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Research Paper

Functional interplay of certain pathogenicity determinant factors governing virulence of the entomopatogenic fungal species *Beauveria*

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Abstract

Different pathogenicity parameters like hydrophobin protein content, spore protein content, rate of germination, in vitro extra cellular enzymes production and bioassays against 2nd instar larval stage of two polyphagous pests viz., Spodoptera litura and Helicoverpa armigera were performed for 30 isolates of Beauveria. There was significant positive correlation and apparent interplay of all the factors studied. However, extracellular proteases played a major role in determining virulence of the fungal isolates.

Keywords: Hydrophobin proteins, Spore proteins, spore germination, extracellular enzymes, bioassays, *S. litura* and *H. armigera*

Introduction

Virulence of biocontrol agent is a key aspect in crop pest management programs, governed by a large number of factors interacting with the target pest right from the time of physical contact till mortality. Conidia or spores form the main resources for transmission and persistence of the fungus. Entomopathogenic fungi defer from other insect pathogens in their mode of infection through insect cuticle. This unique feature is of major importance in the formation, germination and survival of various types of infective spores or conidia that attach to the host cuticle germinate and subsequently penetrate the insect cuticle [1]. Spore germination depends on limited endogenous resources present within the resting spores for completing all the developmental stages that precede host penetration.

These processes, which occur on the host surface, must therefore be highly efficient to ensure successful penetration into the host tissues within a short period of time [2]. Initiation of the infection process in *B. bassiana* starts with adherence of conidia to the cuticle of a susceptible host by hydrophobic/enzymatic mechanism [3]. Hydrophobins are surface active proteins produced by conidia of filamentous fungi which consist of a base protein rich hydrophobic rodlet layer that interacts with insect epicuticle for attachment [4]. Such hydrophobic conidia are most likely to germinate when they contact a carbon or nitrogen source and moreover the conidial wall and the insect epicuticle mediate the adhesion process [5]. Under *in vitro* conditions an utilizable, exogenous carbon energy source is required for germination of *B. bassiana* conidia, while a nitrogen source is further required to sustain hyphal growth. Although conidia apparently possess nitrogen reserves to germinate, the supply soon depletes and without an exogenous nitrogen source, lysis of the germ tube occurs [6].

Beauveria is known to produce extracellular proteolytic, chitinolytic and lipolytic enzymes which aid in the hydrolysis of insect cuticle and help in the entry of hyphae into the haemocoel [7]. When *B. bassiana* was *grown* in medium containing insect cuticle as the sole carbon and nitrogen source, extracellular protease appears first followed by chitinases [8]. Relationship between enzyme and virulence could prove useful in developing enzyme based screening methods to identify new fungal isolates with desired virulence characteristics [9]. The differences in pathogenicity are signs of naturally occurring genetic variations during co evolution of the fungus which can be taken into account for its development as a potential mycopesticide [10].

The objectives of the present study are quantitative estimation of spore protein and hydrophobin protein, rate of germination, in *vitro* extracellular enzyme production i.e. protease, chitinase, lipase and bioassays of 30 *Beauveria* isolates against 2nd instar larvae of two polyphagous pests viz., *S. litura* and *H. armigera* in order to asses genetic variation among the isolates and to generate biochemical markers for virulence. Such information facilitates understanding of the infection process and aid in selection of truly promising isolates for development as mycopesticide.

Materials and Methods

Fungal cultures

A total of thirty isolates were studied out of which, six were exotic (B6, B7, B8, B12, B33 and B35) and the rest were endemic isolated from local farmer's fields. The isolates were routinely sub cultured on SDAY (Sabouraud's Dextrose Yeast Agar) slants maintained at 26ºC ± 1ºC.

Rate of germination

Sterilized glass slides were coated with 0.2 ml autoclaved SDAY medium in a laminar air flow cabinet and allowed to solidify. 10µl of $1x10^6$ conidia /ml suspension was inoculated on the slides which were immediately transferred into pre sterilized petri plates lined with moistened filter papers for providing the required relative humidity and incubated at 26ºC ± 1ºC. Rate of germination was observed from the 8th hour of incubation onwards with every two hour interval. Spores were considered to be germinated only when the germ tube was longer than the conidia. 300 conidia were examined randomly for germination count on each slide and percent germination was calculated.

Spore protein and Hydrophobin protein extraction and estimation

Spore proteins and Hydrophobin proteins were extracted [12, 13] and further protein estimation for both was also performed [14].

In vitro extra cellular enzymatic studies

Detection of extra cellular enzymes by halo plate methodology [15] followed. For protease plate assay, gelatin (1%) in minimal media (0.003 % NaCl, 0.03 % $MgSO_4$ and 0.015 % K_3PO_4) and pH 7.0 was used. For chitinase plate assay, colloidal chitin (1%) was first prepared [16] and added to minimal medium (0.003 % NaCl, 0.03 % $MgSO_4$ and 0.015 % K_3PO_4) and pH 7.0 was used. For lipase plate assay, the medium consisted of Tween 20 (1%) along with peptone (1%), 500 mg NaCl, 10 mg $CaCl_2$ and agar (2%) and pH 6.0.

Autoclaved media were poured into pre sterilized petri plates and allowed to set overnight. For lipase, Tween 20 was not mixed with other components but sterilized separately and added to the autoclaved medium before pouring into the plates. Wells of diameter 2 mm were made at the centre of each petri plate with a sterilized cork borer and 50 µl of 2 $x10^8$ / ml spore suspension was inoculated in the wells following incubation for 10 days at 26ºC± 1ºC. The level of enzyme production was evaluated by the halo diameter, measured in centimeters, in the reverse of the petri plates. For detection of protease production, 10 ml solution containing mercuric chloride (15%) in 2N HCl was flooded on each culture plate and allowed to stand for 5 min after which gradually, a halo zone could be observed around the

wells. For chitinase and lipase, no particular solution was used as the halos were visible. The enzymatic index values were calculated or measured as the ratio of diameter of the zone of clearing to that of the colony [17].

Bioassays on *Spodoptera litura*

The culture of *S. litura* was raised from field collected larvae and maintained under laboratory conditions. Egg patches laid on the surface of castor leaves by adult females were incubated in a growth chamber separately in sterilized containers and allowed to hatch and develop to 2nd instar stage. Fifteen larvae for each *Beauveria* isolate were topically inoculated with 50µl of conidial suspension at a concentration of 2x108 conidia /ml. Three replicates for each isolate were maintained along with controls. The treated larvae were placed in separate containers provided with castor leaves as diet. The larvae treated with only 0.02% Tween 80 were considered as controls.

Bioassays on *Helicoverpa armigera*

The culture of *H. armigera* was raised from field collected larvae and maintained under laboratory conditions. The larvae were fed with artificial diet prepared [18]. As the larvae were cannibalistic in nature, they were maintained in separate containers till pupation. The emerged moths were allowed to lay eggs on chick pea plantlets in special cages. The newly hatched neonate larvae were immediately transferred individually into plastic vials containing a piece of artificial diet described previously until they reach 2nd instar. Fifteen larvae for each *Beauveria* isolate were topically inoculated with 50µl of conidial suspension at a concentration of 2x10^{8} /ml conidia. Three replicates for each isolate were maintained along with controls. The treated larvae were placed in separate containers provided with artificial diet. Larvae treated and fed with artificial diet only with 0.02% Tween 80, served as the control for each replicate.

All the treatments were arranged in a randomized complete block design and replicated 3 times. The experimental set up was maintained at a temperature of 26±1ºC and larval mortalities were recorded from the 1st day of post treatment with 24hr interval. The treated and control insects were observed every day post treatment and the mortality recorded. The cadavers were surface sterilized with 1% mercuric chloride solution and washed thrice with sterile double distilled water [19]. They were placed in moist chambers at 26±1ºC to facilitate mycosis [20]. The cadavers of each replicate experiment were placed in one moist chamber and the number of cadavers, which expressed mycosis, was noted. The cadavers were observed for mycelial growth and sporulation in the next 48 hrs to confirm death due to infection by *Beauveria*.

Statistical analysis

Data pertaining to spore protein, hydrophobin protein and extracellular enzymes viz., protease, lipase and chitinase were analyzed by SPSS version 11.0. The values indicated are mean ± s.e.m of three individual experiments with triplicates for each experiment subjected to statistical analysis by Tukey's HSD test with mean sample size n = 3 and level of significance (P≤ 0.05). TG50s were calculated using SYSTAT version 11.0. From the bioassay data, mean lethal time (LT50) was calculated using Probit analysis [21]. Percent mortality and percent mycosis were analyzed for assessing variance applying general linear model analysis of variance (ANOVA) using STATISTICA 6.0. Significance was accepted at P = <0.05. Percent mortality and percent mycosis data was log transformed before subjecting to analysis. There was no mortality in the controls in any of the replicates so no Abbott correction [22] was therefore required.

Results and Discussion

Rate of germination

Out of 30 *Beauveria* isolates studied, germination could be observed from 8th hr in only 12 isolates and TG50 range was from 11th to 25th hr (Table 1). Germination was delayed and could be seen from 16th and 18th hour onwards in B33 and B37 isolates. The least TG50 was recorded in B27 isolate at the 11th hr. On the other hand, the highest TG50 values were recorded at 25th hr in B33 isolate followed by B37 at the

23rd hr. 100% spore germination was recorded in all the isolates starting between 22nd hr (B22, B27, B28, B29 and B26) and 40th hr (B33).

Spore and Hydrophobin protein

The spore proteins started with a maximum value of 1.32 µg / mg in B27 followed by 1.28 µg/ mg in B19. Minimum value of 0.32 was scored by B38 followed by 0.44 µg / mg in B28. The hydrophobin proteins of the isolates ranged from a maximum of 0.51 µg / mg in B32 and followed by 0.38 µg / mg in B31. Lowest value of 0.03 µg / mg was recorded in B20 followed by 0.04 µg / mg in B8.

Bioassays

Spodoptera litura

Pathogenicity test showed differences in mortality rates among the 30 isolates studied. The LT50s ranged from 3 to 7 days and were within the range of fiducial limits (Table 2) Least LT50 values of 3.80, 3.98, and 3.96 days were observed in B19, B22 and B40 isolates whereas highest LT50 of 7.3 in B8 and 6.5 days in B29 were observed and rest of the isolates displayed a range from 4.1 to 6.0 days. The isolates which were more effective towards 2nd instar *S. litura* larvae are B19, B22 and B40 therefore considered being more aggressive. The isolates which were less effective towards the 2nd instar larvae of *S. litura* were B8 and B29. Highest percent mortality of 95% in B19 and lowest of 57% in B33 were observed in 2nd instar larvae of *S. litura*. Mycosis was not recorded in four isolates of *Beauveria* i.e., B8, B14, B20 and B35. A highest of 86.33% in B19 and lowest of 5.47% in B16 was observed.

Helicoverpa armigera

The pathogenicity of *Beauveria* on 2nd instar *H. armigera* showed differences in the mortality rates (Table 3). The least LT50 values were in the range of 4.27, 4.4 to 4.8 days in B19, B7 and B32. The highest LT50 values were observed in B41 (9.3 days) followed by 7.71 days in B37, 7.6 days in B30 and 7.2 days in B20 and B31. LT50 for rest of the isolates fell in the range of 7.7 days to 5.39 days. LT50 values were within the fiducial limit ranges. The isolates which were more aggressive towards 2nd instar larvae were B7, B15, B16, B19, B32 and B38. The isolates which were less aggressive were B41, B37, B30, B33*, B31 and B20. Highest percent mortality of 95.67 in B33and lowest of 53.33 was observed in B37 in second instars larvae of *H. armigera*. Mycosis was not observed in B8, B14, B15, B16, B20, B28, B29, B30, B33, B35, B39, B40 and B41 isolates. Highest of 43.67 in B19 and lowest of 4.43 in B25 was observed.

Hydrophobins were found to play important role in morphogenesis and pathogenesis in various fungi [22]. B32 isolate, which showed maximum quantity of hydrophobins, demonstrated lower values of LT50 in bioassays against *S. litura* larvae and hence categorized as virulent. On the other hand, isolates B20 and B8, which recorded lower values for hydrophobins, correspond to the low virulent category against the *S. litura* and *H. armigera*. Mycosis was observed in a large number of dead insects in the present study. Larval mortality and time mortality response were found to exist between the different strains of *P. fumosoroseus* and one of the strains had an LT50 of 2.6 days [23]. In our observations, 100% mortality was also observed in several isolates with varying LT100s among the isolates. LT50 of *B. bassiana* and *M. anisopliae* against fruit flies also ranged between 3-4 days where as the LT90 values in all the isolates did not exceed four days [24].

There appears a somewhat linear relationship between spore protein and hydrophobin proteins up to certain extent in the case of adhesion and germination of the spore. The least TG50 and TG100 along with the highest of 1.32 µg/mg spore protein were recorded in B27 where as hydrophobin protein was medium with 0.25 µg / mg. The highest TG50 as well as TG100 was observed in B33. If both spore protein and hydrophobin protein levels are to be correlated with germination, then the two parameters were medium. Lowest hydrophobin protein was found in B20 followed by B8 but this was not the case with spore protein where medium spore protein was recorded. Un- germinated spores of *P. fumosoroseus* isolates attached to diamondback moth cuticle quickly and firmly enough to withstand brief,

Table 1: Germination, spore protein, hyrdrophobin protein and extra cellular enzyme Production in *Beauveria* sp

Isolate number	Germination (%) at 8th hr post inoculation	TG_{50} in hours	Spore protein (µg/mg)	Hydrophobin protein (µg/mg)	Protease	Chitinase	lipase
B6	9.3	15.07±0.01	1.176±0.000	0.12±0.004	1.63	1.04	0
B7	0	18.59±0.00	1.166±0.040	0.19±0.004	1.69	1.06	2.14
B8	0	18.59±0.01	0.820±0.000	0.04±0.007	1.40	1.04	0
B12	0	13.43±0.02	1.120±0.010	0.35±0.004	1.50	1.03	1.84
B13	5.3	15.67±0.00	1.146±0.000	0.27±0.004	1.58	1.05	2.20
B14	0	16.44±0.00	0.713±0.00	0.24±0.014	2.00	1.01	2.00
B15	4.6	12.46±0.01	1.156±0.000	0.26±0.003	1.61	1.06	1.28
B16	0	16.26±0.03	0.840±0.070	0.12±0.004	1.86	1.03	2.27
B18	0	17.49±0.00	1.130±0.008	0.06±0.004	1.62	1.04	0
B19	18	13.58±0.01	1.280±0.004	0.35±0.004	2.20	1.08	1.22
B20	0	15.68±0.00	0.640±0.010	0.03±0.004	1.30	1.03	2.43
B22	0	17.60±0.00	1.130±0.000	0.34±0.004	1.55	1.08	1.67
B23	0	14.77±0.00	1.130±0.008	0.32±0.004	1.16	1.04	1.50
B24	17.6	11.98±0.01	1.180±0.004	0.12±0.004	1.80	1.05	0
B25	0	17.99±0.03	0.880±0.004	0.35±0.004	1.50	1.03	0
B26	9.3	13.30±0.00	1.070±0.004	0.29±0.004	1.69	1.08	0
B27	12	11.82±0.02	1.320±0.000	0.25±0.004	1.18	1.03	2.00
B28	4.3	14.01±0.04	0.440±0.004	0.21±0.007	1.66	1.05	1.67
B29	3.9	14.12±0.00	0.940±0.000	0.32±0.004	1.80	1.05	2.03
B30	0	20.20±0.02	0.920±0.004	0.18±0.004	1.50	1.05	0
B31	0	14.79±0.03	1.180±0.004	0.38±0.004	1.50	1.04	1.60
B32	3.3	13.90±0.00	1.080±0.004	0.51±0.007	2.00	1.07	2.07
B33	0	24.97±0.05	1.080±0.004	0.14±0.004	1.50	1.06	1.87
B35	0	18.15±0.00	0.750±0.004	0.26±0.004	1.60	1.04	0
B37	0	22.46±0.06	0.850±0.004	0.28±0.004	1.50	1.05	2.15
B38	0	19.96±0.01	0.320±0.018	0.24±0.004	1.80	1.04	0
B39	0	14.13±0.02	0.530±0.022	0.23±0.039	2.00	1.04	1.86
B40	0	14.64±0.00	0.830±0.022	0.12±0.004	1.80	1.04	1.65
B41	19.3	11.91±0.03	0.860±0.004	0.17±0.004	1.40	1.06	2.00
B42	0	16.87±0.02	0.820±0.017	0.17±0.004	1.70	1.05	0

vigorous washing 10 min after inoculation [25]. If the germination data in our findings are taken into account, no correlation between the rate of germination and virulence was observed as the few virulent isolates were not the fastest germinating ones. In accordance to our results, slower germinating *N. rileyi* isolates were found to be more virulent against two species of larval noctuidae [26].

Table 2: Bioassay of *Beauveria* isolates against 2nd instar *S. litura* larvae

Isolate number	LT50	Fiducial Limit	% Mortality 2nd instar	% Mycosis 2nd instar
B6	4.101 ± 0.638[a]	3.511 to 4.791	83.33 ± 0.27	40.00 ± 0.47
B7	5.32 1± 0.623	4.739 to 5.988	76.64 ± 0.20	41.67 ± 0.72
B8	7.340 ± 0.794	6.592 to 8.183	66.67 ± 0.22	0
B12	5.210 ± 0.533	4.704 to 5.771	75.33 ± 0.21	57.00 ± 0.47
B13	5.706 ± 0.542	5.189 to 6.274	84.33 ± 0.20	56.33 ± 0.27
B14	4.648 ± 0.447	4.222 to 5.117	73.67 ± 0.22	0
B15	4.761±0.4730	4.097 to 5.542	88.67 ± 0.27	33.00 ± 0.47
B16	4.977 ± 0.535	4.470 to 5.044	86.67 ± 0.20	5.470 ± 0.07
B18	4.955 ± 0.461	4.516 to 5.438	77.67 ± 0.22	43.33 ± 0.54
B19	3.808 ± 0.401	3.428 to 4.230	95.00 ± 0.23	86.33 ± 0.27
B20	5.921 ± 0.442	5.495 to 6.380	63.33 ± 0.21	0
B22	3.983 ± 0.312	3.682 to 4.307	93.33 ± 0.22	38.57 ± 0.03
B23	5.921 ± 0.442	5.495 to 6.380	83.33 ± 0.21	38.83 ± 0.10
B24	4.190 ± 0.381	3.826 to 4.589	86.33 ± 0.20	36.33 ± 0.27
B25	5.134 ± 0.376	4.771 to 5.524	91.33 ± 0.22	44.00 ± 0.47
B26	4.450 ± 0.400	4.067 to 4.869	91.33 ± 0.23	68.67 ± 0.27
B27	5.404 ± 0.640	4.800 to 6.083	72.33 ± 0.24	51.67 ± 0.00
B28	5.428 ± 0.818	4.668 to 6.310	70.33 ± 0.25	38.33 ± 0.27
B29	6.535 ± 0.561	5.998 to 7.120	86.33 ± 0.20	11.67 ± 0.27
B30	4.673 ± 0.381	4.307 to 5.069	82.67 ± 0.26	30.67± 0.54
B31	6.025 ± 0.477	5.567 to 6.521	93.67 ± 0.22	28.33 ± 0.27
B32	4.689 ± 0.426	4.282 to 5.132	84.33 ± 0.20	71.67 ± 0.27
B33*	5.087 ± 0.388	4.713 to 5.489	57.33 ± 0.21	38.10 ± 0.05
B35	4.273 ± 0.405	3.887 to 4.698	82.33 ± 0.23	0
B37	4.845 ± 0.488	4.382 to 5.358	91.33 ± 0.24	41.40 ± 0.05
B38	4.755 ± 0.190	4.290 to 5.271	91.33 ± 0.26	43.93 ± 0.07
B39	4.385 ± 0.402	4.001 to 4.806	93.33 ± 0.28	22.70 ± 0.05
B40	3.963 ± 0.562	3.241 to 4.369	85.67 ± 0.24	32.13 ± 0.11
B41	4.320 ± 0.435	3.906 to 4.777	89.33 ± 0.24	33.67 ± 0.27
B42	4.825 ± 0.687	4.184 to 5.563	84.33 ± 0.20	32.33 ± 0.27

* *Beauveria brongniartii*

Table 3: Bioassay of *Beauveria* isolates against 2nd instar *H. armigera* larvae

Isolate number	LT50	Fiducial limit	% Mortality	% Mycosis
B6	5.253±0.713	4.586to6.075	75.67 ± 0.22	9.501 ± 0.22
B7	4.801±0.682	4.169to5.541	85.67 ± 0.27	11.63 ± 0.26
B8	6.144±0.657	5.521to6.837	60.33 ± 0.23	0
B12	5.328±0.638	4.727to6.005	90.33 ± 0.27	18.83 ± 0.10
B13	5.692±0.814	4.934to6.567	87.67 ± 0.27	22.33 ± 0.27
B14	5.522±0.689	4.875to6.256	73.33 ± 0.27	0
B15	5.077±0.621	4.610to5.592	85.33 ± 0.25	0
B16	4.985±0.528	4.484to5.541	79.67 ± 0.27	0
B18	6.661±0.763	5.939to7.468	73.33 ± 0.23	28.53 ± 0.24
B19	4.273±0.806	3.539to5.160	93.33 ± 0.27	55.53 ± 0.24
B20	7.271±1.356	6.218to8.106	65.67 ± 0.27	0
B22	5.911±0.653	5.293to6.601	80.00 ± 0.27	18.77 ± 0.03
B23	5.395±0.968	4.510to6.455	65.33 ± 0.27	24.00 ± 0.47
B24	5.757±1.052	4.796to6.910	59.33 ± 0.27	18.67 ± 0.27
B25	7.113±0.244	5.972to8.472	80.33 ± 0.27	16.33 ± 0.27
B26	5.930±0.841	4.939to7.119	79.33 ± 0.27	43.00 ± 0.47
B27	5.574±0.676	4.938to6.293	79.67 ± 0.27	25.67 ± 0.27
B28	5.326±0.759	4.619to6.142	61.00 ± 0.27	17.67 ± 0.27
B29	6.614±0.987	5.697to7.678	58.67 ± 0.27	0
B30	7.699±0.765	6.649to8.915	62.00 ± 0.27	22.50 ± 0.00
B31	7.292±0.062	6.353to8.370	63.67 ± 0.25	13.50 ± 0.09
B32	4.472±0.695	4.125to5.521	75.67 ± 0.28	47.67 ± 0.27
B33*	7.200±0.943	6.317to8.206	95.67 ± 0.27	16.33 ± 0.27
B35	5.297±1.350	4.106to6.833	58.67 ± 0.27	0
B37	7.727±1.034	6.760to8.833	53.33 ± 0.27	13.67 ± 0.72
B38	5.053±1.099	4.065to6.280	79.67 ± 0.26	12.67 ± 0.72
B39	5.466±0.851	4.678to6.386	60.67 ± 0.27	8.330 ± 0.27
B40	5.252±1.296	4.104to6.721	65.67 ± 0.25	6.670 ± 0.27
B41	9.337±0.954	8.431to10.34	86.67 ± 0.27	18.33 ± 0.27
B42	6.642±0.071	5.672to7.777	79.33 ± 0.27	25.67 ± 0.27

* *Beauveria brongniartii*

***In vitro* extra cellular enzymes production**

All the *Beauveria* isolates showed clear enzymatic index values for *in vitro* protease production. Maximum enzymatic index value of 2.20 was observed in B19 isolate followed by 2.00 in B14, B32 and B39 while the lowest enzymatic index values of 1.16 in B23 was recorded followed by B20 with 1.30 (Table1). Isolate specific enzymatic index values were observed in thirteen isolates. Extracellular chitinase production was recorded in all the thirty isolates. Maximum chitinase activity was observed in B22 and B26 with an index value of 1.08 followed by 1.06 in B32. On the other hand lowest activity of 1.01 in B14 was recorded followed by 1.03 in B12, B16, B20, B25 and B27. A narrow range of enzyme index values were obtained for *in vitro* chitinase production ranging from 1.01 to 1.08. *In vitro* lipase production was recorded in only 20 out of 30 *Beauveria* isolates. Maximum enzymatic index value of 2.43 was recorded in B32 followed by 2.27 and 2.2 in B16 and B13. Minimum index value of 1.28 was recorded in B15 followed by 1.50 in B23 and 1.54 in B20.

Pearson's correlation

Significant positive correlation of $\alpha = 0.05$ was recorded between spore protein and hydrophobin protein and also between spore protein and chitinase ($\alpha = 0.01$). Significant positive correlation ($\alpha = 0.05$) between hydrophobin protein and chitinase was obtained. While between hydrophobin protein and protease at $\alpha = 0.01$ was observed. Significant positive correlation ($\alpha = 0.01$) between hydrophobin protein and lipase and between protease and chitinase ($\alpha = 0.05$).

The third phase of infection involves the penetration of germ tube through the cuticle and proliferation of mycelium inside the haemocoel of insect host. This is the stage where the extracellular enzymes play a crucial role in the disintegration of cuticle and help in penetration of germ tube into haemolymph. In the present study, *in vitro* protease, chitinase and lipase were studied by plate method for all thirty isolates. The results revealed quantitative differences in the enzymes among the isolates. Protease and chitinase production was found to be higher in the more virulent and less in the less virulent isolates. On the other hand, with respect to lipase production, there was no correlation between virulence and the lipase production. In few isolates, there was no lipase secretion though they demonstrated moderate virulence. On the other hand, few virulent isolates recorded higher lipase activity. Several investigators established the possible role of these enzymes during the process of host penetration [17]. Penetration of the insect cuticle is facilitated by the proteases released during invasion, and then chitinases get activated during later steps of penetration suggesting that they have a minor role in cuticle penetration compared with proteases [27].In our observations, the *in vitro* chitinase enzyme production was least in isolate B14 and this was one of the isolate which did not show mycoses during the bioassays. Low levels of chitinase activity at the time of germination and an increase in the activity during mycelial growth, with maximum activity at the time of sporulation in entomopathogenic fungi can be observed [28].

Conclusion

All the factors viz; hydrophobins, spore proteins, rate of germination, production of extracellular enzymes govern the infection and proliferation of the entomopatogenic fungus in its host. All the factors come into play at the precise time and also interplay in order to infect the host. Of all the parameters studied, extracellular enzymes seem to play a major role in the infection process as their presence is important at the time of germ tube entry through the cuticle and also in breaching of haemolymph, proliferation and completion of life cycle of the fungus by sporulating outside the insect cadaver.

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