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Review Article

ISOLATION, IDENTIFICATION AND MASS PRODUCTION OF SOIL MICROBES AND THEIR POTENTIAL UTILITY FOR BIOCONTROL OF PEST MADHYA PRADESH

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ABSTRACT

Intensive surveys were carried out at different forest ecosystems, including the plantations in Jabalpur, Mandla, and Dindori districts, Madhya Pradesh India, during (2010 to 2013) at different seasons and about 60 soil samples were collected from different forest ecosystem and baited with the insect-pest *Galleria mellonella* for trapping soil microbes particularly the entomopathogenic fungi. Six isolates of Entomopathogenic fungi were trapped/isolated from 60 soil samples. Pure cultures of these isolates were raised on artificial media and identified, and grouped under different species. Among the isolated fungi of *Fusarium oxysporum*, *Aspergillus flavus*, *Beauveria bassiana*, *Metarhizium anisopliae*, *Fusarium* sp., *Pacilomyces* sp., the two fungi *Beauveria bassiana* and *Metarhizium anisopliae* were found potential and used as biocontrol agent against various insect pests. Mass culturing of soil microbes on different agricultural products like Rice powder, Rice bran, sorghum grain and agricultural waste was also attempted and methods were developed. The fungus grew better on sorghum grain at normal room temperature of 25-28°C with Relative Humidity of 60-65%. The spore load on these media varied from 500 – 550 spores /ml counted from 25 sq.mm squares of the haemocytometer as compared to other media which counted 400-450 spores/ml.

Keywords: *Beauveria bassiana*, *Metarhizium anisopliae*, *Pacilomyces* sp., *Fusarium oxysporum* and entomopathogenic fungi.

INTRODUCTION

Soil is the main reservoir of entomopathogenic fungi which have an essential influence on the occurrence and expansion of insect mycoses (4). The entomopathogenic fungi play a major role in the regulation of pest population (2). Taking into account of the substantial information available on the effectiveness and potential use of the entomopathogenic fungi in control of many insect species and also most of the entomopathogenic fungi are soil borne nature an attempt was made to collect the soil samples from various forest ecosystems and the entomopathogenic fungi were isolated from the infected cadavers of *G.mellonella* through insect bait method (9). Infectivity test was conducted on the test insect *Crocidophora* sp. (Bamboo defoliator) shows that the soil borne fungi *Beauveria bassiana* and *Metarhizium anisopliae* were very much effective in causing mortality of the larvae of the test insect larvae of *Crocidophora* sp.

MATERIALS AND METHODS

Soil samples collected from various locations at Jabalpur, Mandla and Dindori district of Madhya Pradesh India by collecting the topsoil down to 10 - 15 cm depth with help of a scoop like tool made up of stainless steel (Table -1).

Three spot samples were made for each site to homogenize and draw a single sample of ½ a kg. The samples were secured in clean polythene bags separately and brought to the laboratory and stored in refrigerated condition at 5°C. Within five days the samples were subjected to insect bait.

The soil samples were transferred to small containers and *G.mellonella* of about 5-10 third instars larvae were released into the container and incubated for approximately 14 days on condition that favors development of the fungus. The soil was agitated, or the containers were repositioned periodically to ensure that the larvae remain exposed to the soil. Diseased and mummified larvae retrieved from the container for isolation of the fungi.

The fungi isolated from the insect cadavers were subculture on artificial media Sabouraud Dextrose Agar medium (SDA). Further, pure cultures were raised and stored. The fungi were identified mainly based on the morphological characteristics of reproductive structures with the aid of several taxonomic keys (1,7,6,7,9). The test insect *Crocidophora* sp. was subjected to pathogenicity test to confirm the infectivity of the fungus in laboratory condition. The fungal isolates were grown on SDA at room temperature 25-28°C for 8 – 10 days were harvested and crude extract was prepared (stock

solution) with distilled water. The concentration of spores in the final suspension was determined by haemo cytometry.

Estimation of Spores

Dilution: 1 ml of the purified stock fungus suspension was made up to 10 or 100 ml with water

Containing 0.1 % wetting agent, teepol. The teepol solution ensured thorough mixing and uniform distribution of fungal spores.

Dilution factor

1 ml made up to 10 ml = 10 times

1 ml made up to 100 ml = 100 times

Counting

With the Thoma white cell pipette provided along with the haemocytometer, the fungal suspension was drawn to the '1' (One) mark and made up to 11 mark with distilled water containing 0.1%teepol. Shook the pipette vigorously, closing the rubber tube tightly. Discarded the first 3 drops and introduced the fourth drop into the groove of the haemocytometer after placing the standard cover slip over the slide. Introduced only a sufficient quantity of the liquid to fill up the calibrated area in the haemocytometer. Allowed the spores for 2 mins. To settle down and then counted the spores in about 25 of the 1/400 sq. mm squares with the help of a research microscope under 10 x 40 magnification. Care was taken to avoid duplication of counts of spores on the lines of calibrations. This could be best done by counting the spores inside the squares as well as the spores on the top and left side lines only. The spore suspensions of known concentration were prepared from the stock solution by suitable dilution with distilled water.

The EPF isolates sub-cultured in Sabrouaud Dextrose Agar (SDA) medium were utilized for preparation of stock solutions and tested for their pathogenicity on the test insect *Crocidophora* sp. Third instars larvae of *Crocidophora* sp. were used for all screening bioassay procedures.

The spore load of the stock solution of different fungi further, a spore suspension of effective fungal concentration of 5.3×10^{10} was prepared and pathogenicity test was conducted on the test insect *Crocidophora* sp. Healthy third instar larvae of this species reared in the laboratory were surface sterilized with 1-5% sodium hypochlorite and sprayed with the fungal inoculums. The sprayed larvae were air dried on a filter paper. The inoculum sprayed larvae of *Crocidophora* sp. were released in the plastic containers. A control sprayed with only distilled water was maintained. Five replications were maintained for each concentration. 10 larvae were used per replication. Larval mortality was recorded at every 24 hrs. Interval and recording of data was concluded on the seventh day of the experiment.

Mass production of entomopathogenic fungi

As the choice of a suitable medium for multiplication of pathogen at a large quantity is one of the

Basic requirements in a microbial control studies because the composition of the medium exerts a great influence on growth, speculation and spore germination. In the present study attempts were made to mass multiply the isolated entomopathogenic fungi on different agricultural products like vegetable waste, rice powder, and rice bran and sorghum

grain. These are cheaper and useful for a medium scale mass production of fungi.

1. Vegetable waste medium

Vegetable waste (Carrot) - 150 g

Distilled water - 150 ml – 300 ml (depending on the vegetables) Streptomycin - 0.25g

2. Rice powder, Rice bran, sorghum grain medium

Rice powder or Rice bran - 150 g or sorghum grain

Distilled water - 150 ml

Streptomycin - 0.25g

The ingredients were mixed and put into a conical flask (500 ml). The closed conical flask was sterilized for 20 minutes at 120° C and then cooled for at least 6 hours. The inoculums pure cultured on SDA media was transferred to the conical flask by using the sterilized inoculation needle. After inoculation the flasks were tightly plugged with cotton buds and left for a week for growth of the fungus. The spores of the fungi were harvested from the media and used for further evaluation at different concentrations.

RESULTS AND DISCUSSION

The soil samples collected from various forest ecosystems were subjected to insect bait method to trap the entomopathogenic fungi by using the insect *G.mellonella*. There were about 6 fungi were isolated from the infected insects and found identified. The soil borne fungi *Fusarium oxysporum*, *Aspergillus flavus*, *Beauveria bassiana*, *Metarhizium anisopliae*, *Fusarium* sp., *Pacilomyces* sp., were isolated from the cadavers of *G.mellonella*. Microphotography of the fungi species recorded in the study showed clear variations in the spore formation, spore size and structure and development of the hyphae.

Beauveria bassiana

Beauveria bassiana is an aggressive parasite of many different insect host species. Not only it has a wide host range but also attacks the larval or adult stages. The spores are tiny, measuring only a few microns. The hyphae and spores are non pigmented (hyaline) and so colonies appear white in cultures or tufts of white mycelium bearing masses of powdery spores burst out through the body parts of infected insects. The spores were produced sympodially. A spore was produced at the tip of the mother cell and the growth of the mother cell ceased. A new growing point initiated just below the terminal spore, grew past it, and a second spore was produced at a higher level. This was used up by the new growing point and a third growing point was then initiated just below the second spore. Every time a spore was produced at the hyphal tip was used up and a new growing point was produced. In this way a succession of spores was produced with the youngest spore at the tip and the spore head got longer and longer. When all the spores were dislodged the spore-bearing tip of the conidiogenous cell had a zig zag appearance and was referred to as a rachis.

Metarhizium anisopliae

Metarhizium anisopliae is a fungus that grows naturally in soils throughout the world and causes Disease in various insects by acting as a parasite; it thus belongs to the entomopathogenic fungi. It is being used as a biological

insecticide to control a number of pests such as grasshoppers, termites, thrips, etc. The disease caused by the fungus is called green mustarding disease because of the green color of its spores. When these mitotic (asexual) spores (properly called conidia) of the fungus come into contact with the body of an insect host, they germinate and the hyphae that emerge penetrate the cuticle. The fungus then develops inside the body eventually killing the insect after a few days; this lethal effect is very likely aided by the production of insecticidal cyclic peptides (detrains). The cuticle of the cadaver often becomes red. If the ambient humidity is high enough, a white mold then grows on the cadaver that soon turns green as spores are produced. Most insects living near the soil have evolved natural defenses against entomopathogenic fungi like *M. anisopliae*.

Aspergillus flavus

Hyphae were septate and hyaline. The conidiophores originated from the basal foot cell located on the supporting hyphae and terminated in a vesicle at the apex. Vesicle was the typical formation for the genus *Aspergillus*. The morphology and color of the conidiophore varies from one species to another. Covering the surface of the vesicle entirely ("radiate" head) or partially only at the upper surface ("columnar" head) had the flask-shaped phialides which were either uniseriate or attached to the vesicle directly or were biseriate and attached to the vesicle via a supporting cell, metula. Over the phialides were the round conidia (2-5 µm in diameter) forming radial chains.

Fusarium oxysporum

In solid media culture, such as Sabouraud Dextrose Agar (SDA), the different special forms of *F. oxysporum* had varying appearances. In general, the aerial mycelium first

appeared white, and then changed to a variety of colors - ranging from violet to dark purple – according to the strain (or special form) of *F. oxysporum*. If sporodochia were abundant, the culture appeared cream or orange in color. *F. oxysporum* produced three types of asexual spores: microconidia, macroconidia, and chlamydo-spores (Microconidia) were one or two celled, and were the type of spore most abundant and frequently produced by the fungus under all conditions. It is also the type of spore most frequently produced within the vessels of infected plants. Macroconidia were three to five celled, gradually pointed and curved toward the ends. These spores were commonly found on the surface of plants killed by this pathogen as well as in sporodochia like groups. Chlamydo-spores were round, thick-walled spores, produced either terminally or intercalary on older mycelium or in macroconidia. These spores were either one or two celled.

Pacilomyces sp.

Conidiophores erect, mononematous, but species on insects often synnematosus, verticillate, bearing whorls of divergent branches and phialides; phialides flask-shaped or with swollen basal part, abruptly tapering into a distinct neck; conidia one celled, hyaline to slightly pigmented, produced in dry divergent chains, smooth-walled or occasionally spiny. Pathogenicity of the potential soil borne fungi *Beauveria bassiana* and *Metarhizium anisopliae* tested on the test insect *Crocidophora* sp. at the concentration of 5.3 x 10¹⁰ spores/ml the isolate of *Beauveria bassiana* was pathogenic to insect resulting 100% larval mortality over a period of 7 days. Whereas, the isolate *Metarhizium anisopliae* shows 52% larval mortality over at period of 7 days (Table - 2).

Table 1: Details of the soil samples collected in Assam state during the Survey Locations

Locations	Geographical location (Lat. N, long. E)	Habitats	No. of Soil Samples collected	Remarks
Jabalpur	N 26° 46' 59.5" E 094° 17' 35.3"	Forest Plantations	20	Each Soil sample is a constituent of 3 spot samples collected from one site. The spot samples were thoroughly homogenized and one sample containing ½ a Kg soil was collected for analysis
Mandla	N 26° 01' 57.4' E 091° 35' 37.1"	Forest Plantations	20	
Dindori	N 26° 32' 46.7" E 093° 00' 37.8"	Forest Plantations	20	

Table 2: Infectivity of different Entomopathogenic fungi for the control of *Crocidophora* sp. by applying effective fungal concentration 5.3 × 10¹⁰ spores/ml (Lab experiment)

Targeted larval pest	Larval mortality (%) of IIIrd instar			
	4 DAT	5 DAT	6 DAT	7 DAT
<i>Beauveria bassiana</i>	20	48	82	100
<i>Metarhizium anisopliae</i>	8	16	24	52
<i>Pacilomyces</i> sp.	0	6	16	24
<i>Fusarium</i> sp.,	4	10	20	32
<i>Aspergillus flavus</i>	0	12	20	36
Control	0	0	0	0

n=10 Values represent mean of 5 replicates; *significant of 5 percent level by DMRT

CONCLUSION

The two potential isolates of entomopathogenic fungi selected out of six were mass multiplied in media like vegetable waste, rice powder, rice bran and sorghum grain media. The fungus grew better on sorghum grain at normal room temperature of 25 -28oC with relative Humidity of 60 - 65%. The spore load on these media varied from 500 – 550 spores /ml counted from 25 sq.mm squares of the haemocytometer as compared to other media which counted 400-450 spores/ml. For large scale production of conidia, cheaper nutritive substrates such as rice, bran or cereal grains were used. Moistened substrates are autoclaved in wide mouthed jars, autoclavable plastic bags or tin trays. After cooling, the substrate is inoculated with a conidial or blastospore suspension and incubated at room temperature. The fungi grow better and sporulate more at 25o than 15oc. The optimum temperature for mycelial growth of the fungi is recorded at 20-25oC, while pH ranging from 5-11 did not significantly affect the growth (9). The use of rice in polypropylene plastic bags is currently the most widely used methods for production of *M. anisopliae* (5) and *Metarhizium flavoviride* (3). Fungi have demonstrated considerable potential in microbial control of arthropods especially within IPM programs. Their restricted host ranges allow for the control of insect pests with limited harm to non target organisms including predators, parasites, and other pathogens. In addition, fungi are also compatible with some fungicides and many other types of Pesticides. The results of the present study confirm not only the occurrence of the entomopathogenic fungal species in soil but also the richness of diversity of the EPF fungi in the forest soil. The study has also highlighted that *Galleria mellonella* is an ideal host for trapping not only the entomopathogenic fungi but also fungi of plant pathogenic and antagonistic nature.

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REFERENCES

1. Domsch, KH., Gams, W. and Anderson, TH. Compendium of Soil Fungi. Academic Press, London. 1980; (1): 893.
2. Ferron, P. Pest control by the fungi *Beauveria* and *metarhizium*, In: Microbiol control of pest plant diseases, H.D Burges (Eds.). 1981; 465-482.
3. Jenkins, NE. and Goettel, MS. Methods for mass production of microbial control agents of grass hoppers and locusts. *Memoris of the Entomological Society of Canada*. 1997; 171: 37- 48.
4. Ignoffo, CM., et al., Stability of conidia of endomopathogenic fungus *Nomuraea rileyi* in and on soil. *J. Inverteba. Path.*, 1978; 28: 256 - 268.
5. Mendonca, AF. Mass production, application and formulation of *Metarhizium anisopliae* for control of sugarcane frog hopper, *Mahanarva posticata*, in Brazil. In; Lomer, C.J.; Prior, C. (Eds.) Biological control of locusts and grasshoppers. Wallingford, UK; CAB International. 1992; 239-244.
6. Nelson, PE., Toussoun, T.A. and Marasas, W.F.O. *Fusarium Species: an Illustrated Manual for Identification*. Pennsylvania State University Press, University Park and London. 1983; p. 193.
7. Samson, RA., Evans, HC. and Latge, JP. Atlas of Entomopathogenic Fungi. Springer – Verlag, New York. 1988; p.187.
8. Sang-Myeong Lee. et al., Cultural characteristics of Entomopathogenic fungi isolated from southern parts of Korea. *FRI. J. Fr. Sci.*, 1999; 62: 126 - 135
9. Zimmermann, G. The *Galleria* baits method for detection of entomopathogenic fungi in soil. *J. Appl. Entomol.*, 1986; 102: 213 - 215.

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