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ORIGINAL RESEARCH ARTICLE

Impact of Different Relative Humidities on *In vitro* Growth And Sporulation of Entomopathogenic Fungal Isolates of *Beauveria* Species

Padmini Palem P.C[1*] and Padmaja V[2]

[1]*Department of Biotechnology, Dr. L. Bullayya PG College, Visakhapatnam, India.*
[2]*Department of Botany, Andhra University, Visakhapatnam, India.*

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ABSTRACT

Thirty isolates of *Beauveria* species were evaluated for *in vitro* growth and sporulation at five different relative humidities at a constant temperature of 30ºC. Only twenty isolates could grow at 60%, 63%, 75% and 85% relative humidities, while 91% relative humidity did not permit growth of ten isolates. On the other hand, eleven isolates only out of thirty displayed sporulation. Optimum RH for *Beauveria* isolates for growth and sporulation appeared to be 60%.

Key Words: *Beauveria bassiana*, *Beauveria Brongniartii*, Relative humidity *in vitro* growth and sporulation.

INTRODUCTION

Natural epizootics as well as biocontrol strategies require a favourable environment interacting with host and pathogen in many intricate ways and a detailed knowledge of these interactions is important for employing fungi as manipulative tools in biological control. Water availability in the microclimate of the host cuticle is a more vital determinant for conidial germination rather than ambient relative humidity (RH), and conidiogenesis on cadavers is dependent on high moisture in the surrounding environment. According to Milner and Soper (1981), effectiveness of fungus in field is determined by a variety of factors influencing its pathogenicity which include temperature, relative humidity, light, air, nutrient availability and host physiological status. Magan (1997) expressed opinion that development of fungal biocontrol agents in field environments is critically dependent on fluctuations in relative humidity and temperature. Soil temperature, moisture, pH, content of organic matter and conductivity (Lingg & Donaldson 1981), soil types (Storey *et al*, 1989), and antagonistic organisms (Fargues *et al*, 1983) have been found to affect the survival and long term efficacy of these fungi. RH is an essential factor in the development of fungal pathogenesis (Benz 1987; Nahas & Alai 1987) and it is evident from several investigations that most entomopathogenic fungi require at least 95% RH on the insect surface for germination, germ tube extension and infection to occur. Kalvish (1976) observed that entomopathogenic fungi collected from northern areas germinated and sporulated at lower temperatures than the ones procured from southern areas and moreover strains from humid areas were more sensitive to a fall in RH than those from dry areas. Steven and Thomas (2001) assessed the effect of temperature and RH on sporulation of *M. anisopliae* in mycosed cadavers of *Schistocerca gregaria* under laboratory conditions and found that optimum sporulation of $>10^9$ spores/ml were produced at RH>96% and temperatures between 20-30ºC. Junianto and Sukamto (1995) reported that germination, growth and sporulation of several isolates of *B. bassiana* were influenced by temperature and RH. At a temperature range of 20-30ºC all isolates had a high percentage of germination (99-100%). However, germination was inhibited at 15ºC and 35ºC. Inhibition of *in vitro* growth and conidia production at <93% RH and an increase in conidia production with an increase of 93 to 97% RH in two isolates of *Valdensinia heterodoxa* were observed by Zhao and Shamoun (2006). A desirable trait of potential microbial control agent is its ability to attain high levels of disease in the target populations, able to grow and sporulate on the dead insect and

***Corresponding Author: Padmini Palem P.C**, **Email:** padmini49@gmail.com.

establish inoculum for further infections. Therefore it becomes mandatory to evaluate the response of potential entomopathogenic fungal isolates under *in vitro* conditions to different RHs which could be encountered at field level. Present study aims at understanding the effect of different relative humidities on *in vitro* growth and spore output in a collection of 30 isolates of *Beauveria* species which includes endemic as well as exotic ones. The outcome of the study would help in selecting potential isolates which will be targeted on crop pests prevailing in atmosphere at those particular relative humidities.

MATERIALS AND METHODS

Source of *Beauveria* cultures and preparation of inoculum

Twenty four endemic isolates of *Beauveria bassiana* were isolated from diseased larvae from various agricultural fields of Andhra Pradesh, India. Six isolates including one *B. Brongniartii* species were procured from USDA - ARS, ITCC and EMBRAPA. Pure cultures were established and maintained in SDAY (Saboraud's dextrose agar yeast) medium at 26°C. Conidia were harvested by scraping the surface of 15 day old culture plates slightly after flooding with sterile distilled water containing 0.1% Tween 80 and concentration was adjusted to $2x10^8$ conidia /ml.

In vitro growth and sporulation at different RHs

According to Hong et al, (2002) five different salts which generate variable relative humidities (NaBr – 60%, $NaNO_2$ – 63.9%, NaCl – 75%, KCl-84.3% and KNO_3 – 91.3%) and temperature at 30ºC were used to evaluate *in vitro* growth and sporulation of the isolates. For the entire experiment a constant temperature of 30ºC was selected since all the thirty isolates of *Beauveria* spp recorded maximum growth and sporulation when compared to other temperatures tested (Unpublished data). Autoclaved SDAY medium was plated in sterilized Petri plates and allowed to solidify overnight. Wells were made by removing agar discs of 2 mm in diameter from the centre of each petriplate with sterilized cork borer and 50µl of $2x10^8$/ml spore suspension was inoculated into each well. Three replicates per isolate along with controls were also maintained throughout the experiment. Saturated solutions of each salt were prepared in sterile distilled water and 100 ml of the solution was poured in desiccators to maintain the specified RH. Inoculated petri plates were then transferred into respective desiccators, which were then sealed air tight with vacuum grease in order to avoid fluctuations in RH and incubated at 30ºC for 10 days. Data pertaining to radial growth were recorded on 5^{th}, 6^{th} and 7^{th} days with a graduated scale (mm) for measuring the colonies. Whereas data regarding spore output was determined on 10th day by flooding petri plates with 5 ml 0.02% Tween 80 and spore concentration was calculated with the aid of haemocytometer.

DATA ANALYSIS

Statistical evaluation of the data for all the experiments was performed using STATISTICA version 6.0 and the data pertaining to radial growth and spore output were subjected to factorial analysis of ANOVA.

RESULTS

At 60% RH, minimum vegetative growth of 10 mm was observed in B16 and B20 on the 5th day and maximum of 12 mm on 7^{th} day in case of B20. However, B16 maintained the same rate of growth on the 7th day also. Maximum values for radial growth were observed in case of B8 which scored 13.5 mm on 5^{th} day and 17.5 mm on 7^{th} day. On the other hand, a maximum of $1x10^9$/ml spore output was recorded in B25 isolate. At 63% RH, all the isolates responded in terms of both the parameters, B16 and B33 being the isolates which recorded lowest radial growth of 8.3 mm on the 5^{th} day. On the other hand highest radial growth of 13 mm was observed on 7^{th} day in B8 and lowest of 9.6 mm was recorded in B33 (**Table - 1**). Lowest spore output was observed in B8 and B16 with $5x10^6$/ml while highest spore output of $4.62x10^8$/ml was recorded in B25 and B26. At 75% RH, highest radial growth of 10 mm in B18 and B26 on 5^{th} day was observed. Highest spore output of $9x10^8$/ml was recorded in B25 and lowest of $1x10^6$/ml was recorded in B20. At 84.5% RH, highest radial growth of 10 mm was recorded in B13 which continued till 7^{th} day of incubation. Lowest radial growth of 5 mm and 7 mm was observed in B14 on 5^{th} and 7^{th} days of incubation and on the other hand highest spore output of $5.6x10^8$/ml was recorded in B42. At

TABLE - 1
Radial growth and spore output of *Beauveria* isolates at different relative humidities

	60% RH		63% RH		75% RH		85% RH		91% RH	
Isolate number	RG 7th day	SP 10th day	RG 7thday	SP 10th day	RG 7thday	SP 10th day	RG 7th day	SP 10th day	RG 7th day	SP 10th day
B6	14.0 ± 0.00	$3.00x10^7$	9.30 ± 0.02	$3.00x10^6$	9.30 ± 0.02	$3.00x10^6$	9.60 ± 0.02	$3.50x10^7$	-	-
B7	14.0 ± 0.00	$5.00x10^7$	10.0 ± 0.00	$4.75x10^6$	10.0 ± 0.00	$4.75x10^6$	9.00 ± 0.00	$9.60x10^5$	-	-
B8	17.5 ± 0.01	$8.00x10^7$	10.6 ± 0.03	$1.25x10^7$	10.6 ± 0.03	$1.25x10^7$	13.6 ± 0.03	$5.00x10^6$	4.00 ± 0.00	0
B12	13.0 ± 0.00	$3.37x10^8$	10.6 ± 0.03	$3.80x10^7$	10.6 ± 0.03	$3.80x10^7$	8.60 ± 0.02	$1.00x10^7$	3.00 ± 0.00	0
B13	14.0 ± 0.00	$1.00x10^8$	11.6 ± 0.03	$5.50x10^7$	11.6 ± 0.03	$5.50x10^7$	10.0 ± 0.06	$8.00x10^7$	5.60 ± 0.03	$5.00x10^6$
B14	13.0 ± 0.00	$2.00x10^7$	11.6 ± 0.03	$5.25x10^7$	11.6 ± 0.03	$5.25x10^7$	7.00 ± 0.00	$2.00x10^7$	-	-
B15	15.5 ± 0.02	$5.00x10^8$	10.0 ± 0.00	$1.22x10^8$	10.0 ± 0.00	$1.22x10^8$	10.6 ± 0.06	$1.60x10^8$	4.00 ± 0.00	$1.00x10^7$
B16	10.0 ± 0.00	$2.50x10^7$	8.60 ± 0.03	$7.00x10^7$	8.60 ± 0.03	$7.00x10^7$	7.60 ± 0.01	0	-	-
B18	15.0 ± 0.00	$3.12x10^8$	12.6 ± 0.03	$1.25x10^8$	12.6 ± 0.03	$1.25x10^8$	9.00 ± 0.02	$5.80x10^7$	3.00 ± 0.00	0
B19	14.5 ± 0.01	$3.00x10^8$	9.60 ± 0.00	$5.00x10^6$	9.60 ± 0.00	$5.00x10^6$	9.00 ± 0.03	$2.60x10^7$	7.60 ± 0.00	$5.00x10^6$
B20	12.0 ± 0.00	$4.50x10^7$	8.00 ± 0.00	$1.00x10^6$	8.00 ± 0.00	$1.00x10^6$	8.60 ± 0.03	$8.00x10^7$	4.60 ± 0.03	0
B22	14.0 ± 0.00	$8.00x10^8$	11.0 ± 0.00	$5.00x10^8$	11.0 ± 0.00	$5.00x10^8$	8.70 ± 0.01	$4.50x10^8$	5.60 ± 0.03	0
B23	15.0 ± 0.02	$6.25x10^8$	10.3 ± 0.02	$6.75x10^7$	10.3 ± 0.02	$6.75x10^7$	9.60 ± 0.02	$3.30x10^7$	4.60 ± 0.03	0
B24	14.0 ± 0.00	$8.75x10^7$	10.0 ± 0.00	$4.00x10^7$	10.0 ± 0.00	$4.00x10^7$	9.00 ± 0.02	$6.20x10^6$	-	-
B25	14.0 ± 0.00	$1.00x10^9$	11.3 ± 0.01	$9.00x10^8$	11.3 ± 0.01	$9.00x10^8$	9.00 ± 0.00	$4.60x10^8$	6.00 ± 0.00	0
B26	15.0 ± 0.00	$8.00x10^7$	12.3 ± 0.01	$8.10x10^7$	12.3 ± 0.01	$8.10x10^7$	9.00 ± 0.00	$5.00x10^8$	5.60 ± 0.03	$1.20x10^7$
B27	12.0 ± 0.00	$6.00x10^7$	9.60 ± 0.03	$4.00x10^7$	9.60 ± 0.03	$4.00x10^7$	8.00 ± 0.00	$5.00x10^6$	-	-
B28	14.5 ± 0.02	$4.12x10^7$	10.6 ± 0.03	$8.00x10^5$	10.6 ± 0.03	$8.00x10^5$	8.60 ± 0.02	$9.80x10^7$	6.00 ± 0.01	$7.50x10^6$
B29	15.0 ± 0.00	$5.00x10^8$	10.3 ± 0.00	$4.35x10^8$	10.3 ± 0.00	$4.35x10^8$	8.60 ± 0.02	$3.30x10^7$	3.00 ± 0.00	0
B30	14.0 ± 0.00	$7.00x10^8$	9.00 ± 0.00	$6.00x10^7$	9.00 ± 0.00	$6.00x10^7$	7.00 ± 0.00	$7.50x10^6$	-	-
B31	14.0 ± 0.00	$2.12x10^8$	10.0 ± 0.00	$3.00x10^7$	10.0 ± 0.00	$3.00x10^7$	9.30 ± 0.02	$7.00x10^7$	4.60 ± 0.01	0
B32	17.0 ± 0.00	$4.12x10^8$	11.6 ± 0.03	$1.75x10^8$	11.6 ± 0.03	$1.75x10^8$	9.30 ± 0.01	$3.20x10^8$	5.60 ± 0.03	$1.00x10^7$
B33	16.0 ± 0.00	$8.75x10^7$	6.60 ± 0.03	$2.00x10^6$	6.60 ± 0.03	$2.00x10^6$	5.00 ± 0.00	$2.50x10^7$	-	-
B35	13.0 ± 0.00	$8.50x10^7$	10.3 ± 0.01	$3.25x10^7$	10.3 ± 0.01	$3.25x10^7$	8.60 ± 0.02	$7.50x10^6$	6.60 ± 0.02	$1.00x10^7$
B37	15.0 ± 0.00	$7.00x10^7$	10.3 ± 0.01	$9.75x10^6$	10.3 ± 0.01	$9.75x10^6$	7.60 ± 0.03	$1.00x10^7$	6.60 ± 0.04	$5.00x10^6$
B38	15.0 ± 0.00	$8.75x10^8$	9.60 ± 0.03	$4.50x10^5$	9.60 ± 0.03	$4.50x10^5$	8.60 ± 0.01	$5.80x10^7$	6.00 ± 0.00	$7.50x10^6$
B39	15.0 ± 0.00	$3.12x10^8$	9.00 ± 0.00	$7.50x10^6$	9.00 ± 0.00	$7.50x10^6$	9.30 ± 0.03	$4.80x10^7$	-	-
B40	13.0 ± 0.00	$9.60x10^8$	10.3 ± 0.01	$1.60x10^8$	10.3 ± 0.01	$1.60x10^8$	7.30 ± 0.02	$6.10x10^7$	-	-
B41	14.0 ± 0.00	$3.12x10^8$	10.3 ± 0.01	$7.50x10^7$	10.3 ± 0.01	$7.50x10^7$	11.0 ± 0.00	$8.00x10^7$	4.60 ± 0.02	$1.00x10^7$
B42	16.0 ± 0.00	$2.75x10^8$	11.5 ± 0.02	$6.10x10^8$	11.5 ± 0.02	$6.10x10^8$	9.00 ± 0.00	$5.60x10^8$	5.00 ± 0.00	$3.10x10^7$

RG- Radial growth (mm), SP- Spore output (Per ml)
Empty cell indicate no growth and sporulation

91% RH, only fifteen out of thirty isolates of *Beauveria* showed symptoms of sparse growth from 5th day onwards. Vegetative growth in all the isolates was progressive and maximum growth of 5 mm and 7.6 mm on 5th and 7th days of incubation in B19 isolate. On the other hand lowest radial growth of 3 mm was scored in B12 and B28. Highest spore output of $3.1x10^7$/ml was observed in B42. Few isolates, which showed growth symptoms till 7th day also, did not record sporulation on the 10th day of incubation. Data pertaining to radial growth and spore output were subjected to factorial analysis of ANOVA and growth of each isolate at different temperature conditions were compared (P=<0.05). Prior to analysis the *in vitro* radial growth and sporulation data were log transformed. The factorial ANOVA showed highly significant differences (P = 0.00) among the *Beauveria* isolates for both the parameter and a value of F=34.0 for radial growth and F=600 for spore output were obtained.

DISCUSSION

Results pertaining to the effect of different relative humidities on *in vitro* growth and sporulation revealed significant differences among isolates at a constant temperature of 30ºC. Among the RHs tested, 91.3% proved to be inhibitory for most of the *Beauveria* isolates. In our findings, 10 isolates out of 30 did not show any signs of vegetative growth during incubation period which was in accordance to Luz and Fargues (1998) who also observed that the germination rates of *B. bassiana* conidia were 0% at 93% RH. Contrary to these findings, Fargues *et al*, (1997) observed that for growth and sporulation, various entomopathogenic fungi require >95% relative humidity. In the present study, vegetative growth and sporulation were excellent at 60% relative humidity and 30ºC temperature. Contradictory findings were also reported by Ibrahim *et al* (1999) that conidia of *M. anisopliae* required 98% RH for germination. Magalhaes *et al* (2000a) observed that *M. anisopliae* in *in vivo* conditions sporulated internally within the grasshopper *Rhammatocerus schistocercoides* under ambient humidities 53% and 75% with no external sporulation for either condition. Piatti *et al*, (1995) observed that most suitable moisture conditions for *B. brongniartii* fungal growth was 57%, though 55% to 65% RH also permitted optimal growth and sporulation. Hastuti *et al*, (1999) also reported that *B. bassiana* requires lower humidities for infection and proliferation in the host insect. Eighteen isolates in the present study displayed reduction in radial growth as the RH increased but this was not the case with sporulation where few isolates did not show any correlation with growth. In conclusion 91.3% relative humidity proved to be inhibitory for most of the *Beauveria* isolates and at 30ºC temperature, optimal RH is 60% for profuse growth and sporulation.

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