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Enzymatic comparison and mortality of *Beauveria bassiana* against cabbage caterpillar *Pieris brassicae* LINN

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ABSTRACT

Beauveria bassiana, an entomopathogenic fungus, is the alternative biocontrol agent exploited against major economic crop pests. *Pieris brassicae* L. is an emerging pest of the Brassicaceae family. Therefore, in the present study, fungal isolates of *Beauveria bassiana*, viz. MTCC 2028, MTCC 4495, MTCC 6291, and NBAII-11, were evaluated for their virulence against third instar larvae of *P. brassicae*. Among all these fungal isolates, maximum mortality (86.66%) was recorded in *B. bassiana* MTCC 4495 at higher concentration of spores (10^9 conidia/ml), and the minimum mortality (30.00%) was recorded in *B. bassiana* MTCC 6291 at a lower concentration (10^7 conidia/ml) after ten days of treatment. The extracellular cuticle-degrading enzyme activities of fungal isolates were measured. Variability was observed both in the pattern of enzyme secretion and the level of enzyme activities among various fungal isolates. *B. bassiana* MTCC 4495 recorded the maximum mean chitinase (0.51 U/ml), protease (1.12 U/ml), and lipase activities (1.36 U/ml). The minimum mean chitinase and protease activities (0.37 and 0.91 U/ml, respectively) were recorded in *B. bassiana* MTCC 6291. The minimum mean lipase activity (1.04 U/ml) was recorded in *B. bassiana* NBAII-11. Our studies revealed *B. bassiana* MTCC 4495 as the most pathogenic isolate against *P. brassicae*, which also recorded maximum extracellular enzyme activities, suggesting the possible roles of extracellular enzymes in the pathogenicity of *B. bassiana* against *P. brassicae*.

Introduction

Pieris brassicae (Lepidoptera: *Pieridae*), which is commonly known as cabbage butterfly, affects cole crops and causes damage at all its growth stages.[1] This caterpillar feeds only on plants that releases glucosinolate, which is a characteristic feature of Brassicaceae that includes cabbages, cauliflower, and mustards.[2] *P. brassicae* is a widely distributed lepidopteran pest of crucifers and is the most destructive pest of Brassicaceae,[3] causing up to 40% yield loss per year.[1] Chemical insecticides are usually applied to control *P. brassicae*, but high tolerance to most insecticides and associated environmental problems have jeopardized their continued use.[4]

The cosmopolitan anamorphic fungus *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) is a

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well-recognized microbial agent known to infect hundreds of host insect species. It is also considered as the most appropriate biological control agent in temperate agriculture regions.[5] This ability has promoted extensive research on the potential of *B. bassiana* for biological control of many agriculturally important pests.[6] The success of *B. bassiana* as biopesticides depends not only on its high efficacy against insect pests but also on its non-pathogenic behavior toward non-target pests.[7] *B. bassiana* attaches its fungal spore on the hydrophobic cuticle that subsequently develops into an infectious structure called appressorium and releases an array of cuticle solubilizing enzymes.[6] The fungus infects the host by breaching its cuticle, which is composed of chitin fibrils embedded in a matrix of proteins, lipids, and N-acylcatecholamines.[8] *B. bassiana* secretes extracellular enzymes such as protease, chitinase, and lipase to degrade the major constituents of the cuticle of the insect that allow hyphal penetration into the cuticle. These enzymes play an important role in the pathogenesis and other physiological processes of *B. bassiana*.[9,10] So, the present study aimed to evaluate the pathogenicity of *B. bassiana* isolates against *P. brassicae* and compare their extracellular enzyme activities.

Materials and methods

Sources of fungal isolates and preparation of conidial suspension

Three fungal isolates, viz. *B. bassiana* MTCC 2028, MTCC 4495 and MTCC 6291, obtained from IMTECH, Chandigarh, India and one *B. bassiana* NBAII-11 fungal isolate procured from NBAII, Bangalore, India was used in the present study. The four isolates were maintained and grown in the Sabouraud Dextrose Agar Yeast Extract (SDAY) medium for 10–14 days in flasks, and aqueous spore suspensions were prepared. Spores were inoculated into 100 ml of SDAY medium in 250-ml Erlenmeyer flasks. The seeded flasks were incubated for 15 days at 25 ± 1 °C. The conidia were harvested by scrapping and suspended in sterilized 0.1% Tween 80 solution.[11] The suspension was vortexed for 5 min to produce uniform conidial suspension and filtered through one layer of sterile muslin cloth. A sample of the suspension was quantified in a Neubauer chamber according to Alves.[12] Then, the suspension containing 10^9 conidia/ml was prepared, and concentrations of 10^8 and 10^7 conidia/ml were obtained through serial dilutions. Sterilized water without fungal inoculation was used as a control.

Rearing of P. brassicae in laboratory

P. brassicae larvae were collected from cauliflower fields and reared on its leaves in the laboratory. The pupae formed were transferred to other battery jars having 2.5 cm thick moist sponge at the bottom covered with a circular paper of diameter just fitting jar to avoid their contact with a moist sponge. The open end of the jar was covered with a piece of muslin cloth and tied with a rubber band. The adults that emerged from the pupae were collected, and pairs of male and female butterflies were transferred to a cage having a pot of cauliflower plant for obtaining egg masses. A cotton swab dipped in liquid protein diet was hung from the roof of the cage. The leaves containing egg masses were taken out and kept separately in another clean battery jar. These egg masses constituted the initial culture, which was further used for the rearing of *P. brassicae*. The different instars at particular stages were kept in different battery jars to obtain synchronized culture of the pest for study.

Bioassays against third instar larvae of Pieris brassicae

B. bassiana isolates were evaluated for the pathogenicity against third instar larvae of *P. brassicae*. The viability of conidia was tested at different concentrations as per the methodology of Ibrahim et al.[13] The bioassays were conducted as per the methodology of Akmal et al.[14] with a few modifications. For each fungal isolate, three different fungal concentrations (10^7, 10^8 and 10^9 conidia/ml) were evaluated, and each concentration was replicated thrice. Larvae to be tested were allowed to crawl on different concentrations of fungal suspension in petri plates. For control, larvae were not treated. There were three replicates per treatment and ten larvae per replicate. After the initial treatment, the larvae were fed with fresh and untreated leaves of cauliflower. The cumulative percentage of mortality was recorded on successive days. Dead larvae were shifted to petri dishes with moist filter paper for fungal development and sporulation in order to confirm death due to fungal infection.

Production of cuticle-degrading enzymes

The extracellular cuticle-degrading enzymes (CDEs) such as chitinase, protease, and lipase activities of fungal isolates were evaluated as described by Nahar et al.[14] with some modifications. These fungal isolates at a concentration of 10^7 spores/ml were inoculated into 250 ml Erlenmeyer flasks containing 100 ml of chitin medium, and the flasks were incubated on a rotary shaker at 150 rpm for 12 days at 25 ± 1 °C. The medium composition was (g/L): KH_2PO_4, 3.0; K_2HPO_4, 1.0; $MgSO_4$, 0.7; $(NH_4)_2SO_4$, 1.4; NaCl, 0.5; $CaCl_2$, 0.5; yeast extract, 0.5; bacto-peptone, 0.5; chitin, 5.0; and olive oil, 5 ml/l. The enzyme extractions and assays were carried out after 24 h of incubation up to 12 days. Flasks were removed from incubation at alternative days, and the broth was centrifuged at 8000 rpm for 25 min at 4 ± 1 °C to extract clear supernatant. The supernatant was considered as crude enzyme extract and used for enzyme assays.

Enzyme assays

Chitinase activity was estimated in the culture supernatant by using acid-swollen chitin as the substrate.[16] To prepare acid-swollen chitin, 10 g of chitin powder (Hi media) was suspended in 300 ml of chilled o-phosphoric acid (88%, w/v) at 4 °C for 1 h with occasional stirring. The mixture was then poured into ice cold distilled water and filtered through Whatman filter paper. Acid swollen chitin was repeatedly washed with 1% (w/v) sodium bicarbonate solution, and the pH was adjusted to 7. The solution was homogenized in a Waring blender (1 min), and the concentration of acid swollen chitin was adjusted to 7 mg/ml by adding 50 mM acetate buffer, pH 5.0. The reaction

mixture for chitinase assay contained 1 ml of 0.7% swollen chitin, 1 ml of 50 mM acetate buffer, pH 5.0, and 1 ml of crude enzyme extract that was incubated at 50 °C for 1 h. The N-acetylglucosamine residues (GlcNAc) produced was estimated at 520 nm according to the methodology of Somogyi.[17] One unit of enzyme activity was expressed as 1 μmol of GLcNAc per min.

Protease activity was measured using casein (10 g casein in 100 ml of 0.2 mM sodium carbonate buffer, pH 9.7) as a substrate.[16] The reaction mixture contained 1 ml of casein, 1 ml of sodium carbonate buffer, pH 9.7, and 1 ml of crude enzyme extract. Moreover, the reaction mixture was incubated at 35 °C for 20 min. The reaction was terminated by adding 3 ml trichloroacetic acid (2.6 ml 5% TCA + 0.4 ml 3.3 N HCl). The absorbance of the TCA soluble fraction was measured at 280 nm. One unit of enzyme corresponds to 1 μmol of tyrosine per min.

Lipase activity was determined by using olive oil and gum acacia as substrate according to the methodology of Pignede et al.[18] The substrate emulsion was prepared with olive oil (50 ml) and gum arabic (50 ml, 10% (w/v), Himedia) in the ratio of 1:1. The reaction mixture contained 1 ml of crude enzyme extract, 5 ml of substrate emulsion and 2 ml of 50 mM phosphate buffer, pH 6.8. The reaction mixture was incubated for 1 h at 37 °C with constant shaking, and the reaction was terminated with 4 ml of acetone–ethanol (1:1) containing 0.09% phenolphthalein as an indicator. Enzyme activity was determined by titration of the fatty acids released with 50 mM sodium hydroxide. One unit of lipase is the amount of enzyme that released of 1 μmol of fatty acids per min. All the enzyme assays were carried out in triplicates at alternative days.

Data analysis

One-way analysis of variance (ANOVA) was used to compare the results by using CPCS1 software. Enzymatic activities means were compared by Duncan's MRT *post hoc* test in SPSS 16.00 statistical software. Means with $P < 0.05$ were considered to be significantly different.

Results and discussion

Bioassay with P. brassicae

After three days of treatment, the higher and middle concentrations (10^9 and 10^8 conidia/ml, respectively) of *B. bassiana* NBAII-11 recorded maximum cumulative percentage mortality (26.66%). Comparatively, the higher concentration (10^9 conidia/ml) of *B. bassiana* 6291 recorded the least mortality (3.34%) and was not significantly different from the untreated control. After five days of treatment, the cumulative mortality ranged from 10.00% to 53.33%. The maximum mortality (53.33%) was recorded in *B. bassiana* NBAII-11 at a higher concentration of spores, which was at par with *B. bassiana* MTCC 4495 that recorded 50.00% mortality at the same concentration (Table 1). Moreover, *B. bassiana* NBAII-11 at middle and lower concentrations recorded 43.33% and 33.33% mortality, respectively, and these were at par with each other. The minimum mortality (10.00%) was recorded in MTCC 6291 and MTCC 2028 at a lower concentration (10^7 conidia/ml). However, this was at par with *B. bassiana* MTCC 4495 (13.34%) and MTCC 6291 (16.67%) at lower and higher concentrations, respectively. After seven days of treatment, the maximum mortality (60.00%) was recorded in *B. bassiana* NBAII-11 and MTCC 4495 at higher concentrations, which was at par with *B. bassiana* MTCC 4495 and NBAII-11 at the middle concentration that recorded 53.33% mortality (Table 1). This was followed by *B. bassiana* MTCC 2028, which recorded 43.33% mortality at higher concentration and was at par with MTCC 4495 and NBAII-11 with 60.00% and 53.33% mortality at higher and middle concentrations, respectively. Minimum mortality (16.66%) was recorded in MTCC 6291 at lower concentration (10^7 conidia/ml) and was at par with 26.66% mortality recorded in *B. bassiana* MTCC 2028 at the same concentration. All treatments at different concentrations were statistically significant compared to the untreated control after seven days of treatment.

After ten days of treatment, the mortality ranged from 30.00% to 86.66%. The maximum mortality (86.66%) was recorded in *B. bassiana* MTCC 4495 at a higher concentration of spores and was significantly better than all other treatments at various concentrations. This was followed by MTCC 4495, which recorded 70.00% mortality at middle concentration and was at par with MTCC 2028 and NBAII-11 isolates that recorded 66.66% and 60.00% mortality at higher concentrations (Table 1). These were further at par with NBAII-11 (60.00%) at the middle concentration (10^8 conidia/ml). The minimum mortality (30.00%) was recorded in *B. bassiana* MTCC 6291 at a lower concentration and was at par with NBAII-11 (40.00%) and MTCC 2028 (43.33%) at the same concentration. All treatments were significantly better than the untreated control.

Chitinase assay

The maximum mean chitinase activity (0.51 U/ml) was recorded in *B. bassiana* MTCC 4495 followed by *B. bassiana* MTCC 2028 (0.49 U/ml). In contrast, the minimum mean chitinase activity (0.37 U/ml) was recorded in *B. bassiana* MTCC 6291 (Table 2). A significant difference was recorded between the chitinase activities at alternative days. The maximum chitinase activity (0.65 U/ml) was recorded on the sixth day that was significantly better than that on all other days. Among all days of observations, the minimum mean chitinase activity (0.29 U/ml) was recorded on the 12th day. The chitinase activity increased from 2nd to 6th day and decreased from 8th to 12th day (Fig. 1a).

Protease assay

The maximum mean protease activity (1.12 U/ml) was recorded in *B. bassiana* MTCC 4495, which was significantly better than all other isolates of *B. bassiana*. This was followed by *B. bassiana* MTCC 2028 with 1.02 U/ml (Table 3). The minimum mean protease activity (0.91 U/ml) was recorded in *B. bassiana* MTCC 6291. A significant difference was recorded in the mean protease activities at various days. The maximum protease activity (1.46 U/ml) was recorded on the 8th day of incubation, and the minimum mean protease activity

Table 1 – Bio-efficacy of *B. bassiana* isolates against *P. brassicae* Linn.

Treatments	Concentrations (conidia/ml)	Days of observations			
		3rd	5th	7th	10th
MTCC 2028	10^7	3.34 (6.68)^{c}	10.00 (18.42)^{defg}	26.66 (30.77)^{defg}	43.33 (41.13)^{efh}
	10^8	10.00 (18.42)^{bc}	20.00 (26.05)^{cdef}	36.66 (37.20)^{dfe}	50.00 (44.98)^{cdefg}
	10^9	16.66 (23.84)^{ab}	23.33(28.76)^{bcdef}	43.33 (41.05)^{bcde}	66.66 (54.76)^{bcde}
MTCC 4495	10^7	3.34 (6.68)^{c}	13.34 (17.97)^{defg}	33.33 (35.20)^{cdef}	60.00 (50.83)^{bcdef}
	10^8	16.66 (23.84)^{ab}	36.66 (37.20)^{abcde}	53.33 (46.90)^{abcd}	70.00 (56.97)^{bcd}
	10^9	23.33 (28.76)^{ab}	50.00 (44.98)^{abc}	60.00 (50.74)^{ab}	86.66 (71.99)^{a}
MTCC 6291	10^7	3.34 (6.68)^{c}	10.00 (15.24)^{defg}	16.66 (23.84)^{fg}	30.00 (32.98)^{fgh}
	10^8	13.33 (21.13)^{ab}	16.66 (23.84)^{cdef}	33.33 (34.99)^{cef}	43.33 (41.13)^{dg}
	10^9	3.34 (6.68)^{c}	16.67 (20.18)^{defg}	36.66 (37.20)^{cdef}	46.66 (43.05)^{defg}
NBAII-11	10^7	16.66 (23.84)^{ab}	33.33 (35.20)^{abcde}	40.00 (39.21)^{bcdef}	40.00 (39.21)^{efgh}
	10^8	26.66 (30.98)^{a}	43.33 (41.13)^{abcd}	53.33 (46.90)^{abcd}	60.00 (50.83)^{bcdef}
	10^9	26.66 (30.98)^{a}	53.33 (46.90)^{abc}	60.00 (50.74)^{ab}	60.00 (50.74)^{bcdef}
CONTROL		3.34 (6.68)^{c}	3.34(6.68)^{fg}	6.67 (12.55)^{h}	6.67 (12.55)^{i}
CD(0.05)		11.85	14.42	8.54	10.93

Values represent means of three replicates.
Values in parentheses represent arc sine transformations.
Means followed by the same letter (a, b, c) in vertical column are not significantly different at 0.05% level of probability.

Table 2 – Chitinase activity of fungal isolates at alternative days.

Chitinase activity on different days after inoculation (μg of N-acetylglucosamine produced min/ml of the broth)

Isolate	2nd	4th	6th	8th	10th	12th	Mean
MTCC 2028	0.50 ± 0.014^{dB}	0.53 ± 0.019^{cC}	0.67 ± 0.004^{aB}	0.63 ± 0.004^{bA}	0.32 ± 0.005^{eC}	0.28 ± 0.002^{fC}	0.49
MTCC 4495	0.52 ± 0.013^{dA}	0.64 ± 0.06^{bA}	0.75 ± 0.010^{aA}	0.54 ± 0.011^{cB}	0.35 ± 0.006^{eB}	0.29 ± 0.002^{fB}	0.51
MTCC 6291	0.34 ± 0.005^{cD}	0.29 ± 0.008^{fD}	0.60 ± 0.006^{aD}	0.39 ± 0.006^{bD}	0.31 ± 0.005^{dD}	0.28 ± 0.016^{eC}	0.37
NBAII-11	0.36 ± 0.004^{eC}	0.57 ± 0.002^{bB}	0.61 ± 0.01^{aC}	0.49 ± 0.004^{cC}	0.45 ± 0.025^{dA}	0.31 ± 0.005^{fA}	0.46
Mean	0.43	0.51	0.65	0.51	0.36	0.29	
CD(5%) isolates = 0.008							
CD (5%) days = 0.01							
CD(5%) isolates × days = 0.02							

Values are mean ± standard deviation of three replicates.
Means followed by the same letter (a, b, c) in horizontal column are not significantly different at 0.05% level of probability using Duncan LSD *post hoc* test.
Means followed by the same letter (A, B, C) in vertical column are not significantly different at 0.05% level of probability using Duncan LSD *post hoc* test.

Table 3 – Protease activity of fungal isolates at alternative days.

Protease activity on different days after inoculation (μg of tyrosine produced min/ml of the broth)

Isolate	2nd	4th	6th	8th	10th	12th	Mean
MTCC 2028	0.54 ± 0.023^{dB}	1.22 ± 0.021^{cAB}	1.33 ± 0.016^{bC}	1.51 ± 0.016^{aB}	1.35 ± 0.021^{bB}	0.18 ± 0.008^{eB}	1.02
MTCC 4495	0.58 ± 0.009^{eA}	1.26 ± 0.028^{dA}	1.50 ± 0.021^{bA}	1.63 ± 0.016^{aA}	1.43 ± 0.016^{cA}	0.32 ± 0.001^{fA}	1.12
MTCC 6291	0.25 ± 0.017^{dC}	1.22 ± 0.020^{cAB}	1.40 ± 0.016^{aB}	1.32 ± 0.016^{bD}	1.20 ± 0.021^{cC}	0.10 ± 0.073^{eB}	0.91
NBAII-11	0.52 ± 0.009^{dB}	1.20 ± 0.021^{bB}	1.38 ± 0.046^{aBC}	1.39 ± 0.016^{aC}	1.12 ± 0.020^{cD}	0.28 ± 0.009^{eA}	0.98
Mean	0.47	1.22	1.40	1.46	1.27	0.22	
CD(5%) isolates = 0.02							
CD (5%) days = 0.02							
CD(5%)isolates × days = 0.04							

Values are mean ± standard deviation of three replicates.
Means followed by the same letter (a, b, c) in horizontal column are not significantly different at 0.05% level of probability using Duncan LSD *post hoc* test.
Means followed by the same letter (A, B, C) in vertical column are not significantly different at 0.05% level of probability using Duncan LSD *post hoc* test.

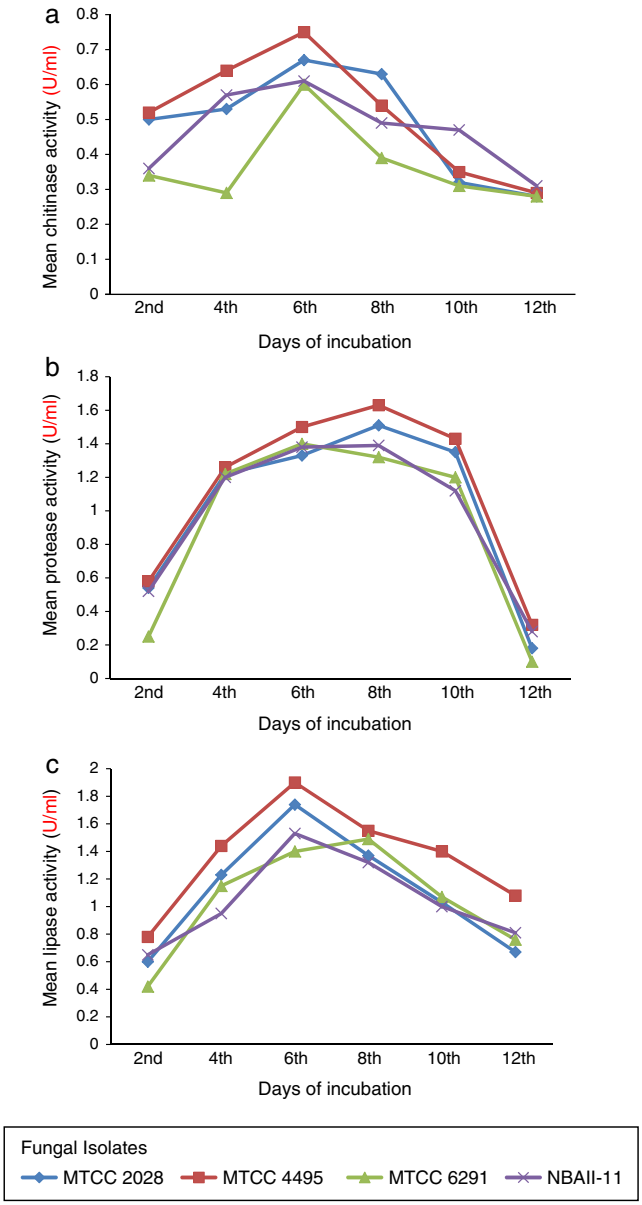

Fig. 1 – (a) Effect of incubation days on Chitinase activity of *B. bassiana* isolates. (b) Effect of incubation days on protease activity of *B. bassiana* isolates. (c) Effect of incubation days on lipase activity of *B. bassiana* isolates.

(0.22 U/ml) was recorded on the 12th day of incubation. Protease activity increased from day 2nd to 8th, and thereafter, it gradually decreased (Fig. 1b).

Lipase assay

A significant difference was recorded between the mean lipase activities of various isolates of *B. bassiana*. The maximum mean lipase activity (1.36 U/ml) was recorded in *B. bassiana* MTCC 4495 followed by *B. bassiana* MTCC 2028 (1.11 U/ml). The minimum mean lipase activity (1.04 U/ml) was recorded in *B. bassiana* NBAII-11 (Table 4). The mean lipase activities of various days were significantly different from each other. The maximum mean lipase activity (1.64 U/ml) was recorded at 6th day, and the minimum mean lipase activity (0.61 U/ml) was recorded on the 2nd day of incubation (Fig. 1c).

Discussion

Mortality variation was observed among various isolates of *B. bassiana*; this could be related to the ability to counter the insect immune response, molecular, and physiological mechanisms such as excretion of extracellular enzymes.[19] In *B. bassiana*, various proteins and enzymes have been characterized that are essential for virulence. Proteins of the P450 family have been identified as important virulence factors involved in cuticle-degradation.[20] It was also reported that Bbslt2, a novel mitogen-activated protein (MAP) kinase has a pivotal role in regulating fungal development, growth, and pathogenicity.[21] Bb*gas1*, a gene encoding a putative transferase (Glycosylphosphatidyl inositol-anchored β-1,3-Glucanosyl transferase) is involved in virulence and thermal regulation.[22] Variation in mortality among different strains of *B. bassiana* can be correlated with differential expression of these virulence governing genes. Study of genetic, molecular and physiological mechanisms of *B. bassiana* can underpin the exact reason responsible for the variation among different isolates of *B. bassiana*.

The cumulative percentage mortality enhanced with the increase in spore concentration. Maximum mortality was recorded at higher concentrations of spores (10^9 Spores/ml) on all days of observations. Butt and Goettel[23] reported a positive correlation between the number of infective spores and mortality by mycosis. They found that susceptibility of most insects depends on spore dosage.

Wright et al.,[24] also reported that susceptibility of target insect to fungal infection is dose dependent and the mortality increases with time. Akmal et al.[14] evaluated different entomopathogenic fungal strains against different species of aphids under laboratory conditions and found that their mortality enhanced with the increase in spore concentration. Tefera and Pringle[25] reported the mortality in their target insect (*Chilo partellus*) increased when treated with *B. bassiana* and *M. anisopliae*. They reported 100% mortality after ten days of the treatment. Similar observations were recorded in our present study against third instar larvae of *P. brassicae*. These larvae recorded higher mortality at higher concentrations that increased with time. Akbari et al.[26] also reported that the cumulative percentage mortality was lower in the first two days but increased thereafter.

The extracellular enzyme activity was considered as an important factor for their virulence. Chitinase is the most important enzyme to degrade the chitin polymer of the insect cuticle. Virulence of entomopathogenic fungi can be correlated with the chitinase activity. Among four isolates of *B. bassiana*, the highest mean chitinase activity was recorded in *B. bassiana* MTCC 4495, which showed maximum mortality against third instar larvae of *P. brassicae*. Pelizza et al.[27] also reported that *B. bassiana* isolate with highest levels of chitinase activity was more pathogenic against *Tropida criscollaris*. Perinotto et al.[28] found that the most virulent strain of entomopathogenic fungi against *Rhipicephalus microplus* showed

Table 4 – Lipase activity of fungal isolates at alternative days.

Isolate	Lipase activity on different days after inoculation (μmol of fatty acids per min/ml of the broth)						
	2nd	4th	6th	8th	10th	12th	Mean
MTCC 2028	0.60 ± 0.12^{dAB}	1.23 ± 0.08^{bB}	1.74 ± 0.08^{aA}	1.37 ± 0.04^{bA}	1.03 ± 0.04^{cB}	0.67 ± 0.08^{dB}	1.11
MTCC 4495	0.78 ± 0.12^{dA}	1.44 ± 0.12^{bA}	1.90 ± 0.08^{aA}	1.55 ± 0.09^{bA}	1.40 ± 0.08^{bA}	1.08 ± 0.08^{cA}	1.36
MTCC 6291	0.42 ± 0.08^{dB}	1.15 ± 0.05^{bB}	1.40 ± 0.08^{aB}	1.49 ± 0.08^{aA}	1.07 ± 0.08^{bB}	0.76 ± 0.16^{cB}	1.05
NBAII-11	0.65 ± 0.08^{dA}	0.95 ± 0.04^{bC}	1.53 ± 0.12^{aB}	1.32 ± 0.25^{aA}	1.00 ± 0.08^{bB}	0.81 ± 0.16^{bcB}	1.04
Mean	0.61	1.19	1.64	1.43	1.13	0.83	
CD(5%) isolates = 0.72							
CD(5%) days = 0.89							
CD(5%)isolates × days = 0.17							

Values are mean ± standard deviation of three replicates.
Means followed by the same letter (a, b, c) in horizontal column are not significantly different at 0.05% level of probability using Duncan LSD *post hoc* test.
Means followed by the same letter (A, B, C) in vertical column are not significantly different at 0.05% level of probability using Duncan LSD *post hoc* test.

the maximum chitinase activity. So, chitinase activity can be correlated with the virulence of *B. bassiana*. Protease was considered as an important enzyme in the infective process of entomopathogenic fungi.[29] Entomopathogenic fungi produced large quantities of protease that degraded the proteinaceous material, and the solubilized proteins were then degraded by amino peptidases and exopeptidases to form amino acids that served as nutrients for the fungi. Extracellular protease produced by *B. bassiana* is suspected to be involved in the pathogenesis. *B. bassiana* MTCC 4495, which was evaluated as the most pathogenic strain against third instar larvae of *P. brassicae*, showed maximum protease activity. Biodochka and Khachatourians[30] recorded marked proteolytic activity associated with virulent isolates of *Beauveria*. *B. bassiana* MTCC 6291 recorded the least mortality and showed the minimum protease activity. Perinotto et al.[28] showed that the isolates of entomopathogenic fungi, which were more virulent against *R. microplus*, exhibited the highest levels of proteolytic activity. Thus, proteases can be considered as the important factor that governs virulence in *B. bassiana* against *P. brassicae*.

Lipases hydrolyze the ester bonds of lipoproteins, fats, and waxes at the interior of the insect integument and significantly contribute to the cuticle penetration and initial release of nutrients.[31] Lipase activity and virulence of entomopathogenic fungi can be correlated. *B. bassiana* MTCC 4495 strain was found to be pathogenic against third instar larvae of *P. brassicae*, which showed the maximum mean lipase activity. The isolate with minimum mean lipase activity was found to be the least effective against *P. brassicae*. Hence, lipases can be considered as an important virulence factor in *B. bassiana*.

The chitinase, protease and lipase activities were found to increase from the 2nd day of incubation up to six days of incubation. After the 8th day of incubation, the extracellular enzyme activities were found to decrease. Proteolytic activity of entomopathogenic fungi decreased with the increase in culture age probably due to nutrient limitation or autolysis of the culture. Braga et al.[32] reported that high caseinolytic activity of *M. anisopliae* during four to six days of culture and a steep decrease in the activity up to 16 days of culture. Ziabeeet et al.[33] reported maximum lipase activity at 6th day and subsequent decrease in incubation lipase activity.

The present results are in corroboration with that of other studies. Nahar et al.[15] reported chitinase activity (0.001 U/ml) in *Metarhizium anisopliae* after 120 h of incubation in chitin-containing medium. Supakdamrongkul et al.[34] reported specific lipase activity (9.41 U/mg) in purified form of lipase after four days of culture. Dhar and Kaur[35] evaluated protease activities of different isolates of *B. bassiana* at different days of incubation and recorded that the protease activity ranged from 0.0804 to 0.7583 U/ml at sixth day of incubation. They also reported variability in enzyme activities and found higher protease activity comparative to other fungal isolates.

Moreover, colloidal chitin, peptone, and olive oil in minimal medium induced the production of chitinase, proteases, and lipase, respectively. Fernandes et al.[36] reported the production of proteases by the entomopathogenic fungi only in the presence of gelatin as the substrate in the medium. In contrast, St. Legar et al.[37] exploited colloidal chitin as the sole source of carbon and nitrogen to induce Pr1 activity and reported that polymeric substances like cellulose and insect cuticle enhance the Pr1 activity either by inducing the protease production. They also reported that colloidal chitin was unable to produce catabolite repression. Our studies suggest that colloidal chitin with peptone may induce the production of chitinase as well as protease. Chitinase and protease act concurrently in these enzymes; this was supported by the fact that the highest levels of chitinolytic and proteolytic activities were recorded on 4th, 6th and 8th day of incubation.

However, the mean lipase activity on the 2nd day of incubation was found to be more than the mean chitinase and protease activities on the same day; thus, this may be considered as the first enzyme secreted by *B. bassiana* for the initiation of infection. Silva et al.[38] also reported that the lipase secreted by entomopathogenic fungi were involved in the initial stages of the adhesion and penetration. In addition, extracellular lipase activity was found to be more as compared to protease and chitinase; hence, lipase may be considered as an important enzyme in metabolic activities of *B. bassiana*. Supakdamrongkul and his coworkers[34] found that fungal isolates amended with purified lipase showed higher virulence against *Spodoptera litura*. Lipase can be considered as the most important enzyme in the physiology and pathogenesis of *B. bassiana*. The difference in enzyme activities and level

of secretion can be correlated with the genetic architecture of *B. bassiana*. In future, these contradictions can be explained by the genomics and proteomics analysis of *B. bassiana*. Moreover, the relationship between enzyme activities and virulence may be useful in developing enzyme-based screening methods to select the most virulent fungal isolates against *P. brassicae*.

Conflicts of interest

The authors declare no conflicts of interest.

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