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Studies on entomopathogenic fungus *Metarhizium anisopliae* isolated from *Spodoptera litura*

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Abstract
The laboratory experiment was carried out at laboratory of Plant Pathology section, College of Agriculture, Nagpur during 2018-19. Isolates of *Metarhizium anisopliae* were collected, studied and evaluated for its morphological and cultural studies on four different media *viz.*, PDA, SDA, SDAY and SMA. The cultural studies revealed that, SDAY media support maximum linear growth (35.00 mm) and abundant sporulation to *M. anisopliae* followed by SMA, SDA and PDA exhibiting 30.83, 29.50 and 25.33 mm growth, respectively. Hence, SDAY media was selected as basal medium for further studies. It was noticed that, the requirement of temperature for maximum growth and sporulation of *M. anisopliae* fungus was observed at 25 ^{0}C (35.00 mm) and 30 ^{0}C (34.50 mm) followed by room temperature (30.50 mm). The fungus could tolerate15-35 ^{0}C. However, it could not grow at 40^{0}C. All the different humidity levels supported growth and sporulation of *M. anisopliae*. The maximum linear growth was recorded at 95 per cent RH (35.50 mm) with abundant sporulation. Among the different pH levels, pH 5.5 was found to be the best level for growth and sporulation (35.33 mm). It indicates that, the maximum biomass at low pH and least biomass growth at higher basic pH.

Keywords: Metarhizium anisopliae, spodoptera, litura, morphology, growth

Introduction
Metarhizium anisopliae is characterized as green muscardine fungus due to green colour of the sporulating colonies. The colony of *M. anisopliae* appeared white when young but, as conidia matured, the color turned to dark green. The infective unit is conidia or blastospores which germinate and forms short germ tube bearing appresoria with infective peg attach to insect cuticle. The death of insect occurs due to obliteration of tissues, also production of toxins (destructxin A, B, C, D, E) and proteolytic enzymes secreted by the fungus. Infected insects shows symptoms like loss of appetite, decreased irritability, general or partial aparalysis, loss of mobility, discoloration and mummification. Metschnikoff (1879) [11] isolated the fungus *Metarhizium anispliae* for the first time from the larvae of grain weevil and also first to demonstrate entomopathogenic nature of the fungus against chrysomelid, curculonid and scarabaeid beetles. In India, *M. anisopliae* was firstly introduced by Kamat and Dhande (1952) [6] as an effective biocontrol agent against *Pyrilla purpusilla* in Maharashtra. *M. anisopliae* capable of infecting more than 100 different insect pests belonging to a variety of insect orders viz., Orthoptera, Homoptera and Lepidoptera (*Helicoverpa armigera, Spodoptera litura*) (McCoy *et al*., 1988) [10]. The survival and pathogenicity of this fungus may vary depending on environmental factors such as, temperature, relative humidity, pH etc. which affecting the entomopathogenic fungi. These factors are also important for the mass production of the entomopathogens in large scale for use in agriculture. This makes it imperative to evaluate the various factors such as temperature, relative humidity, pH etc. keeping all these in view, the present research work was carried out to clarify the best media for growth and influence of different environmental factors on *M. anisopliae.*

Material and Methods
The laboratory study on morphological and cultural characteristics of entomopathogenic fungus *M. anisopliae* isolated from *S. litura* was conducted at the laboratory of Plant Pathology Section, College of Agriculture, Nagpur during 2018-19. The pure culture of the *M. anisopliae* was isolated from naturally infected larvae of *S. litura* collected from different fields of Nagpur district.

Determination of radial growth
Petriplates containing SDAY media were prepared by evenly spreading 0.5 ml of the inoculum over the agar surface. Agar plugs were cut after ten days incubation at 25 ^{0}C using a sterile 5 mm dia. cork borer. The control and treated plates were inoculated with these agar plugs in the center. Mycelial growth was assessed periodically by measuring the colony diameter at right angles on each plate. For calculations, all the observations were recorded on the 10th day of inoculation.

Determination of conidial density
Spore density was estimated on the 10th day of inoculation on solid medium. Discs of 5 mm diameter were cut and shaken for 1 min. in 2 ml of water plus 0.2 per cent tween 80. For each treatment, aliquots of 0.1 ml were taken and conidial concentration was determined with haemocytometer. Conidial count in 1 ml was counted.

Media
Radial growth of isolate of M. anisopliae was estimated on different culture media viz., Potato dextrose agar (PDA), Sabouraud dextrose agar (SDA), Sabouraud dextrose agar plus Yeast extract (SDAY) and Sabouraud maltose agar (SMA). Fungal growth was assessed as colony diameter and conidial density.

Temperature
For studying the effect of temperature on the growth of *M. anisopliae* isolate, seven levels of temperatures i.e., 15, 20, 25, 30, 35 and 40^{0}C were tested. The Petriplates containing the inoculated media were incubated at respective temperatures in an incubator. The fungal growth was assessed on the basis of colony diameter.

pH
To study the effect of pH on growth of isolate of *M. anisopliae*, seven pH levels, 5.5 to 8.5 were tested. The pH of culture medium was adjusted by adding N/10 HCl and N/10 NaOH with the help of indicator paper prior to autoclaving. Fungal growth was assessed in the form of radial growth.

Humidity
The inoculated Petriplates were exposed to 70, 75, 80, 85, 90, 95 and 100 per cent relative humidity levels in the desiccators of uniform size. The humidity levels in desiccators were maintained by using concentrated Sulphuric acid plus water as per method suggested by Soloman (1951) [12].
The experiment was conducted in three replications and inoculated Petriplates were incubated at 27±1 ^{0}C, 85±2 RH and photophase of 12 hours for 10 days (Banu and Rajalaxmi, 2014) [1].

Result and Discussion
Purified culture of *M. anisopliae* was isolated from the larvae of *S. litura* collected from the fields of Nagpur district. Initially growth of fungus was slow on SDAY medium. The mycelium was thick, quite prominently raised with outside concentric rings on medium. White mycelial mat turns malachite green due to sporulation. Detailed microscopic studies revealed that, mecelia were hyaline, branched septate, cinidiophore were in low mounds, erect, branched covered by conidia. Conidia were green, apical long, single celled ovoid to cylindrical in shape with round ends. (Plate 2)

Effect of media (Table 1; Plate 1)
Among the four different media evaluated for growth of *M. anisopliae* revealed that maximum mycelial growth was recorded on SDAY medium (35.00 mm) followed by SMA medium (30.83). SDA medium support good growth and sporulation for *M. anisopliae* (29.50 mm). The growth and sporulation on PDA media was least (25.33 mm). SADY medium has been used for successful culture of *B. bassiana* and *M. anisopliae* as earlier reported by Luz *et al.* (1994) [9]; Kaaya *et al*., (1996) [5]; Hallsworth and Magan, (1999) [3]; and Leland, (2001) [8].

Effect of temperature (Table 2; Plate 3; Graph 1)
The colony diameter was measured after 10th DAI. The measurement was done along the same axis each time. It was observed that, the most favourable temperature for growth and sporulation of *M. anisopliae* was 25 ^{0}C and 30 ^{0}C (35 mm and 34.50 mm). At 35 ^{0}C, there was retarded vegetative radial growth (9 mm) and isolate fail to grow at 40 ^{0}C and above. The similar observations were reported by Chandra Teja and Rahman (2016) [2]. Ambient room temperature has comparatively gives good growth (30.50 mm) as earlier reported by Kotwal *et al.* (2012) [7].

Effect of humidity (Table 3; Plate 4; Graph 2)
The colony diameter and sporulation was recorded after 10 DAI. The fungus showed growth at all humidity levels. The colony growths of *M. anisopliae* were noticed in the range of 27.50 to 35.50 mm. However maximum colony growth and abundant sporulation was recorded at 95 RH (35.50 mm) followed by RH 90, 85, 80 exhibiting 34.40, 34.10 and 32.80 mm respectively. All these treatments were at per with each other. Lowest growth and moderate sporulation was observed in RH 70 recording 27.50 mm radial growth whereas 31.20 mm growth was observed in RH 75 with fair sporulation. Similar observations were recorded by Jairamaiah and Veeresh (1982) [4].

Effect of pH (Table 4)
The highest growth and abundant sporulation was recorded at pH 5.5 (35.33 mm) followed by pH 6.0 recording 33.13 mm growth. These two pH levels were at par with each other. The next promising pH levels were 6.5 to 7.5 exhibiting the growth in the range of 34.13 to 32.46 mm. However, the lowest radial growth was noticed in the pH 8.5 (28.80 mm). These means that as the pH were increases the growth was decreases similar result were reported by Kotwal *et al.* (2012)[7]. The pH is an important abiotic factor influencing not only the survival of the entomopathogenic fungi in the field but also their virulence against the target insect pest (St Leger *et al*., 1999) [13]. Hallworth and Magan (1996) [3] observed that the growth of some entomopathogenic fungi like *B. bassiana*, *M. anisopliae* and *P. farinosus* were optimum at a pH range of 5 to 8. In general the fungus had shown a wide pH range response for their growth but had maximum biomass at low acidic pH and least biomass growth at higher basic pH as recently observed by Chandra Teja and Rahman (2017) [2].

Table 1: Effect of different media on growth and sporulation of *M. anisopliae*

Sr. No.	Particulars of medium	Colony diameter after 10 days (mm)	Sporulation after 10 days	Remark
1	SDA	29.50	++	Fair Sporulation
2	PDA	25.33	+	Moderate Sporulation
3	SDAY	35.00	++++	Abundant Sporulation
4	SMA	30.83	+++	Good Sporulation

Table 2: Effect of temperature levels on growth and sporulation of *M. anisopliae*

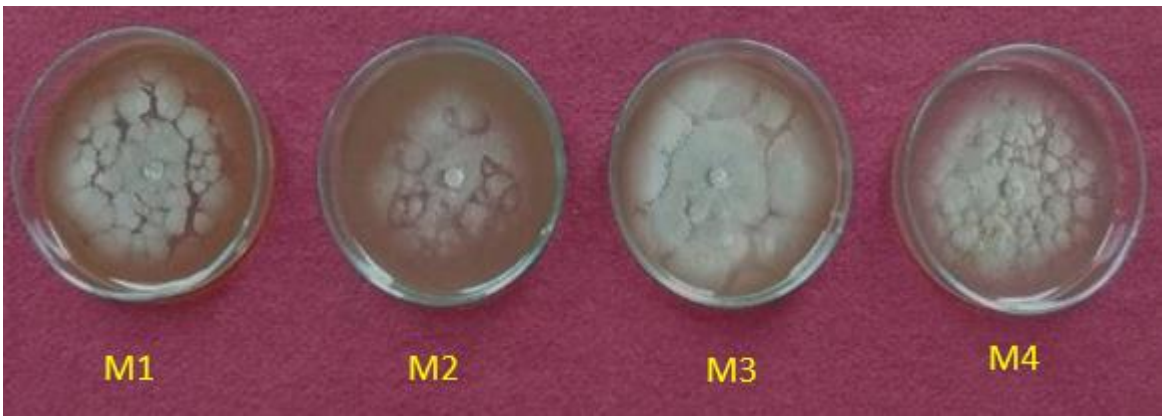
Sr. No.	Temperature (°C)	Colony diameter after 10 days (mm)	Sporulation after 10 days	Remark
1	15	11.33	+	Moderate Sporulation
2	20	20.50	++	Fair Sporulation
3	25	35.00	++++	Abundant Sporulation
4	30	34.50	++++	Abundant Sporulation
5	35	9.00	+	Moderate Sporulation
6	40	0.00	-	No Sporulation
7	Ambient (temp.)	30.50	+++	Good Sporulation
	SE(m)±	1.37		
	CD(P=0.01)	5.54		

Table 3: Effect of relative humidity levels on growth and sporulation of *M. anisopliae*

Sr. No.	Relative humidity levels (%)	Colony diameter after 10 days (mm)	Sporulation after 10 days	Remark
1	70	27.50	+	Moderate Sporulation
2	75	31.20	++	Fair Sporulation
3	80	32.80	+++	Good Sporulation
4	85	34.10	++++	Abundant Sporulation
5	90	34.40	++++	Abundant Sporulation
6	95	35.50	++++	Abundant Sporulation
7	100	33.50	++++	Abundant Sporulation
	SE(m)±	1.11		
	CD(P=0.01)	4.48		

Table 4: Effect of pH levels on growth and sporulation of *M. anisopliae*

Sr. No.	pH	Colony diameter after 10 days (mm)	Sporulation after 10 days	Remark
1	5.5	35.33	++++	Abundant Sporulation
2	6.0	33.13	++++	Abundant Sporulation
3	6.5	34.13	++++	Abundant Sporulation
4	7.0	33.00	++	Fair Sporulation
5	7.5	32.46	+	Moderate Sporulation
6	8.0	29.00	+	Moderate Sporulation
7	8.5	28.80	+	Moderate Sporulation
	SE(m)±	0.58		
	CD(P=0.01)	2.37		



M_1- Sabouraud's Dextrose Agar (SDA) medium.
M_2- Potato Dextrose Agar (PDA) medium.
M_3- Sabouraud's Dextrose Agar + Yeast Extract (SDAY) Medium.
M_4- Sabouraud's Maltose Agar (SMA) medium.

Plate 1: Effect of different media on Growth and sporulation of *Metarhizium anisopliae*.

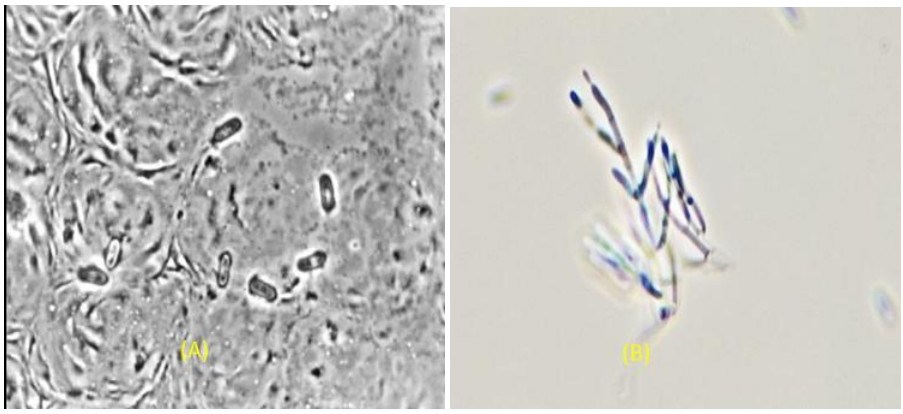


Plate 2: (A) Conidia and (B) Conidiophores of *Metarhizium anisopliae*

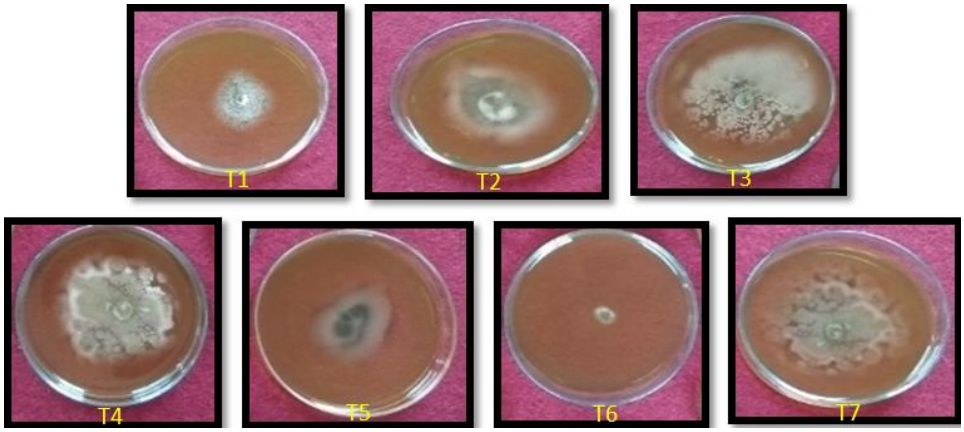


Plate 3: Effect of different temperature levels on growth and sporulation of *M. anisopliae*.

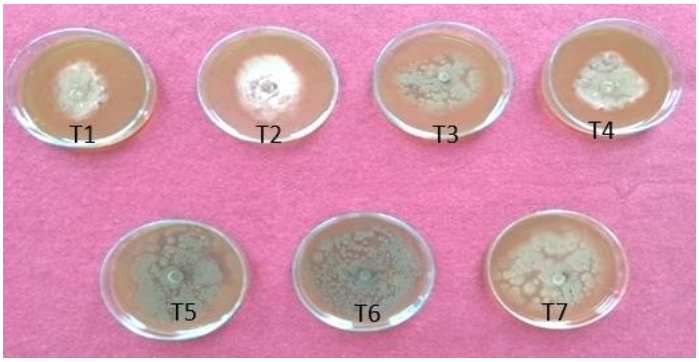
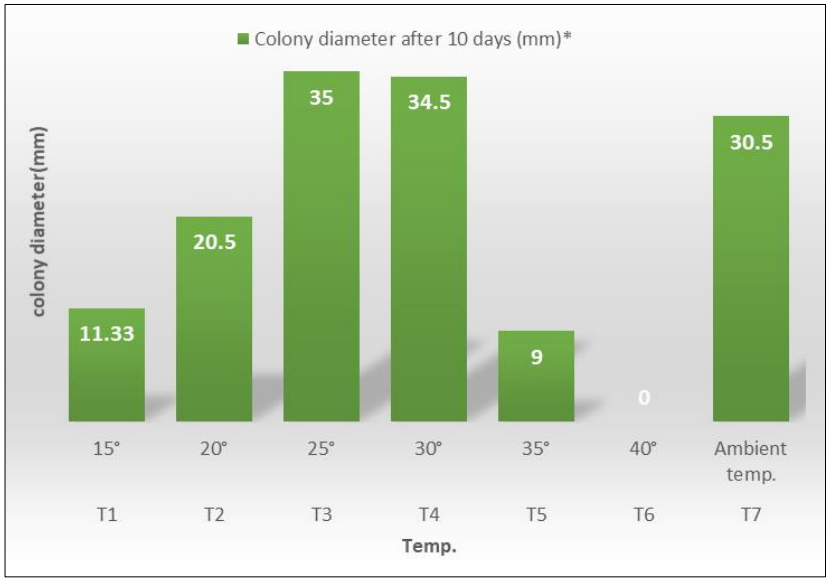
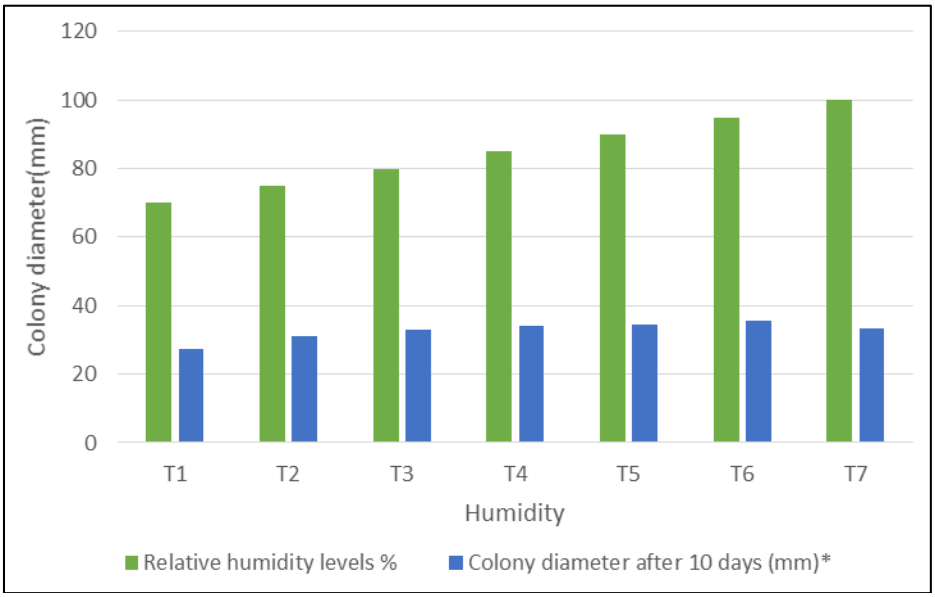


Plate 7: Effect of different relative humidity levels on growth and sporulation of *Metarhizium anisopliae*



Graph 1: Effect of different temperature levels on growth and sporulation of *M. anisopliae*



Graph 2: Effect of different relative humidity levels on growth and sporulation of *M. anisopliae*

Conclusions

SDAY media supports maximum growth and abundant sporulation of *M. anisopliae*. Temperature required for maximum growth and sporulation was 25 ^{0}C to 30 ^{0}C. All humidity levels supports growth and sporulation and pH level of 5.5 was the best growth and sporulation of *M. anisopliae.*

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