressing bacterial growth.

**ENRICHMENT OF FUNGAL SPORE SUSPENSION :** A conidial suspension containing detached spores was prepared from the sporulated culture. The fungal spores were harvested from a 2-3 week-old slant culture or plate. For preparation of fungal spore suspension, grow fungus on PDA. Add 10 ml of distilled water to the plate and shake it well by using a glass rod to remove the spores from the mycelium. Collect the distilled water with spores in a tube and consider it a crude suspension. The whole process is conducted under laminar air flow cabinets. These spore suspensions are used as seed material for mass production of *Metarhizium anisopliae*. Spore density was calculated using a hemocytometer. From this initial suspension, prepare serial dilutions up to 9 dilutions and quantify them by using a hemocytometer. Spore were count by following formula: cells/ml = (n) x $10^4$ or spores /ml where (n) is average of spores calculated in the four 1mm corner squares of the hemocytometer [27] [28] [29].

**MASS PRODUCTION BY SSF :** For mass multiplication of the selected fungi, substrate should be inexpensive and readily available with an appropriate nutrient balance. An inexpensive culture medium is required in order to increase the cost-benefit ratio. Solid-state fermentation (SSF) is an effective method for the mass production of fungal isolates. Various cheap cereal grains like sorghum, rice, millets, ragi, wheat, and green gram are used as substrates. [30] Different grains, such as sorghum,greengram, and rice were used for estimating the sporulation of *Metarhizium anisopliae* at 28 °C. 100 gram of each grain were washed and soaked in a 2% sucrose solution for 6-7 hours, except rice, which was soaked 2–3 hours prior to starting the experiments. The excess water was drained by decanting and shade drying it for half an hour to remove the moisture. The grains were packed separately in a conical flask with a cotton plug and sterilized at 15 psi for 20 minutes in an autoclave after cooling 1 ml of sporulating suspension of *Metarhizium anisopliae* inoculated into each flask under sterile conditions. They were incubated in a BOD incubator at 28 °C for 10 to 15 days. Three replications were maintained for each grain. Mix it at a 3-day interval. After the incubation period, transfer 100 ml of sterilized distilled water into the flask, and shake the flask in a mechanical shaker for 10 minutes. The suspension was filtered through muslin cloth. Spore's counting was made after the serial dilution of suspension [31] [32].

**LIQUID FORMULATION :** Formulations have a significant impact on improving the efficacy of biological insecticide. An appropriate formulation facilitates the use of bio pesticides and lowers the possibility of nontarget organisms coming into contact with fungal spores. Formulation made by active (functional) ingredients combined with non-active (inert) ingredients. Water, oil, polymer, or a combination of these can all be used in liquid formulations [16].

**VIABILITY STUDIES**

1. Utilize several techniques to calculate the obtained spores' viability percentage. Prepare the spore suspensions for this with 0.1% (w/v) Tween-80, and set the spore count to $1\times10^3$ spores/ml.
2. Spread the spore suspensions (0.1 mL) in triplicate onto PDA plates and incubate for 72 hours at 28 °C and 70-80% RH.
3. Calculate the overall viable count for the corresponding sample by manually counting the separated colonies [33].

## III. RESULT

**ISOLATION OF *METARHIZIUM ANISOPLIAE* BY SERIAL DILUTION METHOD:** *Metarhizium* spp. occur naturally in agricultural soil. Different fungal isolates were obtained from soil samples collected at different sites on different Petri dishes. The isolate from each Petri dish was marked and used for further confirmation. As a result, three isolates were determined for the next process. All these strains were transferred to a PDA-agar Petri dish, and the strains were purified for further identification.

**IDENTIFICATION OF *METARHIZIUM ANISOPLIAE***

**Morphological Identification of Fungal Isolates. *:*** The colony of *Metarhizium* species has green pigmentation according to colony character showed in Hussien et al. 2021 [34]. Here we found a total of three green colonies

from a mixed population. The morphological characteristics of all three colonies are given below. Selected colonies are shown in Fig.1

Colony: 1 At the initial stage, the colonies were white and hairy. A white mass of mycelium grows first. In the sporulation stage, there were clumps of green conidia in the middle of the colony.

Colony: 2 Colonies are small, circular, and flat. After about 72 hours, colonies appear coarsely granular, powdery, or dusty. The colour is white when young, but slowly it turns an olive green.

Colony: 3 Colonies are rapid-growing, loose, and cottony. The colour is white when young, but slowly it turns green, and sporulation is extremely high. The entire plate is filled within 3–4 days.

**Microscopic Examination :** All these isolates were microscopically examined for conformation. A microscopic examination of selected isolate is presented in Fig.2.These isolates were used in further process for mass production.

**PURIFICATION OF DESIRED FUNGI BY USING SELECTIVE MEDIUM :** Potato dextrose agar and sabouraud dextrose agar were used for the purification of species for further processing. After 7 days of incubation, morphological and microscopic examination of previously selected colonies we can prove that colony 1 and colony 2 were *Metarhizium spp.* Lown growth of *Metarhizium spp. on* PDA was shown in Fig.3 and Single-isolate colonies of *Metarhizium* were shown in Fig.4

**ENRICHMENT OF FUNGAL SPORE SUSPENSION :** In fungal spore suspension, spore concentration was determined by using a hemocytometer. Count all of the spores in each of the four 1 $mm^2$ corner squares on both sides of the hemocytometer. Calculate the total spores counted in the four corner squares and average of spores count counted in following formula: cells/ml = (n) x $10^4$.

**MASS PRODUCTION BY SSF :** There was a significant difference in the growth of *Metarhizium anisopliae* on different solid carrier materials which is showed in Fig.5, 6, 7. The highest stabilization potential was recorded for *Metarhizium* after 9 days of fermentation for *Metarhizium*. At the end of the fermentation period, the conidia in the medium are harvested. A common way of separating the conidia from the medium is by filtration. The colony-forming units of the concentrated *Metarhizium anisopliae* solution that were obtained after filtering. A mycelia formulation of *M. anisopliae* was developed for the liquid product [35].

**LIQUID FORMULATION :** In the final formulation, Demineralized water is used as a non-active (inert) ingredient, and 0.1% sugar is used as an active ingredient in the current formulation. Add previously made stock cultures of *Metarhizium anisopliae* for the final formulation that have at least $2\times10^6$ CFU. The final formulation of the product is shown in figure this formulation was determined to be acceptable for *Metarhizium spp*. growth and viability during storage. Higher survival stabilization rates are subject to change. The inoculant must have at least $2\times10^6$ CFU/ml. The final formulation of *Metarhizium* in a liquid carrier medium is shown in Fig. 8.

**VIABILITY STUDIES :** The viability was assessed each time by recording the percentage germination of spores on Potato dextrose agar. At 5°C, 80–90% of spores remained viable in the 4:1 ($H_2O$: spores w/w) formulation after 3 Months. As the temperature increased, the survival of the spores decreased. Storage temperature inversely affects the survival of spores in final formulations. Survival of *M. anisopliae* spores at 4-5°C and the reduced shelf life with increased storage temperature.A minimum shelf life of about 12 months is required for commercial success. Improved drying techniques do exist, and we believe that other processes, such as vacuum packing or controlled atmospheres, would further increase the viability of spores over longer time periods. [36]Up to 120 days, viability and survival were assessed.

## IV. DISCUSSION

The optimal nutrient medium is necessary for the adequate growth of microorganisms. *Metarhizium* is added to a list of fungi that have bifunctional lifestyles as an insect pathogen as well as a plant endophyte. Potato dextrose agar and sabouraud dextrose agar are the best mediums for the growth and sporulation of *Metarhizium spp*., but PDA and SDA mediums are highly expensive. In the present study, three types of substrate were used for mass multiplication of selected fungi. The highest sporulation,radial growth, and biomass production were recorded for Rice. Rice is the best substrate because it has all the necessary nutrients, which are badly needed

for the growth of fungi. This may be the reason for the good performance of this medium over other substrates. The fungus *M.anisopliae* also reached the maximum level of biomass in the rice soaked with a 2% sucrose solution. So, Rice soaked with a 2% sucrose solution is considered an ideal and budget-friendly medium for the mass multiplication of *M. anisopliae*. Another method is high-density polyethylene (HDPE) bags, also used for the mass production of *Metarhizium anisopliae*.

Fifty gram of rice were mixed with 50 ml of water in bags measuring 23 x 17 cm. The bags were sealed and thoroughly shaken bags for uniform distribution of spores. Then autoclaved at 121˚C at 15psi for 15 minutes. After autoclaving, the bags were cooled to room temperature. The corners of the bags were clipped and placed in an aseptic condition, and 5 ml of spore suspension of *M. anisopliae* was inoculated. The inoculated bags were then resealed on the burner flame at 25 to 28˚C after that, we can harvest spore in bulk, either in liquid or powder form. For liquid, add sterile distilled water to bags and separate liquid from bags under sterile conditions. For preparation of the spore powder, rice grain covered with the spore mass were collected from the bags and oven-dried at 40˚C for 24 hours. The dried grains were then macerated to make powder formulations. The powder formulation was dissolved in 0.1% Tween 80 solutions to calculate the spore count [31]. This study showed that *M. anisopliae* is a plant endophyte with plant-root-promoting properties. Further research is needed to confirm the role of *M.anisopliae* and the coordinated communication between the fungus and plant. *Metarhizium spp.* is a fungus that could be used in multiple roles, from protecting plants from pests to promoting plant growth.

## V. CONCLUSION

In conclusion, *Metarhizium anisopliae* is a naturally occurring Entomopathogenic fungus with great potential as a biocontrol agent against insect pests. Its unique mode of action and environmental safety make it an attractive alternative to chemical pesticides in agricultural and forestry systems. Ongoing research aims to harness its full potential and further refine its application for effective pest management. Taking a long time to kill compared with synthetic insecticides is the only drawback to the use of Entomopathogenic fungi, but these biopesticides are safe for use [5]. In recent years, the possible roles of a biocontrol agent as a plant growth promoter, plant endophyte, and plant pathogen antagonist may also provide an avenue for further understanding of fundamental biological processes. The discoveries about the ecological roles displayed by *M. anisopliae* with multiple effects will help its eventual commercialization.

## AKNOWLEGMENT

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## FIGURES:

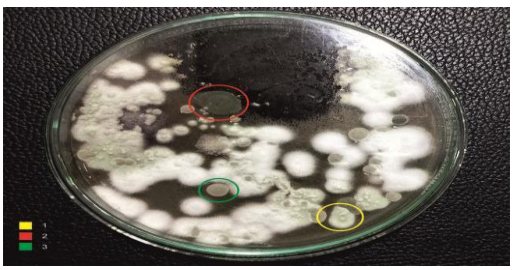

*Figure 1 Mix population of fungi from soil sample*

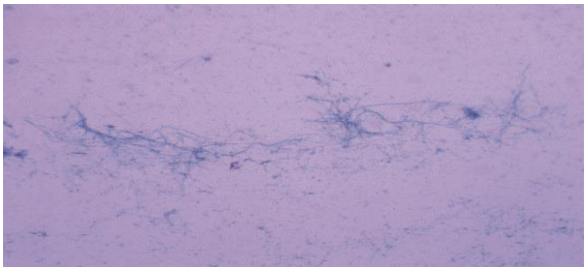

*Figure 2 Microscopic view of Metarhizium spp.*

*Figure 3 Lown growth of Metarhizium spp.*

*Figure 4 Single isolates of Metarhizium spp.*


*Figure 5 Mass production of Metarhizium in Rice*

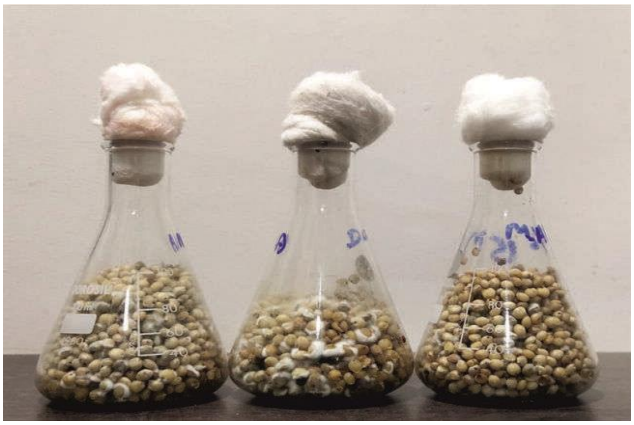

*Figure 6 Mass production of Metarhizium in Sorghum*

*Figure 7 Mass production of Metarhizium in green gram*

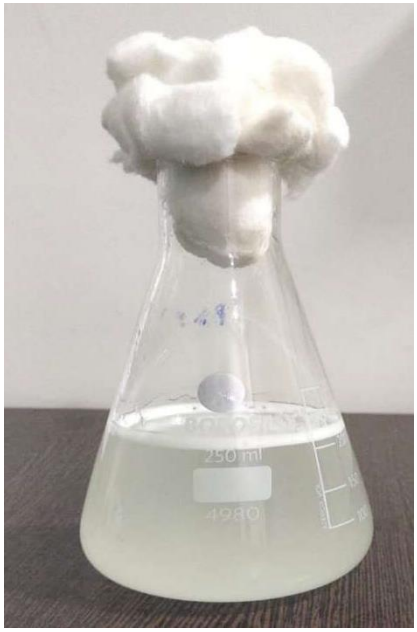

*Figure 8 Final formulation of Metarhizium anisopliae*

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