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Evaluation of economic medium for mass multiplication of entomopathogenic fungus *Metarhizium anisopliae*

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Abstract
The present investigation was carried out during *Rabi* 2013-2014 at the Entomology laboratory, College of Agriculture JNKVV, Jabalpur at Completely Randomized Design (CRD) to select a suitable and economic medium for mass multiplication of *Metarhizium anisopliae*. The experiment on mass production studies was undertaken on fifteen substrates for determining a suitable medium for growth and sporulation. The observations were recorded on 10th, 20th and 30th days after inoculation. Among the different substrates evaluated highest conidial count (12.68×10^7 spores/ml) was observed on cowpea media followed by pea (11.84×10^7 spores/ml). It was also clear that *M. anisopliae* is able to grow on a variety of cheap and easily available grains; hence they can be used for the mass multiplication of the fungus and produced in bulk and can be made available at the doorstep of the farmers.

Keywords: Mass multiplication; *metarhizium anisopliae;* economic medium; days after inoculation

Introduction
Entopathogenic fungi are ideal for Integrated Pest Management (IPM) programs because they are relatively safe to use and have a narrow spectrum of activity than chemical insecticides [1-2].
The integration of microbial pesticides with chemical pest management practices requires detail compatibility studies. Data from such studies would enable farmers to select appropriate compounds and schedule microbial and chemical pesticide treatments such that benefits from compatible sets can be acquired and with non compatible pairs the deleterious effects of the chemical on the microbe in the biopesticide can be minimized.
The ready availability of the mycoinsecticides unlike chemical insecticides is a challenging factor in testing the pathogenecity of the fungal pathogens against target insect hosts. For the evaluation of the entomopathogenic fungus under field conditions, mass multiplication of the fungus on suitable substrate is necessary [3-5].
Lack of reliable substrates was found to be another major constraint in the mass production and utilization of the mycoinsecticides. Hence an attempt was made to determine the most suitable and locally available solid and liquid substrate for the mass multiplication of the fungus.

Material and methods
The experiments were carried out during *Rabi* 2013-2014 at the Entomology laboratory, College of Agriculture JNKVV, Jabalpur under Completely Randomized Design (CRD). There were fifteen substrates for determining a suitable medium for growth and sporulation of mass multiplication of *Metarhizium anisopliae*. (Table 1)

Media preparation
1. Solid substrates
(a) Pulses and (b) Oil seeds
Pulses *viz.* black gram, cowpea, gram, green gram, lentil, pea and rajma and oilseed *viz.* groundnut and soybean were used for estimating the sporulation of *Metarhizium anisopliae* at 25ºC. For this purpose 100g of each grain was washed and soaked in water overnight and cooked till it became soft. The excess water was drained by decanting and shade dried. The 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 *M. anisopliae* was inoculated into each flask under laminar air flow chamber. Flasks were incubated in BOD incubator at 25ºC. Two replications were maintained for each 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.

(c) Cakes

100g of each substrate was taken in a 250ml conical flask and to it 50 ml of sterile distilled water was added. The substrates were sterilized in an autoclave at 15 psi for 20 min. After cooling, 5 mm fungal disc of *M. anisopliae* was inoculated into each flask under laminar air flow chamber. Flasks were incubated in BOD incubator at 25ºC. Two replications were maintained for each cake. To avoid clumping, after 7 days of inoculation, the flasks were shaken vigorously to separate the cake pieces and also to break the mycelia mat.

2. Liquid substrates

(a) Vegetables

250 ml of distilled water was taken in 1l beaker and 100g of peeled and sliced carrot / okra was added. The vegetables were boiled till they became soft. The contents of the beaker were filtered through muslin cloth and all the liquid was squeezed out. 10 g dextrose was dissolved in water, was added to this extract and the volume was made to 500 ml. Dispensed 100ml to each conical flask and plugged with non-absorbent cotton. The flasks were sterilized in autoclave at 15 psi for 20 min. After cooling, 5 mm fungal disc of *M. anisopliae* was inoculated into each flask under laminar air flow chamber. Flasks were incubated in BOD incubator at 25ºC. Two replications were maintained for each vegetable. To avoid clumping, after 7 days of inoculation, the flasks were shaken vigorously to separate and also to break the mycelia mat.

3. Artificial media

Potato dextrose broth

100g of peeled and sliced potato was added in 250 ml distilled water in 1l 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. 10g 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 for 20 min in an autoclave. After cooling, 5 mm fungal disc of *M. anisopliae* was inoculated into each flask under laminar air flow chamber. Flasks were incubated in BOD incubator at 25ºC. Two replications were maintained.

Sabouraud's dextrose broth

250 ml of distilled water was taken, in which 10 g of dextrose and 2.5g of peptone was added, and dispensed 100 ml media into 250 ml conical flask and plugged with nonabsorbent cotton. The flasks were sterilized at 15 psi pressure for 20 min in an autoclave. After cooling, 5 mm fungal disc of *M. anisopliae* was inoculated into each flask under laminar air flow chamber. Flasks were incubated in BOD incubator at 25ºC. Two replications were maintained.

Effect of substrate on sporulation of *M.anisopliae*

Observations on spore counting were done on 10th, 20th and 30th day after inoculation. The spores of the fungus grown on various substrates were estimated using haemocytometer. For this purpose 10g or 10ml of homogenous grain or solution sample was drawn from each replicate of uniformly sporulating flasks and was transferred to 100 ml sterilized distilled water containing Tween 80 (0.05%) solution in 250ml conical flasks. The flasks were shaken in shaker for 10 min. The suspension was filtered through double layered muslin cloth. Counting of spore's were made after the serial dilution of the suspension using double ruled Neubauer haemocytometer for determining the number of conidia in 1 g of the substrate.

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

Tr. code	Treatments	
I	**Solid substrates**	
T_1	Pulses	Black gram, *Vigna mungo* (L) Hepper
T_2		Cowpea, *Vigna unguiculata* (L) Walp.
T_3		Gram, *Cicer arietinum* (L)
T_4		Green gram, *Vigna radiata* (L) Wilezek.
T_5		Lentil, *Lens esculentum* (M)
T_6		Pea, *Pisum sativum* (L)
T_7		Rajma, *Phaseolus vulgaris* (L)
T_8	Oil seed	Ground nut, *Arachis hypogea* (L)
T_9		Soybean *Glycine max* (L) Merrr.
T_{10}	Cakes	Gingelly cake, *Sesamum indicum* (L)
T_{11}		Neem cake, *Azadirachta indica* (A) Juss.
II	**Liquid substrates**	
T_{12}	Vegetables	Carrot, *Daucus carota* (L)
T_{13}		Okra, *Abelmoschus esculentum* (L) Moench
III	**Artificial media**	
T_{14}		Potato Dextrose Broth
T_{15}		Sabouraud's Dextrose Broth

Statistical Analysis

All the data were subjected to statistical analysis after appropriate transformation as suggested by [6].

Results and discussion

The experiment on mass multiplication studies was undertaken on fifteen substrates for determining a suitable medium for growth and sporulation of the fungus. During this period the maximum and minimum temperature of the laboratory were 39.55 ± 3.65 ^{0}C and 29.6 ± 6.4 ^{0}C, respectively while morning and evening relative humidity were 51 ± 19 % and 24.5 ± 8.5 %, respectively. The observations were recorded on 10th, 20thand 30th days after inoculation and the data presented in Table 2.

1.1. Sporulation

1.1. a. Ten days after inoculation

Among the different substrates evaluated on the 10th day after inoculation, significantly highest conidial count (8.00 × 10^7 spores/ml) was observed on rajmash media followed by pea (7.90 × 10^7 spores/ml), but both were at par with each other. This was followed by groundnut (6.90× 10^7 spores/ml), cowpea (6.60 × 10^7 spores/ml) but they did not differ significantly from each other. This was followed by soybean (6.00× 10^7 spores/ml) and SDB (5.70× 10^7 spores/ml), but both were at par with each other. The conidial counts were found to be low on black gram (2.90 × 10^7 spores/ml), carrot washing (2.80 × 10^7 spores/ml), gingelly cake (2.80 × 10^7 spores/ml), neem cake (2.80× 10^7 spores/ml),green gram (2.70 × 10^7 spores/ml) and lowest on okra washing (2.50×10^7spores/ml), but all were at par with each other.

1.1. b. Twenty days after inoculation

Among the different substrates evaluated on the 20th day after inoculation, significantly highest conidial count (10.25 × 10^7 spores/ml) was observed on cowpea media followed by pea (9.63 × 10^7 spores/ml), but both were at par with each other.

This was followed by SDB (9.38 × 10^7 spores/ml), soybean (9.13 × 10^7 spores/ml) and rajmash (9.00× 10^7 spores/ml), but all were at par with each other. This was followed by groundnut (8.38× 10^7 spores/ml) and PDB (8.25 × 10^7 spores/ml), but both of them did not differ significantly from each other. The next substrate was gram (7.5 × 10^7 spores/ml) which was followed by lentil (6.38 × 10^7 spores/ml), but they differed significantly from each other. This was followed by green gram (5.63 × 10^7 spores/ml), neem cake (5.50× 10^7 spores/ml), but they did not differ significantly from each other. This was followed by gingelly cake (4.88 × 10^7 spores/ml), black gram (4.38 × 10^7 spores/ml) and carrot washing (4.88 × 10^7 spores/ml), but all were at par with each other while least spore count was recorded in okra washing (4 ×10^7spores/ml).

1.1. c. Thirty days after inoculation

Among the different substrates evaluated on the 30th day after inoculation, significantly highest conidial count (21.2 × 10^7 spores/ml) was observed on cowpea media followed by gram (18.7 × 10^7 spores/ml), soybean (18.1 × 10^7 spores/ml) and pea (18.0 × 10^7 spores/ml), but all were at par with each other. The next substrate was PDB (16.8 × 10^7 spores/ml) followed by rajmash (16.00 × 10^7 spores/ml) but they did not differ significantly from each other. This was followed by groundnut (15.6 × 10^7 spores/ml) and green gram (14.5 × 10^7 spores/ml), but both of them were at par with each other, this was followed by gingelly cake (13.7× 10^7 spores/ml) and lentil (13.2 × 10^7 spore/ml), but both were at par with each other while lowest spore count was recorded on okra washing (10 ×10^7spores/ml).

1.1. d. Mean

Among the different substrates evaluated, overall highest conidial count (12.68 × 10^7 spores/ml) was observed on cowpea media followed by pea (11.84 × 10^7 spores/ml) but they differed significantly from each other. This was followed by soybean (11.08 × 10^7 spores/ml) and rajmash (11.00 × 10^7 spores/ml) but they did not differ significantly from each other. This was followed by SDB (10.86 × 10^7 spores/ml), groundnut (10.29 × 10^7 spores/ml), gram (10.17× 10^7 spores/ml) and PDB (9.85 × 10^7 spores/ml) but they were at par with each other. The conidial counts were found to be low on carrot washing (6.26 × 10^7 spores/ml), black gram (6.53 × 10^7 spores/ml) and neem cake (6.57 × 10^7 spores/ml) but all were at par with each other, while least spore count was recorded on okra washing (5.50×10^7spore/ml).

1.1. e. Sporulation on different group of substrates

The entomopathogenic fungus was mass produced on three different groups of substrates *viz.* solid, liquid and artificial media. The solid media included pulses (*viz.* black gram, cowpea, gram, green gram, lentil, pea and rajmash), oilseeds (*viz*. groundnut and soybean) and cakes (*viz*. gingelly cake and neem cake), while liquid medium included vegetable washings (*viz*. carrot and okra) and artificial medium (*viz*. PDB and SDB). The data is presented in Table 3.

1.1. e. (i) 10 days after inoculations

Mean maximum sporulation (5.10 × 10^7 spores/ml) was recorded on artificial media followed by solid media (4.99 × 10^7 spores/ml) and lowest in liquid media (2.65 × 10^7 spores/ml), respectively.

Among the solid substrates, oilseeds recorded maximum mean sporulation (6.45 × 10^7 spores/ml) followed by pulses (5.2 × 10^7 spores/ml) and lowest on cakes (2.80 × 10^7 spores/ml), respectively.

1.1. e. (ii) 20 days after inoculations

Mean maximum sporulation (8.81×10^7 spores/ml) was recorded on artificial media followed by solid media (7.33 × 10^7 spores/ml) and lowest in liquid media (4.44 × 10^7 spores/ml), respectively.

Among the solid substrates, oilseeds recorded maximum mean sporulation (8.75 × 10^7 spores/ml) followed by pulses (7.54 × 10^7 spores/ml) and lowest on cakes (5.19 × 10^7 spores/ml), respectively.

1.1. e. (iii) 30 days after inoculations

Mean maximum sporulation (17.15× 10^7 spores/ml) was recorded on artificial media followed by solid media (15.70× 10^7 spores/ml) and lowest in liquid media (10.55 × 10^7 spores/ml), respectively.

Among the solid substrates, oilseeds recorded maximum mean sporulation (16.85× 10^7 spores/ml) followed by pulses (16.27 × 10^7 spores/ml) and lowest on cakes (12.55 × 10^7 spores/ml) respectively.

1.1. e. (iv) Mean

Mean maximum sporulation (10.35× 10^7 spores/ml) was recorded on artificial media followed by solid media (9.34 × 10^7 spores/ml) and lowest in liquid media (5.88 × 10^7 spores/ml), respectively.

Among the solid substrates, oilseeds recorded maximum mean sporulation (10.68× 10^7 spores/ml) followed by pulses (9.67 × 10^7 spores/ml) and lowest on cakes (6.85 × 10^7 spores/ml), respectively.

1.2. Rate of increase in growth

Rate of increase in growth of *M. anisopliae* was calculated and the data is presented Table 2 and depicted in Fig. 1.

4.1.2. a. From 10th to 20th day after inoculation

The rate of increase in growth of *M. anisopliae* on different substrates from 10th to 20th day after inoculation were found to be non-significant. The highest rate of increase in growth of *M. anisopliae* was recorded on green gram (52.01%) followed by neem cake (48.94%), PDB (45.56%), gram (42.64%), gingelly cake (42.63%), carrot washing (41.68)%, SDB (39.15%), okra washing (37.50%), lentil (36.95%), cowpea (35.62%), soybean (34.18%), black gram (33.60%), pea (17.92%), groundnut (17.62%) and least was recorded on rajmash (11.11%) but all were at par with each other.

4.1.2. b. From 20 th to 30 th day after inoculation

All the treatments showed significant differences in the rate of growth of *M. anisopliae* on different substrates. Among the different treatments evaluated, the rate of increase in growth for 20 th to 30 th day after inoculation was significantly highest on black gram (64.41%) followed by gingelly cake (64.40%) and green gram (61.17 %), but all were at par with each other. This was followed by okra washing (59.94%) and gram (59.88%), but they did not differ significantly from each other. This was followed by neem cake (51.75%), lentil (51.67%), cowpea (51.65%), PDB (50.88%) and soybean (49.62%), but all were at par with each other. The conidial counts were found to be low on pea (46.50%), SDB (46.45%), ground nut (46.31%) and least on rajmash (43.74 %), but all were at par with each other.

1.3. Economics of mass production of *M. anisopliae* on different substrates

Cost of production of 1×10^7spores was calculated for all the substrates and the data is Table 4 and depicted in Fig. 2.

The cost of production on different substrates significantly varied from each other. Significantly lowest production cost was recorded on pea (T_6) (Rs. 0.83), this was followed by SDB (Rs. 1.39), PDB (Rs. 1.40), soybean (Rs. 1.48), but the latter three were at par with each other. The next substrate was gram (Rs. 1.62), which was significantly superior to cowpea (Rs. 1.92) and groundnut (Rs. 1.98), but the latter two were at par with each other. The next substrate was gingelly cake (Rs. 2.01) which was significantly superior to neem cake (Rs. 2.16) and rajmash (Rs. 2.21), but the latter two did not differ significantly from each other. This was followed by carrot washing (Rs. 2.30) and lentil (Rs. 2.46), and they differed significantly from each other. The next substrate was okra washing (Rs. 2.61) followed by green gram (Rs. 2.68), but they were at par with each other. Highest cost of production was recorded on black gram (Rs.2.97).

Discussion

Among the different substrates evaluated highest conidial count (12.68×10^7 spores/ml) was observed on cowpea media followed by pea (11.84×10^7 spores/ml) whereas no significant difference was observed on conidial count of soybean and rajmash. The present findings are in conformity with the findings of [7-8]. They also reported conidial count of Pantnagar PI isolate of *B. bassiana* was highest on pea. All the pulses are known to have appreciable amount of protein. Chickpea, green gram and black gram contain more of protein (nitrogen) than starch (carbon) and higher nitrogen is important and necessary particularly for mycelia growth of *M. anisopliae* and production of toxins [9] and this could be the possible reason for maximum production of spores on cowpea, pea, soybean, rajmash and gram.

The present study also support the fact that among several naturally available substrates tested for mass multiplication of *M. anisopliae* on pea and in SDB were most suitable for its growth and development and economically cheap. The present findings are in accordance with the findings of [10-12]. They also reported that liquid media SDB, PDB and yeast extract medium supported excellent growth of *M. anisopliae*. It is also clear that *M. anisopliae* is able to grow on a variety of substrates which are easily available, having wide cost range influencing both the production cost and spore yield. These findings are in agreement with that of [5]. They also reported that *M. anisopliae* grows well on a variety of substrates.

Conclusion

Cowpea was found to be the best substrate for mass production of *M.anisopliae* as it produced maximum spores while pea was found to be the cheapest substrate for mass production of *M.anisopliae.*

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

Treatment nos.	Media /Substrates	Spore count (1×10^7spore / ml) at different days after inoculation				Rate of increase in growth of *M.anisopliae* (%) (DAI)	
		10th day	20th day	30th day	Mean	10 th to 20 th	20 th to 30 th
I	**Solid media**						
T_1	Black gram, *Vigna mungo* (L) Hepper.	2.90	4.38	12.30	6.53	33.60 (43.60)	64.41 (48.74)
T_2	Cowpea, *Vigna unguiculata* (L) Walp.	6.60	10.25	21.20	12.68	35.62 (36.77)	51.65 (45.23)
T_3	Gram, *Cicer arietinum* (L)	4.30	7.50	18.70	10.17	42.64 (41.61)	59.88 (50.30)
T_4	Green gram, *Vigna radiata* (L) Wilezek.	2.70	5.63	14.50	7.61	52.01(44.86)	61.17 (52.48)
T_5	Lentil, *Lens esculentum* (M)	4.00	6.38	13.20	7.86	36.95 (38.16)	51.67(44.97)
T_6	Pea, *Pisum sativum* (L)	7.90	9.63	18.00	11.84	17.92(39.70)	46.50(47.21)
T_7	Rajmash, *Phaseolus vulgaris* (L)	8.00	9.00	16.00	11.00	11.11(40.86)	43.74(46.03)
T_8	Ground nut, *Arachis hypogea* (L)	6.90	8.38	15.60	10.29	17.62 (38.14)	46.31 (45.58)
T_9	Soybean, *Glycine max* (L) Merrr.	6.00	9.13	18.10	11.08	34.18 (35.92)	49.62 (49.58)
T_{10}	Gingelly cake, *Sesamum indicum* (L)	2.80	4.88	13.70	7.13	42.63(44.25)	64.40 (50.64)
T_{11}	Neem cake, *Azadirachta indica* (A) Juss.	2.80	5.50	11.40	6.57	48.94 (43.23)	51.75 (47.18)
II	**Liquid media**						
T_{12}	Carrot *Daucus carota* (L)	2.80	4.88	11.10	6.26	41.68 (39.01)	56.12(49.14)
T_{13}	Okra, *Abelmoschus esculentum* (L) Moench.	2.50	4.00	10.00	5.50	37.50(45.92)	59.94 (58.08)
III	**Artificial media**						
T_{14}	Potato Dextrose Broth	4.50	8.25	16.80	9.85	45.56 (44.60)	50.88 (51.66)
T_{15}	Sabouraud's Dextrose Broth	5.70	9.38	17.50	10.86	39.15 (41.56)	46.45 (50.83)
	SEm ±	0.26	0.20	0.33	0.18	3.33	1.10
	CD at 5%	0.78	0.62	0.99	0.54	NS	3.30

Max. Temp. 39.55 ± 3.65 °C, Min. temp. 29.6 ± 6.4°C, Morning RH (%) 51 ± 19, Evening RH (%) 24.5 ± 8. 5, DAI-Days after inoculation
() = Figures in the parentheses are arcsin transformed values, NS = Non significant

Table 3: Mass production of *Metarhizium anisopliae* on different groups of substrates- at a glance

Media	Substrates	Mean spore count (1×10^7spores /ml) at different days after inoculation on different groups of substrates			
		10 DAI	20 DAI	30 DAI	Mean
Solid	Pulses	5.20	7.54	16.27	9.67
	Oil seed	6.45	8.75	16.85	10.68
	Cakes	2.80	5.19	12.55	6.85
	Mean	4.99	7.33	15.70	9.34
Liquid	Mean	2.65	4.44	10.55	5.88
Artificial media	Mean	5.10	8.81	17.15	10.35

DAI- Days after inoculation

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

Treatment Codes	Substrates	Mean spore count (1×10^7spore / ml)	Cost of substrate / 100g (Rs)	Cost of production of *M.anisopliae* 1×10^7spores / ml (Rs)
T_1	Black gram, *Vigna mungo* (L) Hepper.	6.53	7.00	2.97
T_2	Cowpea, *Vigna unguiculata* (L) Walp.	12.68	12.00	1.92
T_3	Gram, *Cicer arietinum* (L)	10.70	5.00	1.62
T_4	Green gram, *Vigna radiata* (L) Wilezek	7.61	8.00	2.68
T_5	Lentil, *Lens esculentum* (M)	7.86	7.00	2.46
T_6	Pea, *Pisum sativum* (L)	19.84	4.00	0.83
T_7	Rajmash, *Phaseolus vulgaris* (L)	11.00	12.00	2.21
T_8	Ground nut, *Arachis hypogea* (L)	10.29	8.00	1.98
T_9	Soybean, *Glycine max* (L) Merrr	11.08	4.00	1.48
T_{10}	Gingelly cake, *Sesamum indicum* (L)	7.13	2.00	2.01
T_{11}	Neem cake, *Azadirachta indica* (A) Juss.	6.57	1.80	2.16
T_{12}	Carrot *Daucus carota* (L)	6.26	2.00	2.30
T_{13}	Okra, *Abelmoschus esculentus* (L) Moench.	5.50	2.00	2.61
T_{14}	Potato Dextrose Broth	9.85	1.44	1.40
T_{15}	Sabouraud's Dextrose Broth	10.86	2.75	1.39
SEm ±		0.18	-	0.04
CD at 5%		0.55	-	0.13

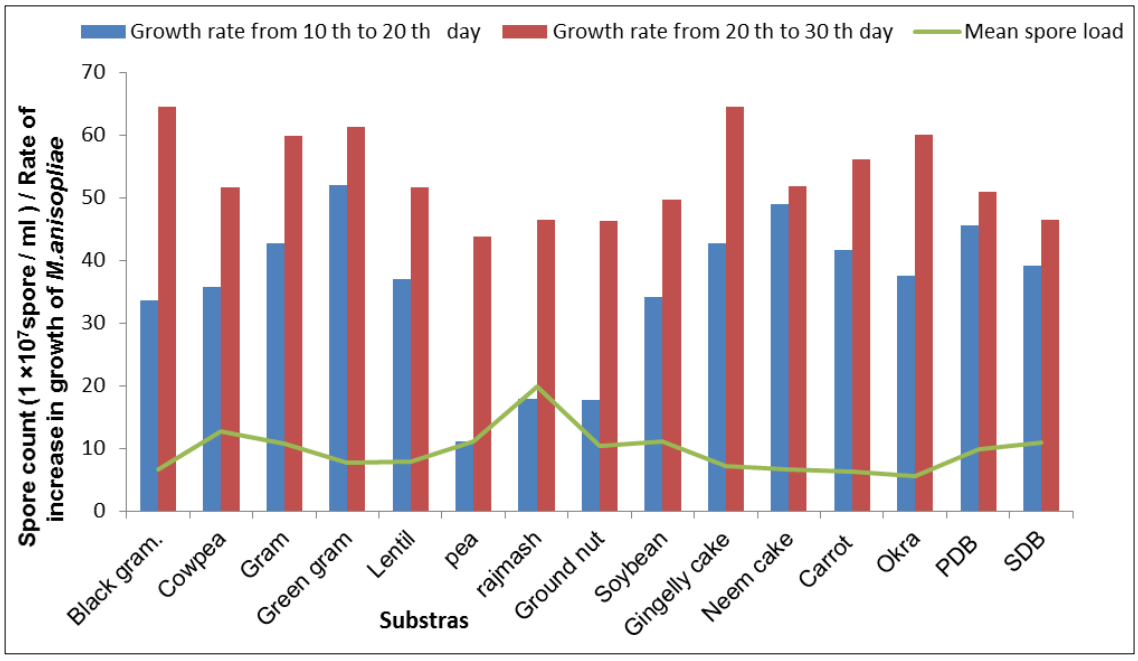

Fig 1: Summary of growth rate and spore production of *Metarhizium anisopliae* on / in different substrates

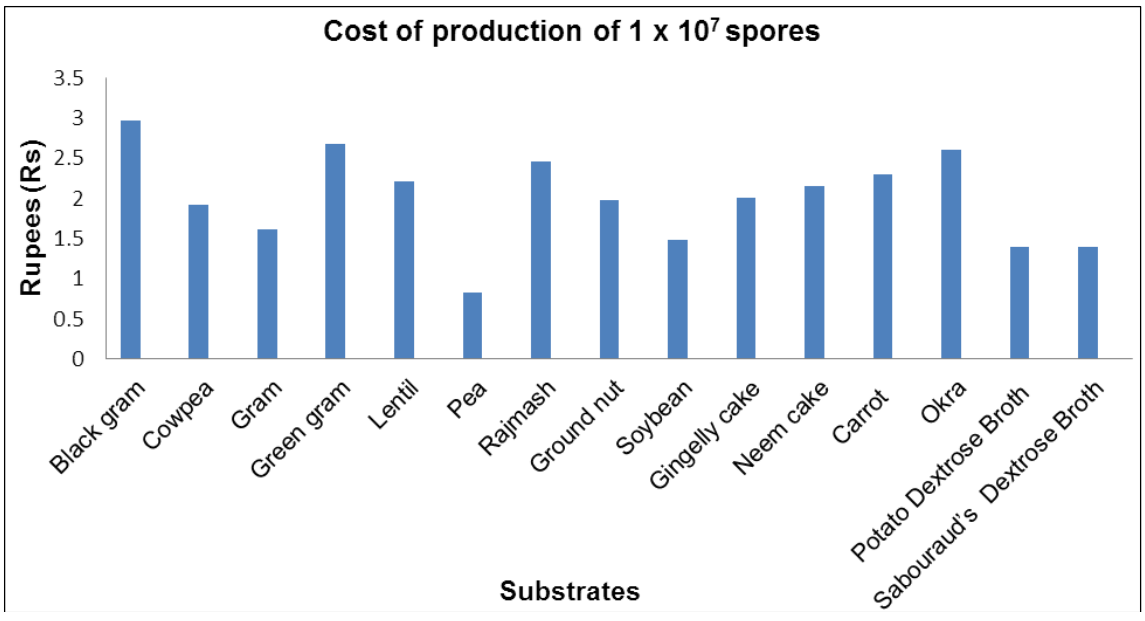

Fig 2: Economics of mass production of *Metarhizium anisopliae* on / in different substrates

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