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Talc formulation of *Metarhizium anisopliae* (Metsch.) Sorokin and its viability on storage

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Abstract
Four *Metarhizium anisopliae* isolates *viz.* NBAIR (Ma-12), NBAII- 11, MTCC-3872 and PDBC (Ma-13) were evaluated for their growth parameters on different cereal substrates *viz.* maize, rice, sorghum and wheat. Maximum spore count (60.75 $X10^5$ $CFUg^{-1}$) and biomass (3.79g $100ml^{-1}$) was obtained on rice. Among all four *M. anisopliae* isolates, NBAII- 11 was significantly better than MTCC-3872, NBAIR (Ma-12) and PDBC (Ma-13). Optimum temperature and relative humidity for production of *M. anisopliae* formulation was 25±2 ℃ and 90% RH, respectively. *M. anisopliae* formulation viability on storage at two different temperatures indicated that refrigeration temperature (4±2 ℃) was better as compared to room temperature (25±2 ℃) till six months. The present studies were conducted during 2017 at Punjab Agricultural University, Ludhiana

Keywords: *Metarhizium anisopliae*, storage, talc, temperature, viability

1. Introduction
Metarhizium anisopliae Sorokin (Metchnikoff) commonly known as green muscardine fungus is an important filamentous entomopathogenic fungi used as biocontrol agent against many crop pests [6]. *M. anisopliae* contributes to the natural regulation of insect populations and is pathogenic to more than 200 species from different insect orders [22]. The application of *M. anisopliae* has several advantages over the conventional chemical pesticides, such as limited harm to humans, honey bees, livestock, and crops [1]. This fungus has the ability to directly penetrate the insect cuticle [23] through cuticle-degrading enzymes [4]. Mycoinsecticides are one of the important components of IPM programmes and success of IPM also depends on economics and good quality inoculums of its components. Thus, there is need for making use of cheap substrates for quality production of biopesticides [5]. The critical environmental factors that affect the mass production of *M. anisopliae* are temperature [15, 7] and relative humidity. There is need to optimise these abiotic conditions so as to identify their effect to improve performance and reduces time, materials and labour [8]. An optimum formulation must ensure the biological and chemical stability as well as the viability of the product at storage. [21].

Large scale production of *M. anisopliae* on different substrates depends on identification of a suitable substrate which is cheap, stable and easily available. So, the present study was planned to produce *M. anisopliae* using cereal products and to optimize their growth conditions for its large scale production and formulation viability on storage.

2. Materials and Methods
The present study was conducted at Dr Gurcharn Singh Kalkat laboratories, Entomological Research Farm, PAU, Ludhiana during the year 2017.

2.1 Isolates
Four isolates of *M. anisopliae viz* NBAII-11, PDBC (Ma-13) and NBAIR (Ma-12) were procured from National Bureau of Agricultural Insects Resources (NBAIR) Bengaluru. Whereas, one isolate MTCC-3872 was procured from IMTECH, Chandigarh was used in present study. These isolates were cultured on PDAY media (Peptone 2%, Dextrose 2%, Yeast 1% and Agar 5%, chloramphenicol 0.5%). The PDAY media was inoculated and incubated with these isolates for 14 days. The stock cultures of these isolates was maintained at 4 ℃ until used

2.2 Evaluation of the growth parameters of *Metarhizium anisopliae* on different cereals by solid state fermentation

Sorghum, rice, maize and wheat grains were used as substrate for recording best growth of *M. anisopliae* following the methodology of Mehta *et al.* [17] hundred grams of coarsely crushed grains were soaked in water along with 0.1% yeast extract and kept overnight. Next day water was drained soaked grains were sieved. These grains were then packed separately in autoclavable polythene bags and autoclaved at 15 psi pressure at 121 °C for 30 minutes. They were then inoculated with 5 ml fungal cultures of *M. anisopliae* under aseptic condition and incubated at 25±2 ^{0}C in BOD humidifier. Sporulated polythene bags were thoroughly mixed daily to avoid clumping. Full sporulated fungal bags were cut open after two weeks and grounded in the rotator mixer to form a finely grounded powder and mixed aseptically with talc powder in the ratio of 2:1 and dried under laminar conditions. This *M. anisopliae* bio-formulation was further used for the estimation of colony forming units. For biomass estimation hundred grams of previously dried substrate was taken and finely ground with blender. Then to each grounded substrate, 100 ml of double distilled water was added and filtered with muslin cloth. The filtrate was collected and transferred to volumetric flask and upto one litre media was made with sterilized distilled water. The pH was adjusted to 7.0 before autoclaving. After cooling, 1 ml of the spore suspension of fungi was aseptically inoculated into each flask. The media was incubated at 25±2 ^{0}C for 15 days. After 15 days, the mycelia mats were collected by filtering on pre weighed Whatman 100 filter papers, and dried in the hot air ovenat 60 ^{o}C for 24 hours and weighed again. The difference in weight gave the biomass produced which was calculated by applying the formula:

Biomass (g) = Final weight of filter paper- Initial weight of filter paper

2.3 Optimization of temperature and RH for *Metarhizium anisopliae* formulation production

The best cereal substrate was studied for optimization of temperature and relative humidity according to methodology of Kotwal *et al.* [12]. *M. anisopliae* isolates were inoculated and incubated at three different temperatures *viz.* 20±2 ℃, 25±2 ℃ and 30±2 ℃ for 15 days. There were five replicates per treatment and growth parameter was recorded. Similarly, the effect of relative humidity on *M. anisopliae* growth on best cereal substrate was also recorded for their growth at three different relative humidity *viz.* 50, 75 and 90%.

2.4 Effect of storage temperature on viability of *Metarhizium anisopliae* formulation

The formulations prepared was packed into sterilized polyethylene bags and stored at room temperature (25±2 ℃) as well as at refrigeration temperature (4±2 ℃) for six months. The viability of these formulations of *M. anisopliae* isolates (NBAII-11) and PDBC (Ma-13) was calculated by determining the conidial viability at monthly interval till six months.

2.5 Statistical analysis

The data was recorded as colony forming units (cfu) and biomass (grams) averaged over five replicates and means were compared using ANOVA.

3. Results and Discussion

3.1 Growth parameters of *Metarhizium anisopliae* on different substrates

The colony forming units recorded after monthly interval revealed that among all substrates, rice gave the highest conidial yield (60.75X 10^5cfu/g) as well as biomass (3.79g/ 100ml) for all the isolates (Table 1). Among isolates *M. anisopliae* isolate NBAII-11 recorded maximum colony forming units (63.33X 10^5 cfu/g) followed by (PDBC (Ma-13), MTCC-3872 and NBAIR (Ma-12) which recorded (55.58X 10^5 cfu/g), (48.66X 10^5 cfu/g) and (44.41X 10^5 cfu/g). Biomass production in PDBC (Ma-13) (2.61g/100ml) and NBAII-11 (2.60g/100ml) was also at par with each other. The lowest yield was obtained with maize. The production cost of *M. anisopliae* formulation on rice was (Rs. 54.60/ kg), sorghum (Rs 53.30 / kg), wheat (Rs 56.60/ kg) and maize (Rs 56.00/ kg).

Our results are in corroborate with the findings of Rachappa *et al.* [21] who also reported that broken rice was ideal substrate for mass production of *M. anisopliae* with higher conidial productivity and yield followed by sorghum and other cereal grains. Chandra and Rahman [6] also recorded that in the solid-state fermentation, rice was found to be best grain for biomass production of the *M. anisopliae* followed by barley. Yadav *et al.* [23] used different solid substrates such as grains, vegetable wastes, maize, bran, cotton seed, rice husk, wheat and liquid media such as coconut water for mass production of two entomopathogenic fungi: *B. bassiana* (Bals.) Vuellemin and *M. anisopliae* and concluded rice as best solid substrate for spore production. Latifian *et al.* [14] evaluated sugar cane, corn, barley, rice, millet and sorghum for production *M. anisopliae* by solid state fermentation. They compared performance characteristics of these by wet weight, dry weight, conidia concentration and germination. and reported maximum spore production (2.8 X10^6spores/ml) on rice followed by sorghum seeds (2.45X10^6). They further reported that the moisture content of the rice grain and its surface to volume ratio is important for conidial yield than the actual nutritional composition of the grains.

Table 1: Viable count and biomass production of *Metarhizium anisopliae* isolates on different cereal substrates

Treatments	Colony forming units (1x10^5 cfu g^{-1}) Mean±S.E					Biomass (g 100 ml^{-1})				
	M. anisopliae NBAII-11	*M. anisopliae* PDBC(Ma-13)	*M. anisopliae* NBAIR-12	*M. anisopliae* MTCC-3872	Mean	*M. anisopliae* NBAII-11	*M. anisopliae* PDBC (Ma-13)	*M. anisopliae* NBAIR-12	*M. anisopliae* MTCC-3872	Mean
Sorghum	67.00±1.15	58.00±1.15	48.00±0.57	52.00±1.15	56.25	3.87	3.58	3.53	3.27	3.56
Wheat	59.33±1.45	54.00±1.15	40.33±0.88	46.33±1.20	50.00	2.17	2.34	1.06	1.41	1.74
Rice	62.00±1.15	74.66±2.60	51.33±2.40	55.00±1.52	60.75	4.01	4.36	3.21	3.60	3.79
Maize	52.33±1.20	48.33±0.66	38.00±1.52	41.33±0.88	45.00	0.37	0.16	0.56	0.31	0.22
Mean	63.33	55.58	44.41	48.66		2.60	2.61	1.96	2.15	
CD (p=0.05)	Isolate (A) = 2.12 Substrate (B) = 3.01 AXB = NS					Isolate (A) = 0.17 Substrate (B) = 0.13 AXB = 0.35				

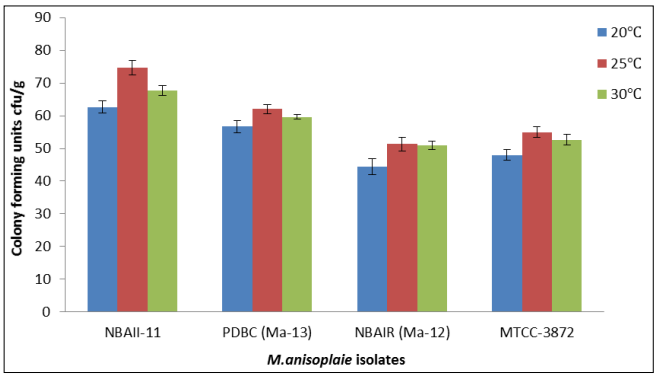

Fig 1: Viable count of *Metarhizium anisopliae* formulation at different temperatures

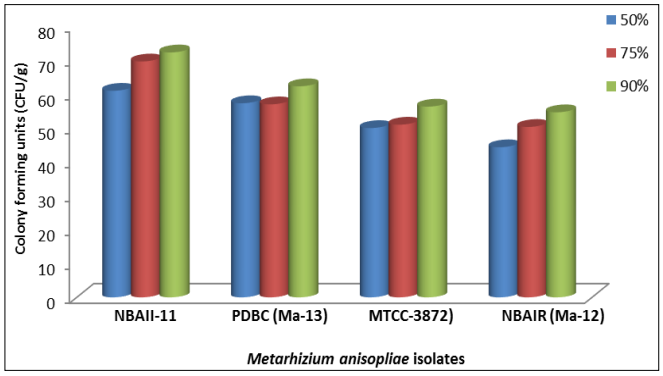

Fig 2: Viable count of *Metarhizium anisopliae* formulation at different relative humidity

Table 2: Viability of *Metarhizium anisopliae* isolate PDBC (Ma-13) and NBAII-11 rice based formulation on different storage temperatures

Days	Colony Forming Units ($1X10^5$cfu g^{-1})			
	***M. anisopliae* NBAII-11**		***M. anisopliae* PDBC (Ma-13)**	
	25 ℃	**4 ℃**	**25 ℃**	**4 ℃**
0 (control)	55.66 (7.52)	51.33	58.00 (7.68)	61.33
30	55.00 (7.48)	51.00	57.33 (7.63)	61.66
60	54.00 (7.41)	50.00	56.66 (7.59)	60.00
90	53.33 (7.37)	48.66	56.00 (7.54)	59.66
120	53.33 (7.37)	49.66	56.00 (7.54)	58.33
150	51.33 (7.23)	48.66	55.33 (7.50)	57.66
180	50.00 (7.14)	48.66	48.33 (7.02)	57.66
CD (p=0.05)	0.21	NS	0.25	NS

3.2 *Metarhizium anisopliae* growth at different temperatures

M. anisopliae recorded maximum viable count at temperature 25±2 ℃ (60.75 $X10^5$ cfu/g) and 30±2 ℃ (57.75 $X10^5$ cfu/g) which were also at par with each other and significantly better than lower temperature 20±2 ℃ (52.6610^5 X cfu/g) (Fig1). Alves [2] too reported that *M. anisopliae* favorable temperature ranged from 24 to 30 °C. Bucarei *et al.* [3] who also reported 25±2 ℃ was optimum temperature to achieve high levels of conidial production in all the substrates. Ouedraogo [20] showed that optimum temperature for vegetative growth of *M. anisopliae* isolates ranged between 25 °C to 32 °C, with 25 °C being the optimum for most isolates. Kumawat [13] studied the effect of temperature and reported that the optimal temperature for mycelial growth was 25 ℃.

3.3 *Metarhizium anisopliae* growth at different relative humidity

M. anisopliae formulation recorded maximum viable count at 75% (57.00 $X10^5$ cfu/g) and 90% RH (61.41 $X10^5$ cfu/g). However, at 50% RH, the growth was significantly lower. Similar observations were recorded by Jairamaiah and Veeresh [10]. This finding was also supported by Namasivayam [19] who reported maximum count of fungal colonies obtained at moisture content 80% and above.

3.4 Storage of *Metarhizium anisopliae* formulations at different storage temperatures

Storage of rice based formulation of two *M. anisopliae* isolates PDBC (Ma-13) and NBAII-11, at room temperature (25±2 ℃) and at refrigeration temperature (4 ℃±2 ℃) was compared. In NBAII-11 when formulation was stored for 180 days the viable count recorded on 0 day (control) was significantly at par with all treatments till 120 days at 25±2 ℃ whereas, at refrigeration temperature (4 ℃±2 ℃) all treatments were non- significant means at par with each other. In PDBC (Ma-13) again at refrigeration storage (4 ℃±2 ℃) all the treatments were non-significant whereas at room temperature, viable counts recorded at 0 day (control) (58.00 X 10^5 cfug^{-1}) was at par with all treatments till 150 days at 25±2 ℃. So, it was concluded that storage at refrigeration temperature (4 ℃±2 ℃) was best as compared to room temperature (25±2 ℃). Similar, observations were recorded by several scientists. Kaur and Joshi [11] also reported talc formulation of *Beauveria bassiana* at refrigeration temperature was best and colony forming units were at par with control (0 day) upto three months of storage. Hong *et al.* [9] developed a model to quantify the effect of temperature and moisture content on the longevity of conidia of the fungi *Metarhizium flavoviride*. This model incorporated a negative semi-logarithmic relation between longevity and a temperature. Morley-Davies [17] who worked on isolates of *B. bassiana* and *M.anisopliae* reported markedly lower tolerance of myco formulations to high temperatures.

4. Conclusion

From our studies it was concluded that broken rice was best cereal substrate for production of *M. anisopliae* formulation at optimum temperature of 25±2 ℃ and 90% RH. Storage of formulation at refrigeration temperature (4 ℃±2 ℃) was better as compared to room temperature (25±2 ℃). Thus, rice is best substrate for large scale production of *M. anisopliae* and the formulation produced could be used for evaluation against crop pests in organic farming

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