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Research Article

Mass production of insect pathogenic hypocreale fungi, *Metarhizium anisopliae* on solid substrates and liquid media

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ABSTRACT: Mass production of *Metarhizium anisopliae* on different locally available solid substrates *viz*., pearl millet, maize, sweet corn, cowpea, rice bran, wheat bran, press mud + yeast extract (1%) and bagasse + yeast extract (1%) and Pulses *viz*., green gram, red gram, chick pea and cowpea and liquid media *viz*., sabouraud's dextrose broth, potato dextrose broth, sabouraud's maltose broth, sabouraud's sucrose broth and sabouraud's glucose broth was taken up. Significantly highest number of CFU were recorded in green gram, 94.00, 91.00 and 97.00 CFU per ml with 13.24 × 10^8, 16.42 × 10^8 and 15.56 × 10^8 conidia per ml in 3 promising strains, UASRBC-Ma2, UASRBC-Ma31 and ICAR-NBAIRMa4, respectively followed by wheat bran, 78.00, 73.00 and 93.33 CFU per ml with 22.00 × 10^8, 26.23 × 10^8 and 24 × 10^8 conidia per ml and maximum dry mycelia recorded in Sabouraud's maltose yeast broth, 127.33 mg, 219.67 mg, 325.67 mg and 386.00 mg per 100 ml of liquid broth on 4th, 6th, 8th and 10th days after inoculation. The present study revealed that solid substrates namely green gram, rice and wheat bran and liquid media, sabouraud's maltose broth provide a simplest productive medium for mass production of *Metarhizium anisopliae* spores.

KEYWORDS: Mass production, solid substrates, liquid media

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INTRODUCTION

Insect pathogenic fungi under the phylum Deuteromycota are widely distributed (Zimmermann, 1986; Humber, 1997). There are around 250-300 such fungal isolates infecting Coleoptera, Dermaptera, Hemiptera, Lepidoptera and Orthoptera (Moore *et al*., 1996) and these provide biological control alternatives for insect control. These include *Metarhizium anisopliae***,** *Beauveria bassiana***,** *Metarhizium rileyi***,** *Lecanicilium lecanii***,** *Isaria fumasorosea* and *I. farinosa***.** In 1883, Metchinikoff cultured such insect pathogens for experiment with two beetle pests. The current study evaluates certain solid substrates and liquid media *viz*., pearl millet, maize, sweet corn, cowpea, rice bran, wheat bran, press mud + yeast extract (1%) and bagasse + yeast extract (1%) and Pulses *viz*., green gram, red gram, chick pea and cowpea and liquid media *viz*., sabouraud's dextrose broth**,** potato dextrose broth, sabouraud's maltose broth, sabouraud's sucrose broth and sabouraud's glucose broth for mass culturing *Metarhizium anisopliae*.

MATERIALS AND METHODS

Evaluation of solid substrates for mass production of *M. anisopliae*

Seven different solid substrates namely pearl millet, maize, sweet corn, cowpea, rice bran, wheat bran, press mud + yeast extract (1%) and bagasse + yeast extract (1%) and Pulses *viz*., green gram, red gram, chick pea and cowpea (Table 1) were used. 100 g of each of the substrates except rice bran, wheat bran press mud and bagasse were soaked in sterile distilled water overnight in 500 ml polypropylene bags. Rice bran, wheat bran, press mud and bagasse were moistened with sterile distilled water. All the substrates were sterilized in autoclave at 121°C at 15 lb pressure for 30 min. After cooling each of the substrate was inoculated with 1 × 10^6 spores of individual isolates of *M. anisopliae*. For assessing the spore count homogenous conidial suspension was prepared by adding Tween 80 (0.02 %) as wetting agent to get uniform spore suspension. Spores were further extracted by passing through muslin cloth. The number of conidia produced per gram of the solid substrate was carried out by serial dilution by pour plate technique to count the colony forming units (CFU/gram/ml). The conidia produced per g of medium were calculated at 15 days after inoculation.

Evaluation of liquid media for mass production of *M. anisopliae*

Potato dextrose broth

200 g of peeled and sliced potato was added in 500 ml distilled water, the potatoes were boiled till they became soft. The contents of the beakers were filtered through muslin

Table 1. Details of substrates (food grains) evaluated for mass production of *M. anisopliae*

Sl. No.	Common Name	Scientific name	Plant part	Quantity	Spore suspension concentration (CFU/ ml)
1	Rice	*Oryza sativa*	Grain	100 g	1×10^8
2	Sorghum	*Sorghum bicolor*	Grain	100 g	1×10^8
3	Maize	*Zea mays*	Grain	100 g	1×10^8
4	Green gram	*Vigna radiata*	Grain	100 g	1×10^8
5	Black gram	*Vigna mungo*	Grain	100 g	1×10^8
6	Bengal gram	*Cicer arietinum*	Grain	100 g	1×10^8
7	Molasses	-		100 g	1×10^8
8	Press mud	-		100 g	1×10^8
9	Sweet corn	*Zea mays*	Grain	100 g	1×10^8
10	Rice bran	-		100 g	1×10^8
11	Wheat bran	-		100 g	1×10^8

cloth and squeezed out all liquid. 20 g dextrose was dissolved in water and added to the extract and made the volume to 1000 ml. Dispensed 250 ml to each conical flask and plugged with non-absorbent cotton. The flasks were sterilized at 15 psi pressure for 20 min in an autoclave. After cooling, 5 mm fungal disc of entomopathogenic fungus was inoculated into each flask under laminar air flow chamber and replicated thrice.

Sabouraud's dextrose broth

1000 ml of distilled water was taken, in which 10 g of dextrose and 2.5g of peptone was added, and dispensed 250 ml media into 500 ml conical flask and plugged with non-absorbent cotton. Sterilized the flasks at 15 psi pressure for 20 min in an autoclave. After cooling, 5 mm fungal disc of entomopathogenic fungus was inoculated into each flask under laminar air flow chamber and three replications were maintained.

Sabouraud's Maltose broth

1000 ml of distilled water was taken, in which 20 g of maltose and 10 g of peptone was added, and dispensed 250 ml media into 500 ml conical flask and plugged with non-absorbent cotton. Sterilized the flasks at 15 psi pressure for 20 min in a autoclave. After cooling, 5 mm fungal disc of entomopathogenic fungus was inoculated into each flask under laminar air flow chamber and replicated thrice.

Sabouraud's Sucrose broth

1000 ml of distilled water was taken, in which 20 g of sucrose and 10 g of peptone was added and dispensed 250 ml media into 500 ml conical flask and plugged with non-absorbent cotton. Sterilized the flasks at 15 psi pressure for 20 min in an autoclave. After cooling, 5 mm fungal disc of entomopathogenic fungus was inoculated into each flask under laminar air flow chamber and three replications were maintained.

Jaggery broth

1000 ml of distilled water was taken, in which 40 g of jaggery and 5 per cent yeast extract and dispensed 250 ml media into 500 ml conical flask and plugged with non-absorbent cotton. Sterilized the flasks at 15 psi pressure for 20 min in an autoclave. After cooling, 5 mm fungal disc of entomopathogenic fungus was inoculated into each flask under laminar air flow chamber. The observations on per cent surface coverage and biomass developed by fungus on 10^{th} days after inoculation were noted. The experimental data were subjected to statistical analysis (ICAR-WASP version 2.0 and PAST software). These experiments were carried out in CRD with three replications. The drawn samples were also tested for its Colony Forming Unit (CFU) per gram simultaneously with growth and biomass development.

RESULTS AND DISCUSSION

I. Evaluation of solid media for the mass production of of *M. anisopliae*

Inexpensive and high speed mass production of UASRBC-Ma2, UASRBC-Ma31 and ICAR-NBAIR Ma4 strains, the provincially obtainable solid substrate such as rice, sorghum, maize, green gram, black gram, bengal gram, molasses, press mud, sweet corn, rice bran and wheat bran were evaluated (Table 2) (Figure 1 a and b). Numbers of colony forming units are varied among the different solid substrates, rice, sorghum, maize, green gram, black gram, bengal gram, molasses, press mud, sweet corn, rice bran and wheat bran. Significantly highest number of CFU were recorded in green gram, 94.00, 91.00 and 97.00 CFU per ml with 13.24×10^8, 16.42×10^8 and 15.56×10^8 conidia per ml in 3 promising strains, UASRBC-Ma2, UASRBC-Ma31

and ICAR-NBAIRMa4, respectively followed by wheat bran, 78.00, 73.00 and 93.33 CFU per ml with 22.00 × 10^8, 26.23 × 10^8 and 24 × 10^8 conidia per ml, Rice, 69,00, 66.00 and 73.33 CFU per ml with 22.0 × 10^8, 24.26 × 10^8 and 21.36 × 10^8 conidia per ml. Latifian *et al.* (2014) noticed the highest conidia production (2.8 × 10^6 conidia per ml) on rice followed by sorghum seeds (2.45 × 10^6). Rice waste also supported more conidia as well as biomass production. Tincilley (2000) recorded that carrot was found to be the inexpensive and best suitable media for the large-scale production of Deuteromycete fungi. Rice husk along with 2 per cent dextrose solution recorded more sporulation of *M. anisopliae* (Puzari, 1997). This holds good with the current study with respect to wheat bran recorded more sporulation of mycopathogen *M. anisopliae.* From this investigation it was clear that the *M. anisopliae* was able to grow on both cereals and pulses in solid state and this can be useful to farmers to culture these fungi easily in farm on press mud, molasses and rice bran. According to Ibrahim and Low (1993) and Sharma (2002), rice was found to be the suitable media for the mass culture of *B. bassiana*. This cereal was also used for the mass production of other Deuteromycete fungi. Gopalakrishnan (1999) recorded that sorghum was the perfect cereal for the mass production of *Paecilomyces farinosa*. In the case of *V. lecanii,* sorghum was found to be the ideal substrate for mass production, which is in confirmation with the findings of Lakshmi (2001). Sharma *et al*. (1999) and Bhide (2001) obtained the maximum sporulation of *M. anisopliae* on sorghum grains. Thus the results of current investigation are in line with above. Banu (2012) studied solid state fermentation, with five grains *viz*., Rice, Wheat, Sorghum, Pearl millet and Finger millet along with PDA for multiplication of *L. lecanii* and recorded maximum sporulation of fungus *L. lecanii* on solid state fermentation with rice grains (9.84 × 10^8 conidia per g) followed by wheat grains (9.12 × 10^8 conidia per g).

II. Evaluation of different liquid media for the mycelial production of promising strains of *M. anisopliae*

One of the key factors in enhancing the population of mycopathogens propagules in the inoculum is the availability the nutrients. Several attempts have been made to multiply these mycopathogens using semi synthetic and solid substrate primarily to cut down cost of production. Additionally, the availability of nutrients also influences the survival of these organisms in nature. Carbon and nitrogen are the most essential nutrients required for growth and sporulation (Campbell *et al.,* 1983). Therefore, the effect of different carbohydrates and nitrogen on the growth of *M. anisopliae* was assessed at different concentrations in Sabouraud's broth. The growth of the fungal strains, UASRBC-Ma2, UASRBC-Ma31 and ICARNBAIR-Ma4 was studied on five different media *viz*., SMYB, SDB, PDB, SGB and SSB. The growth of the fungal isolate Ma2 in terms of dry mycelial weight per 100 ml of broth was recorded and the findings are represented in the Table 3. Different liquid media evaluated for mass production of *M. anisopliae* (UASRBC-Ma2), significant maximum dry mycelia recorded in Sabouraud's maltose yeast broth, 127.33 mg, 219.67 mg, 325.67 mg and 386.00 mg per 100 ml of liquid broth on 4^{th}, 6^{th}, 8^{th} and 10^{th} days after inoculation, respectively followed by sabouraud's sucrose yeast broth recorded 125.00 mg, 167.67 mg, 206.67 mg and 261.67 mg on 4^{th}, 6^{th}, 8^{th} and 10^{th} days after inoculation, respectively. Minimum mycelia recorded in Sabouraud's glucose yeast broth, 94.33 mg, 131.00 mg, 188.33 mg and 220.67 mg on 4^{th}, 6^{th}, 8^{th} and 10^{th} days after inoculation, respectively (Table 3). Different liquid media evaluated for mass production of *M. anisopliae* (UASRBC-Ma31), significantly highest dry mycelia recorded in Sabouraud's maltose yeast broth, 131.00 mg, 184.67 mg, 217.67 mg and 270.00 mg per 100 ml of liquid broth on 4^{th}, 6^{th}, 8^{th} and 10^{th} days after inoculation, respectively followed by Sabouraud's sucrose yeast broth recorded 97.00 mg, 128.67 mg, 132.33 mg and 176.67 mg on 4^{th}, 6^{th}, 8^{th} and 10^{th} days after inoculation. Lowest dry mycelia recorded in Potato dextrose broth, 68.67 mg, 99.33 mg, 128.33 mg and 193.33 mg per 100 ml of liquid broth on 4^{th}, 6^{th}, 8^{th} and 10^{th} days after inoculation, respectively (Table 3). Similarly, in *M. anisopliae* (ICAR-NBAIRMa4), significantly highest dry mycelia recorded in sabouraud's maltose yeast broth, 136.33 mg, 206.67 mg, 288.33 mg and 320.67 mg per 100 ml of liquid broth on 4^{th}, 6^{th}, 8^{th} and 10^{th} days after inoculation, respectively followed by Sabouraud's glucose yeast broth recorded 128.33 mg, 167.33 mg, 221.67 mg and 308.00 mg on 4^{th}, 6^{th}, 8^{th} and 10^{th} days after inoculation (Table 3).

Current results are in accordance with that Pandey *et al.,* (2010) revealed that Sabouraud's dextrose yeast broth medium was the best with regards to biomass production, conidial count (4.80 × 10^7 conidia per ml) of *M. anisopliae* and *B. bassiana.* In earlier studies, the most effective carbon sources for sporulation of *N. rileyi* were reported to be dextrose (Im *et al*., 1988) and maltose (Balardrin and Loch, 1989). Edelstein *et al.,* (2004) on the other hand found that media with potato extract induced higher growth rate.

The fungus, *B. bassiana* grow very well in sabouraud's agar medium + yeast extract (Knudsen *et al*., 1991), sabouraud's dextrose both with 1 per cent yeast (SDBY) was used for the production of *B. bassiana* (Bextine and Thorvilson, 2002). In all the media used in the present study had carbohydrate source as one of its ingredients. The addition of glucose, maltose or complex sugar-rich media increased the bio control effect (Druvefors *et al*., 2005). Sugars can improve the viability of freeze-dried cultures that are important component of the formulation of bio control agent.

Table 2. Evaluation of different substrate for mass production of promising isolates of *Metarhizium anisopliae* during 2019-20

Sl. No.	Substrates	Number of CFU/ml per gm of solid substrate ($x10^6$) (14 DAI)			No. of conidia per gram of solid substrate ($\times10^8$) (14 DAI)		
		UASR BC-Ma2	UASR BC-Ma31	ICAR-NBAIR-Ma 4	UASR BC-Ma2	UASR BC-Ma31	ICAR-NBAIR-Ma 4
1	Rice	69.00 (8.31)^{c}	66.00 (8.15)^{c}	73.33 (8.59)^{c}	22.00	24.26	21.36
2	Sorghum	41.00 (6.40)^{g}	36.67 (6.09)^{f}	52.33 (7.26)^{f}	11.56	12.62	10.23
3	Maize	53.00 (7.28)^{f}	51.33 (7.19)^{e}	83.33 (9.15)^{e}	12.62	13.00	9.00
4	Green gram	94.00 (9.69)^{a}	91.00 (9.56)^{a}	97.00 (9.87)^{a}	23.00	20.00	24.12
5	Black gram	64.33 (8.02)^{d}	61.00 (7.84)^{d}	69.00 (8.33)^{d}	13.24	16.42	15.56
6	Bengal gram	59.33 (7.70)e	57.33 (7.60)^{d}	42.33 (6.54)^{d}	12.63	14.00	13.65
7	Molasses	29.00 (5.38)^{h}	25.67 (5.11)^{g}	36.00 (6.04)^{g}	8.23	11.00	10.00
8	Press mud	21.00 (4.58)^{j}	19.33 (4.45)^{h}	42.33 (6.54)^{h}	3.78	4.23	5.26
9	Sweet corn	27.33 (5.23)^{h}	24.67 (5.01)^{g}	45.00 (6.74)^{g}	7.80	8.00	12.32
10	Rice bran	24.00 (4.89)i	20.67 (4.59)^{h}	36.33 (6.06)^{h}	8.82	13.00	9.52
11	Wheat bran	78.00 (8.83)^{b}	73.00 (8.57)^{b}	93.33 (9.68)^{b}	22.00	26.23	24.00
12	Control	0.00 (0.70)^{i}	0.00 (0.70)^{i}	0.00 (0.70)^{i}	0.00	0.00	0.00
SEm±		**0.07**	**0.14**	**0.13**	**-**	**-**	**-**
CD at P=0.01		**0.22**	**0.42**	**0.40**	**-**	**-**	**-**

Figures in the parenthesis indicate √x+0.5 transformed values.
CD @ P = 0.01 significant at (0.01) per cent level of significance
Values in the column followed by common letters are non-significant at p = 0.05 as per Tukey's HSD (Tukey, 1965)

Table 3. Liquid media evaluation for mass production of *Metarhizium anisopliae*

Sl. No	Treatments	Yield of dry mycelia (mg/100ml of liquid media)											
		UASR BC-Ma2				UASR BC-Ma31				ICAR-NBAIR-Ma4			
		4th	6th	8th	10th	4th	6th	8th	10th	4th	6th	8th	10th
1	SDB	117.67 (10.87)^{b}	216.67 (14.72)^{a}	319.33 (17.88)^{a}	356.67 (18.89)^{b}	78.33 (8.82)^{d}	109.00 (10.42)^{d}	194.33 (14.52)^{d}	241.00 (15.47)^{b}	83.00 (9.14)^{c}	114.67 (10.71)^{c}	221.67 (14.89)^{b}	284.33 (16.86)^{b}
2	PDB	108.33 (10.43)^{c}	133.00 (11.53)^{c}	196.33 (14.03)^{c}	242.00 (15.57)^{d}	68.67 (8.13)^{e}	99.33 (9.99)^{e}	128.33 (11.57)^{e}	193.33 (13.92)^{d}	73.00 (8.57)^{d}	110.67 (10.52)^{c}	156.67 (12.52)^{c}	197.00 (14.04)^{d}
3	SMYB	127.33 (11.44)^{a}	219.67 (14.82)^{a}	325.67 (18.06)^{a}	386.00 (19.66)^{a}	131.00 (11.37)^{a}	184.67 (13.64)^{a}	217.67 (15.00)^{a}	270.00 (16.44)^{a}	136.33 (11.69)^{a}	206.67 (14.37)^{a}	288.33 (16.83)^{a}	320.67 (17.90)^{a}
4	SSYB	125.00 (11.64)^{a}	167.67 (12.95)^{b}	206.67 (14.39)^{b}	261.67 (16.19)^{c}	97.00 (10.27)^{c}	128.67 (11.45)^{c}	132.33 (11.95)^{c}	176.67 (13.38)^{d}	67.00 (8.21)^{e}	103.33 (10.16)^{c}	127.00 (11.22)^{d}	234.00 (15.29)^{c}
5	SGYB	94.33 (9.84)^{d}	131.00 (11.45)^{c}	188.33 (13.74)^{d}	220.67 (14.87)^{e}	117.67 (11.09)^{b}	135.67 (11.75)^{b}	153.00 (12.75)^{b}	211.00 (14.88)^{c}	128.33 (11.35)^{b}	167.33 (13.00)^{b}	221.67 (14.89)^{b}	308.00 (17.55)^{e}
6	Control	0.00 (0.70)^{e}	0.00 (0.70)^{d}	0.00 (0.70)^{e}	0.00 (0.70)^{f}	0.00 (0.70)^{f}	0.00 (0.70)^{f}	0.00 (0.70)^{f}	0.00 (0.70)^{f}	0.00 (0.70)^{f}	0.00 (0.70)^{d}	0.00 (0.70)^{e}	0.00 (0.70)^{f}
F		**	**	**	*	*	*	*	*	*	*	*	*
S Em±		0.15	0.06	0.09	0.18	0.13	0.08	0.19	0.72	0.14	0.32	0.06	0.28
CD at P = 0.01		0.32	0.17	0.27	0.52	0.39	0.26	0.55	2.14	0.42	0.96	0.20	0.84

*significant at 5 per cent level of significance
**significant at 1 per cent level of significance
Figures in the parenthesis indicate √x+0.5 transformed values
CD at P = 0.01 significant at 1 (0.01) per cent level of significance
SDB: Sabouraud's dextrose Broth, PDB: Potato Dextrose Broth, SMYB: Sabouraud's Maltose Yeast Broth, SSYB: Sabouraud's Sucrose Yeast Broth, SGYB: Sabaurd's Glucose Yeast broth

Figure 1 (a) and (b). Preliminary screening of different solid substrates for mass production of promising isolates of *Metarhizium anisopliae*.

CONCLUSION

Solid substrates and liquid media evaluation methodology allows to optimize the mass production of *M. anisopliae.* Solid substrates namely green gram, rice and wheat bran and liquid media, sabouraud's maltose broth provide a simplest productive medium for mass production of *M. anisopliae* spores.

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