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Entomopathogenic Fungi for the Management of *Calopepla leayana* on *Gmelina arborea*

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Abstract

Entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae* were isolated and identified as natural pathogens of *Calopepla leayana*. Both the species of fungi were effective against the larval and adult stages of *C. leayana* causing white and green muscardine diseases respectively. It was also found that *B. bassiana* was more pathogenic to the pest as compared to *M. anisopliae*. The susceptibility of larvae and adults was negatively associated with the age and positively associated with the fungal spore concentration. In all the stages from I instar to adult, the highest mortality was observed in 10% of $1x10^9$ spores/ml concentration of *B. bassiana* among 1, 2.5, 5 & 10% of $1x10^9$ spores/ml concentration. It was also reflected in probit analysis that the concentration of spores required to kill fifty percent of the population was ranges from 0.09% to 5.98% of $1x10^9$ spores/ml concentration of *B. bassiana* and 1.02% to 9.72% of $1x10^9$ spores/ml concentration of *M. anisopliae*. The larvae of I instar have shown highest mortality, which gradually decreased with the increase in age or decrease in the concentration of fungal spores. Mass production of *B. bassiana* using different substrates was attempted to harvest ample amount of spores. This study revealed that spore production was directly proportional to the mycelial weight and incubation period. From the seven substrates tested, wheat flour was identified as one of the suitable substrates for the mass production of *B. bassiana*. A field trial was conducted to test their efficacy under field conditions. 72-93% mortality was observed in field conditions.

1. Introduction

Gmelina arborea (Roxb.) is a commercially important fast growing deciduous tree species. *G. arborea* is considered to be one of the most reliable timbers found in India. The wood of this species is an excellent source for pulp and paper industries as vessels and fiber configurations are most suitable for pulping. It has also a distinguished acceptability as fodder and medicinal plant, and used in treatment of various diseases like gonorrhea, catarrh of bladder and cough. It is also suitable for manufacture of matchboxes splints. In India and particularly in northeast, it is grown extensively both in government and private lands, and has substantially been contributing in timber, fodder and industrial wood. However, low productivity, poor bole form and susceptibility to various insect-pests and diseases are some of the reasons for its non deployment at commercial scale. The chrysomelid beetle, *Calopepla leayana* Latr., is the most destructive defoliator of this tree species. It is known to destroy more than 2000 acres of young plantations in a single year in India. The pest is active from March/April to November/December in NE region. The trees are defoliated completely two times in this region – once during May/June and secondly in August/September. Earlier insecticides were thought to be panacea for an insect attack, and used indiscriminately. Their deleterious effects on the environment had limited their use, especially in forestry. Application of microbial control measures of insect pest is safe in plantations and nurseries. So an attempt is made for the management of *C. leayana* using entomopathogenic fungi.

2. Materials and Methodology

2.1. Study area

The study was carried out in plantation forest of *G. arborea* at Naharani, Jorhat district of Assam (India) located between 26° 40′ - 26° 45′ North latitude and 94°20′ -94°25′ East longitude covers an area of 19.49 square kms of tropical semi evergreen forest on the flat plains of Brahmaputra River, Assam. The altitudinal range is 100-120 M above sea level. Average temperature ranges from 27.9°C to 18.95°C and average humidity ranges between 64.5% and 94.5 %. Annual rainfall of the study site is 249cm.

2.2. Rearing of Calopepla leayana

Different stages of *C. leayana* were reared in the laboratory in its natural host, *G. arborea*. For maintaining the laboratory culture of *C. leayana*

adults and eggs were collected from the field station and reared in the laboratory at room temperature 28±2°C and 70-80% RH. The adults were reared in plastic jars (20.5cm in height and 15.5cm in diameter). Mouth of each jar was covered with muslin cloth with the help of a rubber band. Fresh leaves of *G. arborea* were provided to the larvae after every 24 hrs. This was considered as a stock. All experiments were conducted from this stock only.

2.3. Laboratory bioassay

The fungal pathogens viz., *B. bassiana* and *M. anisopliae* were isolated from the diseased larvae collected from the field. They were cultured on Potato Dextrose Agar (PDA) medium and were incubated at 26-27° C for 6-8days in the dark. Spores were harvested in 0.1% Tween – 80 solution in double distilled water. The pure culture was stored in sterilized double distilled water in capped 30 ml vials at 4° C. Concentration of spores/ml in the stock solution was determined by haemocytometer method. Different concentrations of spores suspension of *B. bassiana* and *M. anisopliae* were prepared and sprayed to I to V instars larvae and adults. The larval and adult mortality was observed continuously after spraying. The dead insects were kept in petriplates lined with moist blotting paper for sporulation of the infecting fungi. The cause of the death was confirmed by examining the cadavers for spores and associated mycelia of concerned pathogen under a phase contrast microscope.

2.4. Pathogenicity test

Pathogenicity test was carried out in the laboratory to study the effect of two entomopathogenic fungi, *B. bassiana* and *M. anisopliae* on different larval stages and adults of *C. leayana.* Newly hatched Ist, IInd, IIIrd, IVth , Vth instar larvae and adults were used. The fungal pathogens viz., *B. bassiana* and *M. anisopliae* were isolated from diseased adults *C. leayana.* They were cultured on Potato Dextrose Agar (PDA) medium. Spores were harvested in 0.1% Tween – 80 solution in double distilled water. Concentration of spores/ml in the stock solution was determined by haemocytometer method. Different concentrations of spore suspension, as used in the experiment were made from the stock solution. The freshly prepared spore suspensions were sprayed on I-V larval instars and adults. Five treatments (0, 1, 2.5, 5 & 10 % of 1x10^9 spores/ml of *B. bassiana* and *M. anisopliae*)) were given to each stage. Control where (0% spore suspension) 0.1% Tween – 80 solution in sterile double distilled water was used. Each treatment was replicated five times in a Completely Randomised Design (CRD) having 20 insects in each replicate. Observations were taken at every 24 hrs interval for the mortality of insects. Cause of death was confirmed with mycelial growth on the cadavers by visual observation. The mortality data obtained were converted into percent mortality by using Abbot's formula. The values obtained were tested through ANOVA using SPSS 10.1 software for ascertaining treatment differences. Data were subjected to probit analysis through SPSS software (version 10.1) for LC_{50}.

2.5. Mass production of entomopathogenic fungi

In order to screen suitable substrates for the mass production of *B. bassiana*, a preliminary attempt was made in the laboratory. Different substrates such as Wheat bran, rice bran, wheat flour, rice flour, bakery waste, Potato dextrose & sabraoud agar medium were used as carbon sources in this study. In this method, 200 gms of substrates were placed in 1 li capacity of conical flask and moistened with 100 ml of distilled water. Mouth of the conical flasks was plugged with non absorbent cotton and autoclaved at 15psi for 15 min. Homogeneous suspension of *B. bassiana* (1x10^8 spores/ml) was inoculated into each flask. Flasks were incubated in BOD incubator at 25±1°C for 15 days. Mycelial mat was taken out from the flasks after 15 days of incubation and kept in another flasks and air dried under laminar air flow for 72 hrs. The spores were removed by adding 100 ml of 0.05% sterile Triton X-100 to each flask containing dried mycelial mat, shaken well, then the suspension was filtered into sterile container and the total volume of the suspension was also measured. The spore count per ml of recovered spore suspension was determined with the help of haemocytometer. Each treatment was replicated five time before arrive at any conclusion.

2.6. Field evaluation

Experiment was carried out in the *G. arborea* field at Deovan, RFRI campus to study the effect of entomopathogenic fungi, *B. bassiana* and *M. anisopliae* on adults of *C. leayana.* Newly hatched adults were used. A sum of hundred (100) adults was released into each tree and was covered with nylon net to avoid insect movement from one tree to another. Different concentrations of spore suspension were made from the stock solution. The freshly prepared spore suspensions were sprayed on adults of *C. leayana.* Five treatments (0, 1, 2.5, 5 & 10 % of 1x10^9 spores/ml of *B. bassiana* and *M. anisopliae* were given to adults. Control

where (0% spore suspension) 0.1% Tween – 80 solution in sterile double distilled water was used. Each treatment was replicated three times in a Completely Randomised Design (CRD) having 100 insects in each replicate. Observations were taken at every 24 hrs interval for the mortality of insects. Cause of death was confirmed with mycelial growth on the cadavers by visual observation. The mortality data obtained were converted into percent mortality by using Abbot's formula. The values obtained were tested through ANOVA using SPSS 10.1 software for ascertaining treatment differences.

3. Results and Discussion

The commercially important, multipurpose tree species, *G. arborea* is facing serious problem of defoliation by *C. leayana* every year, resulting retardation of growth and development. Earlier insecticides were thought to be panacea for an insect attack, and used indiscriminately. Their deleterious effects on the environment had limited their use, especially in forestry. Therefore, an attempt has been made to study the management of *C. leayana* on *G. arborea* using entomopathogens, *B. bassiana* and *M. anisopliae*.

3.1. Exploration of entomopathogenic fungi

Entomopathogenic fungi namely, *Beauveria bassiana* and *Metarhyzium anisopliae* collected from dead and diseased larvae of *Calopepla leayana* were taken for further detailed study. It was also found that both the fungi were pathogenic to *C. leayana*. The pathogenecity studies showed differential percent mortality with respect to different concentrations of the spore suspension (**Table 1**.). Both the fungi were found to be significantly pathogenic to *C. leyanana* causing white and green muscardine disease respectively. The similar disease condition was reported by Sandhu *et al*., (1993) on teak pest, *Hyblaea purea*. The results clearly indicate the effective role of *B. bassiana* and *M. anisopliae* in causing the mortality to both larvae and adults of *C. leayana*. Dadwal and Jamaludin (1989) identified an entomogenous fungus, *B. bassiana* as a potential biocontrol agent on *Atteva fabricella*. It is evident from the results that *B. bassiana* was more pathogenic to the pest as compared to *M. anisopliae*. Misra (1993) also reported that the entomopathogenic fungus, *B. bassiana* is a promising pathogen for the biological control of *Hypsiphylla robusta*, a serious pest of *Toona ciliata* and *Sweietenia macrophylla* in Uttar Pradesh, India. However, all the four fungal treatments were found highly significant to the control. The susceptibility of larvae and adults was negatively associated with the age and positively associated with the fungal spore concentration. In all the stages from I instar to adult, the highest mortality was observed in 10% of $1x10^9$ spores/ml concentration of *B. bassiana* (**Table 2.**). The observation made in this study were also confirmed by Ramarethinam *et al*., (2000), who have reported a mortality rate as high as 95.68% due to infection caused by the entomogenous fungus, *B. bassiana* on hairy caterpillar *Eupterote* sp. on Tea. Similar observation was also made by Sankaran *et al*., (1989) with *B. bassiana* at concentrations of $1x10^5$ spores/ml causing mortality in the *Myllocerus viridanas* on Teak in Kerala. Such effect of high mortality due to *B. bassiana* on the shot hole borer, *Euwallacea fornicatus* infesting tea in south India was also reported (Selvasundaram and Muraleedharan, 2000). Larvae of I instar have shown highest mortality, which gradually decreased with the increase in age or decrease in the concentration of fungal spores.

Table 1. Effect of different concentrations of *Beauveria bassiana* and *Metarhizium anisopliae* on different stages of *C. leayana*.

Conc. of $1x10^9$ spores/ml stock	Mortality (%)					
	Larval instars					Adult
	I	II	III	IV	V	
Beauveria bassiana						
0 (control)	19±4.18a	13±2.73a	11±6.51a	7±5.7a	3±4.47a	5±3.53a
1	57±5.70b (23.67)	50±3.53b (24)	46±6.51b (22.09)	40±5.34b (22.50)	34±4.18b (25.18)	28±4.47b (10.14)
2.5	66±9.61b (37.21)	60±7.90c (38.33)	57±8.36c (37.70)	49±12.94b (34.71)	43±5.70c (36.02)	38±10.36c (24.84)
5	78±11.51c (53.64)	71±4.18d (52.69)	65±7.90c (48.08)	58±2.73c (45.93)	52±5.70d (46.23)	42±4.47c (30.10)
10	96±4.18d (76.21)	92±5.70e (77.87)	85±6.12d (72.06)	71±9.61c (61.14)	67±4.47e (62.52)	61±7.41d (52.80)
Metarhizium anisopliae						
0 (control)	19±4.18a	13±2.73a	11±6.51	7±5.7a	3±4.47a	5±3.53a
1	54±4.18b (18.18)	45±3.53b (16.11)	41±4.18b (14.17)	34±4.18b (13.41)	28±6.70b (17.29)	23±5.70b (1.26)
2.5	62±5.70b (31.35)	57±8.36c (34.19)	51±4.18c (29.43)	45±7.90c (29.44)	40±5c (32.50)	37±7.58c (23.49)
5	73±10.36c (46.97)	67±7.58d (47.60)	62±5.70d (44.26)	56±4.18d (43.50)	48±4.47d (41.75)	44±2.23cd (32.64)
10	91±4.18d (70.12)	87±5.70e (72.06)	76±9.61e (61.53)	64±6.51e (53.06)	56±7.41e (50.64)	48±9.08d (37.58)

All values are mean ± SD of five replicates with 20 insects in each replicate (total 100 insects), values followed by the same alphabets are not significantly different at $P<0.05$ (DMRT) Figures in the parenthesis are corrected mortality based on control (Percent mortality in experiment-Percent mortality in control / percent mortality in experiment * 100)

Table 2. Probit analysis to test pathogenicity of *B. bassiana* and *M. anisopliae* on different stages of *C. leayana.*

Stages	Heterogeneity ($\chi 2$)	Regression Equation	LC50 1x10^9 spores/ml
B. bassiana			
I Instar	7.209	Y = -0.04865 + 1.29533 x	0.09
II Instar	6.285	Y = -0.11696 + 1.22436 x	1.25
III Instar	3.503	Y = -0.17931 + 1.03604 x	1.49
IV Instar	0.618	Y = -0.29626 + 0.78963 x	2.37
V Instar	0.873	Y = -0.46314 + 0.833 x	3.59
Adult	1.927	Y = -0.62932 + 0.81057 x	5.98
M. anisopliae			
I Instar	4.869	Y = -0.00975 + 1.10459 x	1.02
II Instar	3.532	Y = -0.20982 + 1.14659 x	1.52
III Instar	0.842	Y = -0.27994 + 0.91506 x	2.02
IV Instar	0.057	Y = -0.41814 + 0.78392 x	3.42
V Instar	0.059	Y = -0.56608 + 0.72985 x	5.96
Adult	0.876	Y = -0.67346 + 0.68182 x	9.72

Y = Probit Kill; x = log (concentration x 10^9); LC_{50} = Concentration to give 50 per cent mortality, *All data were found to be significantly Heterogeneous at 5% level

3.2. Mass production of entomopathogenic fungi

A preliminary attempt was made to screen suitable substrates for the mass production of *B. bassiana* and *M. anisopliae* in the laboratory. Different substrates such as Wheat bran, rice bran, wheat flour, rice flour, bakery waste, Potato dextrose & sabraoud agar medium were used as carbon sources in this study. Results from this study revealed that spore production was directly proportional to the mycelial weight and incubation period (**Table 3.**). 500mg of mycelia of *B. bassiana* gave 9.5x10^6 spores/ml followed by rice flour (450mg of mycelia gave 8.4x10^{6}spores/ml). Similar observation was also made by Niranjana (2004) who reported 16.76X10^9 conidia of *B. bassiana* cultured on wheat flakes followed by 6.8 X10^9 conidia on wheat bran whereas, Nelson *et al.*,(1996) achieved 7.8x10^9 conidia/g dry rice. Among the seven substrates used, wheat flour gave more mycelial biomass & conidia when compared to the rest of the substrates used. Finally wheat flour was found to be a suitable media for mass production of *B. bassiana* and *M. anisopliae.*

Table 3. Effect of different substrates as carbon sources for the mass production of *B. bassiana* and *M. anisopliae*

S.No.	Substrate	Mycelial dry Wt. (mg)	Conidia (1x10^6/ml)
B. bassiana			
1	Wheat flour	500a	9.5a
2	Rice bran	225g	2.8g
3	Rice flour	450b	8.4b
4	Wheat bran	405d	6.9d
5	PDA medium	425c	7.8c
6	Sabouraud agar medium	280f	4.2e
7	Bakery waste	300e	3.2f
M. anisopliae			
1	Wheat flour	530a	9.5a
2	Rice bran	365g	3.0g
3	Rice flour	460b	8.8b
4	Wheat bran	410d	7.2d
5	PDA medium	420c	8.0c
6	Sabouraud agar medium	380f	3.5f
7	Bakery waste	400e	4.0e

Figures followed by same alphabets within column do not vary significantly at the 95% confidence level LSD

3.3. Field evaluation

A field trial was conducted to test the efficacy of entomopathogenic fungi, *B. bassiana* and *M. anisopliae* under field conditions. All treatments including *B. bassiana* and *M. anisopliae* applied exhibited significant mortality of *C. leayana*. A range between 72.1 and 93.7% mortality was observed in field conditions **(Table 4**.). Similar observation was also made by Hazarika and Puzari (1997) with *B. bassiana* causing 88.68 to 9.31% mortality in the *Dicladispa armigera* on rice in Assam. Bhagat *et al*., (2003) observed 28.8 to 56.5% reduction in population of white grubs infesting potato in Himachal Pradesh due to *B. bassiana* and *M. anisopliae* under field conditions. Highest mortality of 93.7% was observed in $10x10^9$ spores/ml concentration of *B. bassiana*. Such effect of high mortality due to *M. anisoplaie* on the grey back canegrub, *Demolepida albohirtum* infesting sugarcane in Australia was also reported (Logan *et al*., 1999). Similar observation was also made by Niranjana (2004) with *B. bassiana* causing 74% mortality of coffee berry borer, *Hypothenemus hampei* in Mysore.

Table 4. Field evaluation of different concentrations of *B. bassiana* and *M. anisopliae* against adults of *C. leayana*

S. No.	Concentration of $1x10^9$ spores/ml stock	Average percent mortality of adults of *C. leayana* treated with *B. bassiana*	Average percent mortality of adults of *C. leayana* treated with *M. anisopliae*
1	0.0	11.2±7.0a	11.2±7.0a
2	1.0	72.3±5.6b	72.1±5.7b
3	2.5	86.0±2.4c	75.0±5.8c
4	5.0	89.5±2.7d	79.0±8.2d
5	10.0	93.7±2.2e	91.1±3.5e

100 adults /tree/con – 3 replicates (Figures followed by same alphabets within column do not vary significantly at the 95% confidence level LSD)

4. Conclusion

Entomopathogenic fungus, *Beauveria bassiana* spore suspension at the concentration of 0.09 to $5.98x10^9$ spores/ml should be sprayed to kill fifty percent of the population if population is about 100 individuals of I-V instar larvae and adults per tree. The freshly prepared spore suspension will be very effective on adults in the field. Rainy season, preferably just after a spell of rain, is suitable for field application of fungal spores when temperature and humidity conditions are optimum for pathogenesis. The mortality of larvae and adults may be observed within 10 days after application. Observation of mycelial growth on the cadavers is the confirmation of the cause of death due to fungal attack. Based on the present investigation, it may be suggested that *B. bassiana* showed a promising biocontrol agent against early developmental stages of *C. leayana* on nursery and young plantations of *G. arborea.*

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