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Evaluation of different media for the mass multiplication of entomopathogenic fungus *metarhizium anisopliae* (metschn.) sorokin

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Abstract
An experiment entitled "Evaluation of different media for mass multiplication of entomopathogenic fungus *Metarhizium anisopliae* (Metschn.) Sorokin" was conducted at the Biocontrol Laboratory, Department of Entomology, Post Graduate Institute, MPKV, Rahuri, Dist: Ahilyanagar (Maharashtra). During the course of study, Seven treatments (substrate) were evaluated in Completely Randomized Design (CRD) with three replications during Year, 2024-25 for growth and observations were recorded on 10th, 20th and 30th days after inoculation. Among the different substrates evaluated highest conidial count (6.92×10^8 spores/ml) was observed on wheat substrate followed by maize (6.50×10^8 spores/ml). Combining biological efficiency and cost-effectiveness, the study concludes that wheat is the most reliable substrate because protein and nitrogen content responsible for large-scale, economical production of *Metarhizium anisopliae*, with maize and rice as viable alternatives for sustainable biocontrol programs.

Keywords: Mass Multiplication, *Metarhizium anisopliae*, Biological Efficiency

Introduction
Entomopathogenic fungi (EPF) are fungal species that are pathogenic to insects. These fungal pathogens play a vital role in insect population dynamics making it the earliest insect pests control agents. Many insects' pathogenic fungi- based bio insecticides have been formulated and commercially manufactured (Hafiza *et al*. 2014) [2]. *M. anisopliae* is a soil-borne fungus that infects insects by penetrating their cuticle, proliferating in the hemocoel and ultimately causing the host's death. It produces distinctive green conidia, which aid in its identification and also contribute to its role in biological control. Its placement within Clavicipitaceae reflects its evolutionary relationship with other fungi that often form close associations with hosts and produce secondary metabolites that can be toxic to insects (Kulat *et al.*, 2002) [5]. The two phases of culture (liquid and solid) are the most commonly used technique to mass produce *Metarhizium*, liquid fermentation is used to produce blastopores and mycelium forms (Perira and Roberts, 1991 and Kurger *et al.*, 2014) [8]. The solid phase is carried out in a solid substrate, which has a large surface area for aeration and physically supports the fungus conidia and it is also used as a source of nutrients (Jenkins *et al.*, 1998) [3]. *Metarhizium anisopliae* is commercially produced in solid substrates, but this type of production complicates process automation; it relies on batch production and does not provide a satisfactory economy of scale (Wraight *et al*., 2001) [13]. Different substrates of vegetable origin can be used to mass produce conidia, such as different forms of potato, wheat, soy, rice and bran.

Material and Methods
The present investigation entitled, "Evaluation of different media for the mass multiplication of entomopathogenic fungus *Metarhizium anisopliae* (Metschn.) Sorokin" was conducted at Biocontrol Laboratory, Department of Entomology, Post Graduate Institute, MPKV, Rahuri. during the year 2024-25. There were seven substrates for determining suitable substrate for growth of mass multiplication of *Metarhizium anisopliae.*

Preparation of media

Solid substrates

Rice, wheat, maize, sorghum and PDA were used for estimating the growth of *Metarhizium anisopliae* at 25^{0}C. For this purpose Rice, wheat, maize, sorghum (100g of each) grain was Crushed into ¾ pieces then washed and soaked in water for 2-3 hrs. before starting the experiment and cooked till it becomes soft the excess water was drained by decanting and shade dried. The crushed grains were placed separately in 250 ml conical flask and the mouth of the flask was plugged with cotton and autoclaved at 15 pound per square inch (psi) for 20 minutes (min). After cooling, 5 mm fungal disc of *Metarhizium anisopliae* was inoculated into each flask under laminar air flow chamber. Flasks were incubated at room temperature. three replications were maintained for rice substrate. To avoid clumping, after 7 days of inoculation, the flasks were shaken vigorously to separate the grains and also to break the mycelia mat.

For the PDA, 200 g of peeled and sliced potato was added in 1000 ml distilled water in beaker, the potatoes were boiled till they became soft. The contents of the beakers were filtered through muslin cloth and all the liquid was squeezed out then added 20 grams of dextrose and 20 grams of agar (solidifying agent). Distributed in 100ml to each conical flask and plugged with nonabsorbent cotton. The flasks were sterilized at 15 psi pressure (121^{0}C) for 30 min in an autoclave. After cooling, 5 mm fungal disc of *Metarhizium anisopliae* was inoculated into each flask under laminar air flow chamber. Flasks were incubated at room temperature at 25ºC three replications were maintained.

Liquid substrates

Potato Dextrose Broth (PDB)

200g of peeled and sliced potato was added in 250 ml distilled water in beaker, the potatoes were boiled till they became soft. The contents of the beakers were filtered through muslin cloth and all the liquid was squeezed out. 20g dextrose was dissolved in water and added to this extract and made the volume to 500ml. Dispensed 100ml to each conical flask and plugged with nonabsorbent cotton. The flasks were sterilized at 15 psi pressure (121^{0}C) for 30 min in an autoclave. After cooling, 5 mm fungal disc of *Metarhizium anisopliae* was inoculated into each flask under laminar air flow chamber. Flasks were incubated at room temperature at 25ºC. three replications were maintained.

Modified Fungal Broth (MFB)

40 grams of Jaggery mixed with 4 grams Yeast extract to prepare the modified fungal broth, which was dissolved in 1000 ml of distilled water and heated to boiling point. Dispensed 100ml to each conical flask and plugged with nonabsorbent cotton. The flasks were sterilized at 15 psi pressure (121^{0}C) for 30 min in an autoclave. After cooling, 5 mm fungal disc of *Metarhizium anisopliae* was inoculated into each flask under laminar air flow chamber. Flasks were incubated at room temperature at 25^{0}C. three replications were maintained.

Effect of substrates on growth of *Metarhizium anisopliae*

Observation on spore counting was done on 10th, 20th and 30th days after inoculation. The counting of spores was according to the method reported by (Bias *et al*., 1990) [1] using haemocytometer with slight modification. For this purpose10g/10ml of homogenous grain/solution sample was drawn from each replication of uniformly sporulated flasks and was transferred to 100 ml sterilized distilled water containing tween 80 (0.05 %) solutions in 250 ml conical flasks. The flasks were shaken in mechanical shaker at 500 rpm for 10 minutes. The suspension was filtered through double layered muslin cloth.

Counting of spores were made after the serial dilution of the suspension using a drop of conidial suspension made from solid and liquid culture was placed on the engraved grid and the preparation was allowed to stand for 1-2 minutes to allow the conidia to settle at the bottom. A cover glass was placed over the grid carefully to avoid no air bubble enters between the slide and cover glass. The slide was focused until coloured rings were visible as the two surfaces of cover glass and slide come into close contact. The conidia of fungus were counted in the 4-corner large square (I, II, III, IV) which consist of 16 small square each group has 0.2 mm square. The number of spores/cells per ml of suspension were calculated using the following formulas.

Total number of spores/ml/gm. = No. of spores in square × 10^4 × Dilution factor.

Statistical Analysis

All the data were subjected to statistical analysis after appropriate transformation as suggested by[7]

Table 1: Treatment details for mass multiplication of *Metarhizium anisopliae* on different substrates

Tr. No.	Name of Substrate	Quantity of Substrate (g or ml)
1.	Rice	100
2.	Wheat	100
3.	Sorghum	100
4.	Maize	100
5.	Potato Dextrose Agar (PDA)	100
6.	Modified Fungal Broth (MFB)	100
7.	Potato Dextrose Broth (PDB)	100

Result

The experiment on mass production studies was undertaken on seven substrates for determining a suitable medium for growth and sporulation. During this period the maximum and minimum temperature of the laboratory were 31.6 ± 2.21^{0}C and 26.3 ± 3.28^{0}C, respectively while morning and evening relative humidity were 58 ± 19 per cent and 20.5 ± 8.5 per cent, respectively. The observations were recorded on 10th, 20th and 30th days after inoculation and the data presented in Table 2 and Fig. 1.

Spore count at different days after inoculation

10 days after inoculation

Within the different substrates evaluated on the 10th day after inoculation, significantly highest conidial count (2.75x10^8 spores/ml) was observed on wheat substrate (T_2) in comparison with other substrate followed by maize (T_4) (2.58 x 10^8 spores/ml) both were at par with each other. This was followed by sorghum (T_3) (2.08 x 10^8 spores/ml) and rice (T_1) (1.92 x 10^8 Spores/ml) both were at par with each other. Conidial count was found moderate on modified fungal broth (T_6) (1.50 x 10^8 spores / ml) and potato dextrose broth (T_7) (1.42 x 10^8 spores /ml) this are also at

par with each other. Conidial count of potato dextrose agar (T_5) is low in comparison with other studied substrate (1.00 x 10^8 spores /ml).

Twenty days after inoculation

Among the different substrates evaluated on the 20th day after inoculation, highest conidial count (6.25x10^8 spores/ml) was observed on wheat substrate in comparison with other substrate (T_2) followed by maize (T_4) (6.08x10^8 spores/ml) both were at par with each other. This was followed by sorghum (T_3) (4.58x10^8 spores/ml) and rice (T_1) (4.50x10^8 Spores/ml) both were at par with each other. Conidial count was found moderate on modified fungal broth (T_6) (3.33x10^8 spores / ml) and potato dextrose broth ($_{T7)}$ (3.25x10^8 spores /ml) this are also at par with each other. Conidial count of potato dextrose agar (T_5) (2.25x 10^8 spores /ml) was low in comparison with other substrate.

Thirty days after inoculation

In the context of different substrates evaluated on the 30th day after inoculation, highest conidial count (11.75 x 10^8 spores/ml) was observed on wheat substrate (T_2) in comparison with other substrate followed by maize (T_4) (10.83 x 10^8 spores/ml) both were at par with each other. This was followed by sorghum (T_3) (8.00 x 10^8 spores/ml) and rice (T_1) (7.67 x 10^8 spores/ml) both were at par with each other.

Pooled Mean

In relation to the different substrates evaluated, overall highest conidial count (6.92x10^8 spores/ml) was observed on wheat substrate (T_2) in comparison with other substrate followed by maize (T_4) (6.50x10^8 spores/ml) and both were at par with each other. This was followed by sorghum (T_3) (4.89x10^8 spores/ml) and rice (T_1) (4.69x10^8 Spores/ml) and both were at par with each other. Conidial count was found moderate on modified fungal broth (T_6) (3.30x10^8 spores / ml) and potato dextrose broth (T_7) (3.14x10^8 spores /ml) this are also at par with each other. Conidial count of potato dextrose agar (T_5) (2.31x 10^8 spores /ml) was recorded low as compare to other studied substrate lowest on potato dextrose agar (T_5) (2.31x 10^8 spores /ml).

Rate of increase in growth

Rate of increase in growth of *Metarhizium anisopliae* was calculated and the data is presented in Table 3 and Fig. 2.

From 10th to 20th day after inoculation

The rate of increase in growth of *Metarhizium anisopliae* on different substrates from 10th to 20th day after inoculation. The highest rate of increase in growth of *Metarhizium anisopliae* was recorded on maize (T_4) (57.51 %) followed by rice (T_1) (57.41 %), potato dextrose broth (T_7) (56.41 %), wheat (T_2) (56.00 %), potato dextrose agar (T_5) (55.56 %), modified fungal broth (T_6) (54.95 %) and least was recoded on sorghum (T_3) (54.51 %).

From 20th to 30th day after inoculation

The rate of increase in growth of *Metarhizium anisopliae* on different substrates from 10th to 20th day after inoculation. The highest rate of increase in growth of *Metarhizium anisopliae* was recorded on wheat (T_2) (46.81%) followed by maize (T_4) (43.88 %), sorghum (T_3) (42.75 %), rice (T_1) (41.30 %), potato dextrose agar (T_5) (38.64 %), modified fungal broth (T_6) (34.59 %) and least was recoded on potato dextrose broth (T_7) (31.58 %).

To work out the economics of mass production of *Metarhizium anisopliae*

The cost of production of 1x 10^8 spores was calculated for all the substrates and data is Table 4 and Fig. 3.

The cost of production on different substrates significantly varied from each other. Significantly low production cost was required for Wheat (T_2) (Rs. 2), this was followed by Rice (T_1) (Rs. 4), Maize (T_4) (Rs. 5), Sorghum (T_3) (Rs. 6), Modified Fungal Broth (MFB) (T_6) (Rs. 10.3), Potato Dextrose Broth (PDB) (T_7) (Rs. 44.5). High cost of production was required for Potato Dextrose Agar (PDA) (T_5) (Rs. 108).

Discussion

Amongst the different substrates evaluated highest conidial count (6.92 × 10^8 spores/ml) was observed on wheat substrate followed by maize (6.50 × 10^8 spores/ml). The results of present investigations were discussed in the light of findings of previous workers. Soundarapandian and Chandra (2007) [12] carried out trials for mass production of *M. anisopliae* in the laboratory by using solid media. Four types of solid media such as rice, maize, wheat and Kodo millet were used for the mass production of *M. anisopliae* and highest sporulation was observed in *M. anisopliae* cultured in wheat (8,300 x 10^4). Hence, wheat was recommended as best solid media for the mass production of *M. anisopliae*. The optimum temperature and ideal PH for the mass production of *M. anisopliae* was found to be 25-30°C and 7.0 respectively. Kumar *et al.* (2007) [6] also studied the conidial sporulation of *M. anisopliae* on wheat, sorghum, rice grains. The fungus sporulated on all substrates at 21 days after inoculation. Conidial sporulation reached 2.17 x 10^8/g on wheat, 4.22 x 10^8 /g on rice and 3.97 x 10^8 /g on sorghum. Although conidial production was greatest on rice grains, difficulties in the extraction of conidia from this substrate were observed. The enzyme amylase which presents in *Metarhizium anisopliae* uses amylose sugar efficiently which ultimately results in growth of fungus.

The present findings are in conformity with the findings of (Kumar *et al*., 2007) [7] and Shivakalai *et al* (2015) as they both reported rice also reported that rice grain was the best substrate for spore production of *Metarhizium anisopliae* strain. Rice, wheat, maize and jowar comes under cereal group and poaceae family which posses comparatively higher percentage of carbohydrates (amylose sugar) and protein percentage which affect multiplication of fungus *Metarhizium anisopliae*.

The cost of production on different substrates significantly varied from each other. Significantly low production cost was required for Wheat (T_2) (Rs. 2), this was followed by Rice (T_1) (Rs. 4), Maize (T_4) (Rs. 5), Sorghum (T_3) (Rs. 6), Modified Fungal Broth (MFB) (T_6) (Rs. 10.3), Potato Dextrose Broth (PDB) (T_7) (Rs. 44.5). High cost of production was required for Potato Dextrose Agar (PDA) (T_5) (Rs. 108). The results of present investigations were discussed in the light of findings of Raypuriya *et al.* (2019) and Prasad *et al* (2014) [9-10].

Table 2: Mass production of *Metarhizium anisopliae* on different substrates

Tr. No.	Substrates	10 DAI*	20 DAI	30 DAI	Mean × 10^8 spores/ml
1.	Rice	1.92 (1.55)**	4.50 (2.24)	7.67 (2.86)	4.69 (2.22)
2.	Wheat	2.75 (1.80)	5.92 (2.59)	11.75 (3.50)	6.81 (2.63)
3.	Sorghum	2.08 (1.61)	4.58 (2.25)	8.00 (2.92)	4.89 (2.26)
4.	Maize	2.58 (1.76)	5.58 (2.57)	10.83 (3.36)	6.50 (2.56)
5.	Potato Dextrose Agar (PDA)	1.00 (1.22)	2.75 (1.66)	3.67 (2.03)	2.31 (1.64)
6.	Modified Fungal Broth (MFB)	1.50 (1.41)	3.33 (1.96)	5.08 (2.36)	3.30 (1.91)
7.	Potato Dextrose Broth (PDB)	1.42 (1.38)	3.25 (1.93	4.75 (2.29)	3.14 (1.87)
	S.E. ±	0.04	0.06	0.08	0.06
	CD at 1%	0.13	0.27	0.24	0.21

**Figures in parentheses are ($\sqrt{x} \pm 0.5$) transformations
*DAI = Days after inoculation

Table 3: Mass production of *Metarhizium anisopliae* on different substrates

Tr. No.	Substrates	Spore count (1x 10^8 spores/ml) at different DAI				Rate of increase in growth of *Metarhizium anisopliae* (%)	
		10 DAI*	20 DAI	30 DAI	Mean	10^{th} to 20^{th}	20^{th} to 30^{th}
1.	Rice	1.92	4.5	7.67	4.69	57.41	41.30
2.	Wheat	2.75	6.25	11.75	6.92	56.00	46.81
3.	Sorghum	2.08	4.58	8.00	4.89	54.51	42.75
4.	Maize	2.58	6.08	10.83	6.50	57.51	43.88
5.	Potato Dextrose Agar (PDA)	1.00	2.25	3.67	2.31	55.56	38.64
6.	Modified Fungal Broth (MFB)	1.50	3.33	5.08	3.30	54.95	34.49
7.	Potato Dextrose Broth (PDB)	1.42	3.25	4.75	3.14	56.41	31.58

*DAI- Days after Inoculation

Table 4: Economics of mass production of *Metarhizium anisopliae* on different substrates

Tr. No.	Substrates	Mean spore count (1 x 10^8 spores /ml)	Cost of substrates per 1 kg (Rs.)	Cost of production of 1 x 10^8 spores/ml
1.	Rice	4.69	22	4
2.	Wheat	6.92	16	2
3.	Sorghum	4.89	32	6
4.	Maize	6.50	3.5	5
5.	Potato Dextrose Agar (PDA)	2.31	25	108
6.	Modified Fungal Broth (MFB)	3.30	34	10.3
7.	Potato Dextrose Broth (PDB)	3.14	14	44.5

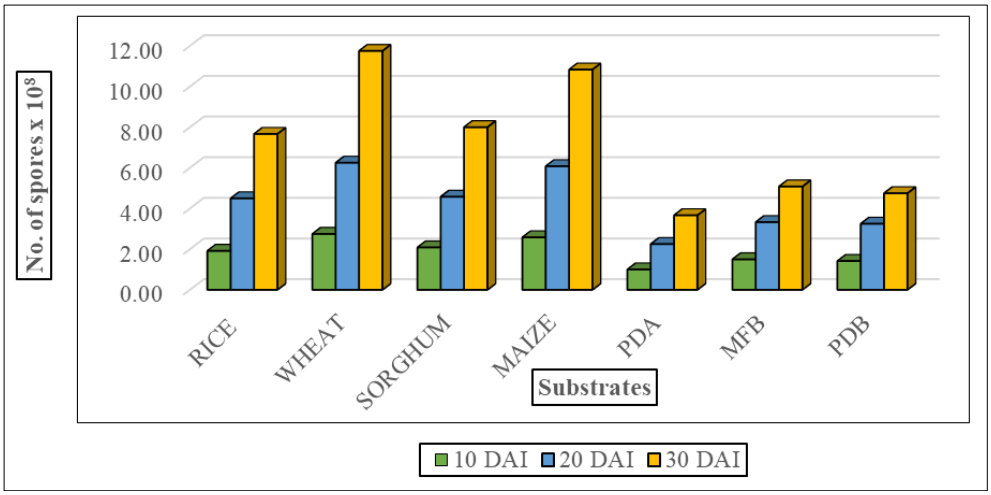

Fig 1: Mass production of *Metarhizium anisopliae* on different substrates

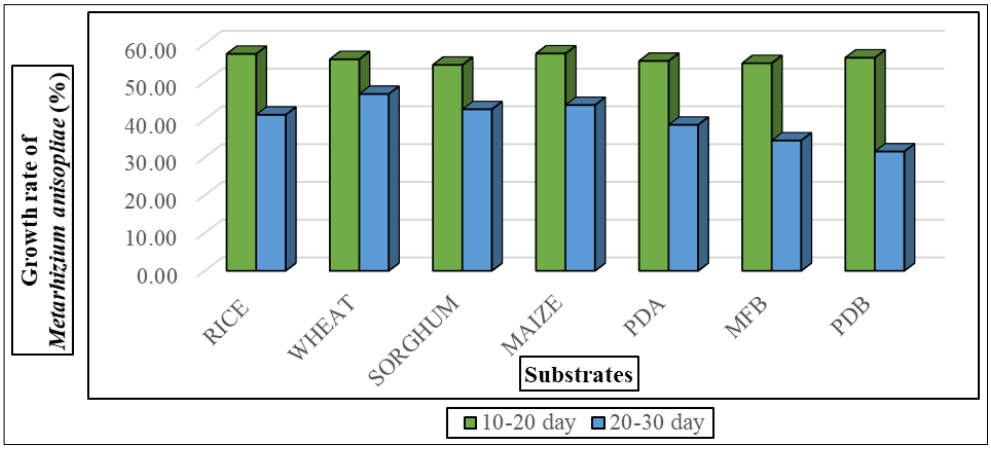

Fig 2: Impact of substrates on growth rate of Metarhizium anisopliae

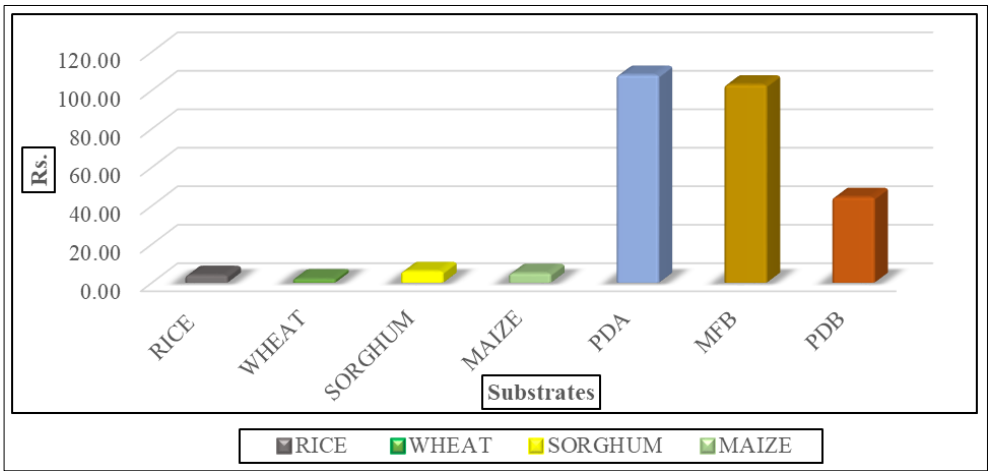

Fig 3: Economics of mass production of Metarhizium anisopliae on different substrate

Conclusion

The study concludes that wheat is the most reliable substrate for large-scale, economical production of *Metarhizium anisopliae*, with maize and rice as viable alternatives for sustainable biocontrol programs.

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