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RESEARCH ARTICLE

Germination rates of *Beauveria bassiana* and *Metarhizium anisopliae* its possible correlation with virulence against *Spodoptera litura* larvae

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Abstract

The present study reports relationship between rate of conidial germination of the two entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* and their virulence against the insect pest *Spodoptera litura* larvae. Rate of germination in terms of TG_{50} (Time taken for germination of 50% of conidia) was estimated by calculating the germinated and ungerminated conidia at every 2 hour intervals up to 48 hours from the SDAY plates inoculated with $3x10^6$ conidia/ml. Virulence of *Beauveria bassiana* and *Metarhizium anisopliae* was elucidated by performing bioassay with *S. litura* larvae at 2nd instar stage. The study demonstrated that the strains of both *B. bassiana* and *M. anisopliae* with low TG_{50} values were more virulent towards *S. litura* larvae, there by paving the way to use germination assay as an aid for screening the fungal strains for virulence.

Introduction

The cutworm *Spodoptera litura* Fab. (Lepidoptera, Noctuidae) is well known cosmopolitan pest with extensive host range of economically important crops such as cotton, groundnut, soybean, tomato, sweet potato, and many other crops (Matsuura and Naito, 1997; Sahayaraj and Paulraj, 1998). *S. litura* has been shown to be resistant to a wide range of insecticides, which has led to sporadic out breaks of the pest and failure of crops (Ahmad et al., 2007a). The pest has developed high resistance against wide variety of insecticides including organophosphate, carbamate, pyrethroids and some selected newer chemistry insecticides with field control failure (Armes et al., 1997; Kranthi et al., 2001; Ahmad et al., 2007a, b, 2008; Saleem et al., 2008). The management of the pest has therefore become increasingly difficult all over the world. Microbial insecticides such as entomopathogenic fungi can provide a more environmentally friendly alternative, to control these insect pests. *B. bassiana* and *M. anisopliae* are promising and extensively researched biological control agents that can infect wide range of economically important insect pests (Coates et al., 2002; McGuire et al., 2005) and differences have been reported in both host specificity and virulence among strains (Ferron et al., 1991).

Asexually produced conidiospores of entomopathogenic Hyphomycetes generally responsible for infection and are prevalent in the agrienvironments in which the insect hosts are present. Conidial attachment followed by germination is the first step in establishing infection of terrestrial insects by most entomogenous fungi including *Beauveria bassiana* (Bals.) Vuill. Alves et. al., (1996) expressed the opinion that there was a strong correlation between the rate of germination and virulence of *B. bassiana* toward *Diatraea saccharalis*, but not toward *Solenopsis saevissim.* The present study was therefore designed to understand the relation between the germination rate and virulence of *B. bassiana* and *M. anisopliae* against *S. litura* larvae.

Materials and Methods

Fungal cultures

Details of the nine strains of *Metarhizium anisopliae* and seven strains of *Beauveria bassiana* used in the present investigation is presented in Table1. The strains were obtained from the ARSEF-USA culture collection.

Pure cultures were maintained on Sabouraud's dextrose yeast extract agar (SDAY) medium. The monosporic cultures were cultured on SDAY in slants and incubated at 25 ± 1°C in an incubator for a period of ten days. After sporulation, cultures were stored at 4°C and sub culturing of fungal strains was done at every two month interval. Freshly renovated single spore cultures of glycerol stocks were sub cultured on Sabouraud's dextrose agar (SDAY) slants in test tubes and conidia from 14-day-old cultures were used for the experiment. Viability of conidia was tested by germination bioassays on SDAY plates prior to experimentation.

Rate of germination

All strains of *M. anisopliae* and *B. bassiana* were screened for the rate of germination. Fifty micro liters of 3 X 10^6 conidia / ml was used for inoculating SDAY plates by spread plate method, and four sterile cover slips were randomly placed on each plate. Plates were sealed with parafilm and incubated at 25±1^{0}C. From 6th hour onwards at 2 hour interval 1ml formaldehyde (0.5%) was transferred onto plate to arrest germination as per the method of David et al. (2008). Each cover slip was removed and placed on glass slide for making germinated / un-geminated conidia count (500 per each cover slip). For each sample three replicates were observed. Time taken for germination of 50% conidia (TG_{50}) was determined for each strain in three replicates. Conidia were counted as germinated when the germ tube was greater than or equal to conidial length.

Life cycle and rearing of *Spodoptera litura*

Spodoptera litura larvae were collected from infested coccinia fields of Visakhapatnam district, Andhra pradesh for rearing in the laboratory. Larvae were transferred into plastic bowls and fed with castor leaves (15 cm in diameter). To avoid crowding, 20 larvae were taken per bowl sealed with muslin cloth for aeration and kept at 26°C, at a photoperiod of 12h + 12h light and dark regimes. Castor leaves were changed daily. After pupation the pupae were kept in plastic boxes, half filled with sterile moist sand for maintaining RH. After 5 to 6 days, the emerged moths were transferred into cages 30 cm^3 volume having bouquets of castor leaves with petioles dipped in water in a conical flask. The adults were fed on artificial nectar (20% honey, 0.4% Vit. E and Vit. B complex 0.5%). Masses of eggs appear in light brown colour and egg patches were covered with felt of pale brown scales on the castor leaves. Egg patches were collected carefully along with a piece of leaf and kept in sterile boxes. Boxes containing egg patches were incubated at 25-28°C for hatching. Larvae that emerge from the eggs after 3 to 5 days were reared at 26°C. Young larvae were fed with fresh leaves of castor. The larvae obtained from a single egg patch were used for each experiment. Second generation larvae were used for conducting pathogenicity experiments in order to get homogeneity. Larvae of same age group were chosen for experimentation.

Bioassay with *B. bassiana* and *M. anisopliae* conidia

To test the virulence of each fungus, twenty second instar larvae (5day old) were chosen at random. The number of conidia/ml of the suspension was estimated through haemocytometer counts. 100 µl of conidial suspensions at 1x10^8 conidia/ml was used for surface treating of the insects using a micropipette. Larvae were placed as a batch of 20 (for each treatment) in perforated plastic boxes (15 cm). Fresh castor leaves were provided as feed every day and containers were cleaned of insect litter daily. They were placed in an environmental chamber set at 25±1°C, 90% relative humidity and a 16 hr/8 hr light/dark cycle. The insects were treated for two consecutive days. The larvae that were fatally injured during inoculation could be identified and removed during the two days treatment period. Bioassays were set up with three replicates for each treatment and controls were treated with an equal volume of water with 0.02% Tween 80®. The dead insects were transferred to moist chambers (autoclaved Petri dishes with a moist filter paper lining) to facilitate mycosis. Before transferring the dead insects into moist chambers they were immediately surface sterilized with 1% sodium hypochlorite followed by three rinses with sterile distilled water. The median lethal time (LT_{50}) was calculated from the cumulative mortality data on each day post treatment, using probit analysis and the whole bioassay experiments were repeated twice.

Result and Discussion

Conidial germination

Time required for 50% germination of the conidia (TG_{50}) was computed from the *invitro* germination data of the conidia of *M. anisopliae* and *B. bassiana* strains. TG_{50} values showed a range of 15.24 - 24.89 hrs and 15.76 – 20.12 hrs for *M. anisopliae* and *B. bassiana* respectively. Initiation of germination was recorded after 6 hrs of inoculation in five strains (M19, M20, M45, B51, B52 and B55), and from 12 hrs in the remaining strains. The maximum rate of germination was attained at 40 hrs. The least TG_{50} of 15.24 hrs was recorded in M45 strain and the highest of 24.89 hrs in M49. Strains with TG_{50} between 15-16 hrs were considered as early germinating, 17-19 hrs were slow germinating and 20 hrs and above were categorized as late germinating (Table 2).

Bioassay with *B. bassiana* and *M. anisopliae* conidia

All the strains tested in the laboratory bioassay at a concentration of 1 x 10^8 conidia ml^{-1} were found to be pathogenic to 2nd instar larvae. No mortality was observed in controls. Difference in virulence against the pest was

observed among the strains. LT_{50} values for 2nd instar *S. litura* larvae varied from 3.93 to 6.4 days (Table 3). *M. anisopliae* strains M20 and *B. bassiana* B55 originally isolated from *Nilaparvata lugens*, Homoptera demonstrated least LT_{50} value of 3.93 and 4.13 days respectively.

The mean cumulative mortality of *B. bassiana* and *M. anisopliae* strains ranged between 71% - 99%. Strain M46 showed the least mortality of 71% and the highest mortality was shown by M20, B51 and B55. Mycosis was found in all the strains. The mycosis percent ranged from 30% - 98% (Table 3). B55 strain showing rapid germination also depicted higher mycosis value as 98% and maximum value as 1.43 relative virulence index. The RVI of the strains ranged between positive and negative values (Table 3). Virulence of *M. anisopliae* and *B. bassiana* strains was understand with respect to the three parameters i.e., median lethal time, total percent mortality and percent mycosis against the 2nd instar larvae of lepidopteran pest *Spodoptera litura*. The study enable characterization of the strains of *M. anisopliae* and *B. bassiana* strains into more and less virulent categories.

The observations on *invitro* germination speed (TG_{50} values) of strains of *M. anisopliae* and *B. bassiana* (Table 2) correlate with LT_{50} values. The more virulent strains (M20, B55 and B51) demonstrated early germination with TG_{50} values of 15.29, 16.2 and 15.76 hrs respectively. While less virulent strain M46, M49, B56 and B57 required longer in *vitro* germination time (17.96, 24.89, 18.14 and 20.12 hrs). The low virulent strain B52 showing low TG_{50} (15.92 hrs) appear to be an exception. Similar observation was given by Samuels et al. (1989) reporting positive correlation between rapidly germinating strains of *M. anisopliae* and pathogenicity against *Nilaparvata lugens* (Homoptera: Delphacidae). On the other hand, Tefera and Pringle (2003) found no consistent relationship between virulence, conidial germination, vegetative growth and sporulation, indicating that there may be other factors governing virulence of the strains. For instance, high temperature and solar UV-B radiation are the principal factors limiting entomopathogenic fungi under field conditions. These factors cause both the conidial inactivation and delay in the germination of the survivors (Fernandes et. al., 2008)

Any attempt to demonstrate correlation between germination characteristics of conidia and virulence could provide a better understanding of the infection process and aid in the selection of most promising strains for development as microbial pesticides. Comparing the infectivity of *Paecilomyces fumosoroseus* strains to the larvae of *Plutella xylostella*, Altre et al. (1999) reported that the correlation of infectivity with spore length and germination speed in broth was highly significant. Penetrant hyphae of virulent strains of *P. fumosoroseus* were visible in larval cuticle cross-sections of *P. xylostella* and *Spodoptera frugiperda* within 22 h after inoculation. Virtually no penetration was observed for an avirulent strain for up to 52 h after inoculation (Altre et.al, 2001). Specific conidial traits have been identified which were considered to be good indicators of virulence including spore size, adhesion, and germination speed. The present study demonstrated that low TG_{50} values in both *M. anisopliae* and *B. bassiana* strains associated with high virulence, against *S. litura* larvae as indicated by low LT_{50} values. Although more detailed studies are needed, it appears that germination rate is an important parameter useful for screening the pathogenic strains for virulence.

Table 1: Origin, original host and accession number of *M. anisopliae* and *B. bassiana* strains.

Strain	Acc. No.	Fungal species	Insect Host	Order	Geographical origin
M19	ARSEF 1080	*M. anisopliae sensu stricto*	*Helicoverpa zea*	Lepidoptera	USA
M20	ARSEF1823	*M. anisopliae sensu lato*	*Nilaparvata lugens*	Homoptera	India
M45	ARSEF 925	*M. anisopliae sensu lato*	*Deois flavopicta*	Homoptera	Brazil
M46	ARSEF 1726	*M. anisopliae sensu stricto*	*Nilaparvata lugens*	Homoptera	India
M48	ARSEF 1882	*M. anisopliae sensu stricto*	*Tibraca limbativentres*	Hemiptera	Brazil
M49	ARSEF 2786	*M. anisopliae sensu stricto*	*Helicoverpa zea*	Orhoptera	Moldova
M50	ARSEF 538	*M. pingshanse*	*Oryctes rhinoceros*	Coleoptera	Thailand
M51	ARSEF 1725	*M. pingshanse*	*Nilaparvata lugens*	Homoptera	India
M52	ARSEF 2575	*M. robertsii*	*Curculio caryae*	Coleoptera	USA
B51	ARSEF 502	*B. bassiana*	*Ostrinia nubilalis*	Lepidoptera	China
B52	ARSEF 504	*B. bassiana*	-----	Coleoptera	Yogoslavia
B54	ARSEF 533	*B. bassiana*	*Ostrinia nubilalis*	Lepidoptera	China
B55	ARSEF 654	*B. bassiana*	*Nilaparvata lugens*	Homoptera	China
B56	ARSEF 679	*B. bassiana*	*Nilaparvata lugens*	Homoptera	China
B57	ARSEF 736	*B. bassiana*	*Chalacodermus aeneus*	Coleoptera	Brazil
B44	ARSEF 1514	*B. bassiana*	*Musca autumnalis*	Diptera	France

Table 2: Rate of conidial germination of *M. anisopliae* and *B. bassiana.*

Strain	TG50	Fiducial Limits	
		Lower	Upper
M19	16.6	16.38	16.82
M20	15.29	15.03	15.55
M45	15.24	15.03	15.44
M46	17.96	17.7	18.23
M48	17.54	17.34	17.74
M49	24.89	24.71	25.06
M50	22.75	22.57	22.93
M51	19.42	19.17	19.67
M52	22.12	21.82	22.56
B44	18.45	18.11	18.97
B51	15.76	15.25	16.21
B52	15.92	15.37	16.55
B54	17.99	17.28	18.38
B55	16.2	15.61	16.89
B56	18.14	17.71	18.69
B57	20.12	19.91	20.55

Table 3: Laboratory bioassay data of strains of *M. anisopliae* and *B. bassiana* at $1x10^8$ conidia/ml concentration against 2nd instar *S. litura.*
* RVI – Relative Virulence Index.

Strain	LT50	Regression	Slope	Fiducial limit		Mortality %	Mycosis %	RVI*
M48	4.46	2.28±4.18	4.467±0.33	4.14	4.81	87.03±1.48	65.9±3.2	0.59
M19	5.13	1.091±5.49	5.139±0.28	4.86	5.43	75.9±1.53	89.7±1.49	0.01
M50	4.53	2.348±4.03	4.536±0.33	4.21	4.88	81.9±2.68	78.0±2.59	0.46
M20	3.93	2.506±4.19	3.935±0.31	3.63	4.26	91.4±3.1	76.2±3.51	1.29
M51	4.6	2.091±4.34	4.607±0.31	4.3	4.93	80.5±1.83	79.3±3.09	0.37
M45	4.71	1.93±4.56	4.713±0.31	4.41	5.03	83.21±1.38	93.3±4.19	0.72
M46	6.4	1.254±4.64	6.405±0.37	6.04	6.78	71.98±0.45	30.7±2.59	-1.84
M49	6.17	1.327±4.64	6.176±0.36	5.82	6.54	73.8±1.59	51.9±2.85	-1.25
M52	5.64	1.875±4.15	5.641±0.36	5.28	6.02	79.8±2.89	67.9±2.59	-0.36
B51	4.5	1.99±4.62	4.62±0.35	4.01	4.9	90.1±3.18	92.3±3.84	0.76
B52	6.31	1.43±4.47	4.47±0.34	5.9	6.75	63.56±2.56	85.3±3.29	-1.34
B54	4.58	2.21±4.22	4.22±0.34	4.26	4.93	90.7±1.93	93.5±4.82	0.77
B55	4.13	2.32±4.34	4.34±0.34	3.84	4.46	99.1±3.45	98.2±3.19	1.43
B56	5.74	1.62±4.45	4.44±0.33	5.4	6.11	78.32±1.48	71.3±2.54	-0.81
B57	5.6	1.74±4.36	4.36±0.32	5.25	5.96	83.3±0.33	76.2±3.95	-0.44
B44	4.62	1.92±4.64	4.64±0.35	4.32	4.94	80.3±1.58	62.1±0.37	-0.38

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