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Full Length Research Paper

Bioassay and enzymatic comparison of six entomopathogenic fungal isolates for virulence or toxicity against green peach aphids *Myzus persicae*

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***Myzus persicae* is an economically important pest infecting more than 40 families of crops throughout the world. Six entomopathogenic fungal isolates, three each of *Beauveria bassiana* and *Verticillium lecanii*, were screened for pathogenicity test against the *M. persicae* to select high virulent isolate with the most suitable application and to determine the role of individual enzyme in its virulence. Two treatments that is, conidial shower (190±23 conidia/mm^2) and filtrate (3 ml filtrate per treatment from six days liquid broth culture of 1.0x10^8 conidia ml^{-1}) were conducted for virulence or toxicity test and a comparison was made between treatments and among fungal isolates against the target pest. The percent mortality rates of filtrate at each day, after inoculation was found higher as compared to percent mortality of conidial showering. *V. lecanii* 3 showed highest virulence or toxicity against the target pest treated either with conidial (80.70%) or filtrate (88.36%) application while *B. bassiana* 70 and *B. bassiana* 76 showed high toxicity (77.14 and 80.86%, respectively) in filtrate application at 6th day of incubation. The aphidicial activities of the fungal isolates were evaluated by correlating the enzymes (Chitinase, protease and lipase) activities with enzymes production. On the basis of enzymatic activities, lipase was assumed to participate more in the total virulence or pathogenicity as compared to protease and chitinase while protease was assumed to participate more than chitinase. The pathogenicity test reveals the selection, effective application of most virulent isolate and the role of individual enzyme to develop an alternative control agent against *M. persicae*.**

Key words: *Beauveria bassiana,* filtrate, conidia, enzymes, *Verticillium lecanii.*

INTRODUCTION

Aphids are serious pests infesting both agricultural and horticultural crops by direct feeding damage and transmission of plant viruses (Harrington and van Emden, 2007). Green peach aphid *Myzus persicae* (Sulzer) is a cosmopolitan and polyphagous pest (Vorburger et al., 2008) which infests various plants, particularly cruciferous vegetables in China (Su-Dan et al., 2005) and transmits plant viruses (Kanavaki et al., 2006). Due to environmental, human and other organism's health hazards effects, the use of some chemical pesticides is banned in the developed countries. Therefore, biological control agents have importance in managing the agricultural and forestry practices (Khachatourians, 2008) and the entomopathogenic fungi is important among all the biological control agents due to its broad host rang, route of pathogenicity and control of sap sucking pests such as mosquitoes and aphids (Thomas and Read, 2007; Fan et al., 2007) as well as pests with chewing mouthparts (Hajek and St. Leger, 1994; de Faria and Wraight, 2007).

Entomopathogenic fungi, like V*erticillium lecanii,*

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Beauveria bassiana and *Metarhizium anisopliae* are intensively studied natural enemies and important epizootics to aphids, and other agricultural pests (Roberts and St. Leger, 2004; Thomas and Read, 2007; Wang et al., 2004; Li and Sheng, 2007; Chen et al., 2008) as well as to plant pathogens (Ownley et al., 2010; Goettel et al., 2008). *V. lecanii* (Zimm. Viegas) infests aphids (Faria and Wraight, 2007) as well as plant pathogenic fungi like powdery mildews (Verhaar et al., 1997, 1998; Askary et al., 1998; Dik et al., 1998; Miller et al., 2004), rusts (Spencer and Atkey, 1981; Leinhos and Buchenauer, 1992), green molds (Benhamou and Brodeur, 2000), *Fusarium* (Koike et al., 2007), *Verticillium dahliae* (Kusunoki et al., 2006) and *Pythium ultimum* (Benhamou and Brodeur, 2001). *B. bassiana* colonize endophitically in plants, inducing the induced systemic resistance against pathogen (Ownley et al., 2010).

Mitosporic fungi are generally environmentally friendly, with negligible or low mammalian toxicity and have no residual toxicity (Copping, 2004) and are successful as mycoinsecticides against aphids (Faria and Wraight, 2007; Milner, 1997; Shah and Pell, 2003). Based on *V. lecanii* and *B. bassiana*, several mycopesticides like Vertalec®, Botanigard® etc. have been developed against aphids and used in several countries including the United Kingdom and the United States (Goettel et al., 2005; Kiss, 2003).

The conidial based application, specificity, abiotic factors like humidity, temperature and sunlight (Burges, 1998; Ownley et al., 2004) and the slow germination of conidia reduce the total market-percentage which is directly proportional to killing speed of the pesticide (St Leger and Wang, 2009). Hence, liquid broth culture application is the alternate method to bypass all these hindrances (Kim et al., 2009). Quesada-Moraga et al. (2006) have demonstrated that entomopathogenic fungi secrete insecticidal or antifeedant bioactive compounds in the cultural broth which may be purified and can be used as antifeedant or insecticides e.g. spinosad and abamectin (Copping and Menn, 2000; Godfrey et al., 2005).

Insect pathogenic fungi infect insects by breaching the host cuticle composed of chitin fibrils embedded in a matrix of proteins, lipids, pigments and N-acylcatecholamines (Richard et al., 2010). The cuticle composition strongly influences conidial germination, resulting in the differential susceptibility of various insect species to a fungal Pathogen (Boucias and Latge, 1988; El-Sayed et al., 1991; Wang et al., 2005). They secrete extracellular enzymes proteases, chitinases and lipases to degrade the major constituents of the cuticle (that is, protein, chitin and lipids) and allow hyphal penetration (Wang et al., 2005; Cho et al., 2006). The outer layers of the epicuticle in insects contain extractable free lipids consisting mainly of aliphatic polar and non-polar compounds (Lockey, 1988). Extracellular lipases are also involved in microbial virulence and play different roles in the infection process (Stehr et al., 2003). Cuticular fatty acids play an important role in spore germination and differentiation. They may be stimulatory in spore germination or fungistatic or toxic (Gillespie et al., 2000).

Campos et al. (2005) demonstrated that addition of chitin or proteins or lipids substrate into the culture medium as inducer can enhance the enzyme production. Similarly the use of transformants by incorporating multiple copies of the gene (St. Leger et al., 1996) can play an effective role to increase the virulence of the strain.

A large number of studies were conducted to potentiate and improve the virulence of entomopathogenic fungi to a greater extent than their alone activity when compared to the wild type (Wang and St. Leger, 2007c; Wraight et al., 2001; Ahman et al., 2002; Fan et al., 2007).

Most of the work is done to increase the virulence by increasing the production of cuticle degrading enzymes either by adding an inducer to the medium or developing transformants by inserting multiple copies of the virulent gene to enhance the virulence. Little is known about the toxicity or virulence of each enzyme (chitinase, protease and lipase) in the total toxicity or virulence of culture filtrate or conidia. Hence it is important to find the degree to which each of the three (chitinase, protease and lipase) enzymes participates in the total virulence of conidia or toxicity of filtrate. This will help us to find the most virulent strain for killing the pest on time and they would have the advantage that a filtrate can be frozen or freeze-dried, resulting in an almost indefinite shelf-life.

Developing different strategies to produce entomopathogenic fungal products and enhancing their efficacy against the target pest will lead to commercialization of these products as biocontrol agents. The objective of the present study was to select the most virulent entomopathogenic fungal strain against *M. persicae*, its appropriate application (conidial or filtrate) and to demonstrate the role of chitinase, protease and lipase enzymes in total virulence.

MATERIALS AND METHODS

Aphid population

Green peach aphids (*M. persicae*) were collected from cabbage leaves in greenhouse conditions at Chinese Academy of Agricultural Sciences (CAAS) Beijing, China. The aphid populations were maintained on potted Chinese cabbage plants (*Brassica rapa)* in cages at 24°C, 45 to 60% relative humidity (RH) and a photoperiod of 16:8 (light : darkness) with the plants replaced every week.

Fungal strains

Three isolates of *B. bassiana* and three isolates of *V. lecanii* were kindly provided by XinMin Li Heilongjiang Academy of Agricultural

Table 1. Names, hosts and origins of the six entomopathogenic fungal isolates.

Isolate	Symbols	Original host	Geographical origin
Beauveria bassiana 70	Bb70	Green peach aphid	Vladivostok (Russia)
Beauveria bassiana 76	Bb76	Green peach aphid	Vladivostok (Russia)
Beauveria bassiana 252	Bb252	Green peach aphid	Vladivostok (Russia)
Verticillium lecanii 2	V2	White fly	Vladivostok (Russia)
Verticillium lecanii 3	V3	White fly	Moscow (Russia)
Verticillium lecanii 5	V5	White fly	Moscow (Russia)

Sciences, Heilongjiang China. The names of isolates, original host, symbols and their origin are listed in Table 1. All the six fungal strains were maintained by culturing on potato dextrose agar Petri plates (90 mm diameter) for 30 days at 26°C in dark.

Conidial suspension

Conidia were harvested from 20 days culture plates. The suspension (0.02% tween 80) was vortexed well and then filtered through sterile cheese cloth. The conidial concentration of the resulting suspension was estimated (1.0 x10^8 conidia mL^{-1}) under a microscope using hemocytometer. The viability of conidia before use in bioassays was confirmed by the method of Hywell-Jones and Gillespie (1990).

Fungal filtrate

Primary cultures of all the fungal isolates were prepared by adding 4 mL of conidial suspension into 100 ml of Adamek's liquid medium. The culture was incubated at 150 rpm for three days. Secondary culture (1%) was prepared by adding 2.5 ml of primary culture into 250 ml of Adamek's liquid medium by incubating at 26°C, 150 rpm for six days. The mycelia were removed by centrifugation at 10,000 rpm for half an hour at 4°C and the resulting supernatant was filtered through 0.45 µm-pore-size filter (Millipore Corp) to get the filtrate. The same process was repeated at different intervals of time (0, three, four, five and six days) during culture incubation to get 2 ml filtrate for enzymatic assays.

Filtrate bioassay

The filtrate (pH 6.0) with tween 80 (0.1% v/v) was filtered through 0.45 µm-pore-size filter (Millipore Corp.) and was sprayed at the rate of 3 ml/treatment. Each treatment consists of two aphids-contaminated-plants (145 to 170 aphids/ plant). The filtrate was sprayed on both sides of the leaves (aphids-contaminated-plants leaves) with Tween 80 (0.1%) sprayed as control. After the filtrate application, the leaves were cut, air dried and placed in Petri dishes at 24°C, 100% RH and a photoperiod of 16L : 8D. The experiment was repeated for all the fungal isolates and corrected mortality rate was recorded daily.

Conidial bioassay

The virulence of the fungal spores against the aphids was determined by the method of Xu and Feng (2002). Cabbage plant leaves having 89 to 121 nymphs were maintained in Petri dishes and were exposed to fungal shower. The Petri plates were placed in a sterilize Petri dish (150 x 25 mm) having 1% agar to maintain 100% RH for conidial germination. The number of spores fall on the aphids or on the leaves were determined by counting the numbers of spores fall on a wet sterilized cover slip (3 fields of cover slip, 0.785 mm^2/field) placed in the Petri plates. The resulting concentration of spores per millimeter square was 190-220. Mortality was recorded after every 24 h for 6 days. Dead aphids from the Petri plates were collected and placed in 100% RH in dark. Only those aphids having fungal growth on their body after several days were counted in dead. The procedure was repeated thrice for all the strains of the fungus and an untreated leaves were used as a control for each isolate.

Enzymatic activities of the filtrate

Chitinase assay

The chitinase activities of all the six fungal filtrates were determined with Chitinase Assay Kit (Sigma-aldrich, MO 63103 USA) according to the manufacturer's instructions. In brief, 10 µl of filtrate was added into 90 µl of the substrate solution in a 96 well plate and incubated at 37°C for 30 min. The reaction was stopped after 30 min by adding 200 µl of stopping solution to each well. The optical density was measured at 405 nm soon after stopping the reaction. One unit will release 1 µmoles of *p*-nitrophenol from the appropriate substrate per minute at pH 4.8 at 37°C.

Protease assay

The method of Campos et al. (2005) was used to determined the subtilisin-like protease Pr1 activities of all the six isolates filtrates with N-Suc-(Ala)2-Pro-Phe-p-nitroanilide (Sigma) as a substrate. The reaction mixture includes 15 µL substrate (2 µM), 10 µl enzyme sample and 75 µl 50 mM Tris–HCL pH 8.0. After incubating for 45 min at 30°C, the reaction was terminated by adding 0.30 ml of 30% acetic acid. Samples were centrifuged at 1000 rpm for 10 min at 4°C. A spectrophotometer was used to measure the optical density at 405 nm. One unit of protease activity was defined as the amount of enzyme needed to release 1 µmoles of nitroanilide (NA) per minute at 37°C.

Lipase assay

The method of Lesuisse et al. (1993) was used for the determination of lipase activity. Chromogenic substrate, *p*-nitrophenyl laurate (8 mM solubilized in isopropanol) was used as a substrate and the amount of *p*-nitrophenol produced was used as lipase activity indication (U/ml). Filtrate volume of 20 µL was added to 880 µl reaction buffer (0.1 M potassium phosphate buffer pH 8.0,

Table 2. Percent mortality rate after 3 and 6 days treatment with conidia or filtrate of entomopathogenic fungi.

Isolate	Filtrate (% mortality)		Conidia(% mortality)	
	Day 3	Day 6	Day 3	Day 6
Beauveria bassiana 70	34.29	77.14	26.55	68.14
Beauveria bassiana 76	36.17	80.85	38.78	57.14
Beauveria bassiana 252	41.48	69.13	28.81	60.17
Verticillium lecanii 2	41.75	71.20	47.19	67.42
Verticillium lecanii 3	49.06	88.36	24.56	80.70
Verticillium lecanii 5	39.38	58.67	26.17	68.22

Table 3. Analysis of variance for the conidial application of the different fungal strains and incubation time interval.

Day	Among days			Among fungi		
	df	F	Sig.	df	F	Sig.
D1	5	3.55	0.034	5	11.84	0.000
D2	5	24.4	0.000	5	10.61	0.000
D3	5	15.43	0.000	5	10.93	0.000
D4	5	34.17	0.000	5	39.44	0.000
D5	5	9.30	0.001	5	38.35	0.001
D6	5	30.96	0.000	5	69.32	0.000

Table 4. Analysis of variance for the Filtrate application of the different fungal strains and incubation time interval.

Day	Among days			Among fungi		
	df	F	Sig.	df	F	Sig.
D1	5	587.90	0.000	5	11.84	0.0003
D2	5	679.55	0.000	5	10.61	0.0004
D3	5	799.61	0.000	5	10.94	0.0004
D4	5	487.09	0.000	5	39.45	0.000
D5	5	666.30	0.000	5	38.36	0.000
D6	5	430.52	0.000	5	69.33	0.000

0.1% gum Arabic and 0.2% sodium deoxycholate) in a tube, and incubated for 3 min at 37°C. The reaction was initiated by adding 100 µl of 8 mM substrate and stopped by adding 0.5 ml of 3 M HCl. The suspension formed was centrifuged for 10 min at 10,000 rpm and 333 µl of the supernatant was taken in a separate tube. 1 ml of 2 M NaOH was added to the supernatant and absorbance was noted at 420 nm. Standard curve was prepared with known concentration of *p*-nitrophenol. The lipase enzyme activities for all the six filtrates were determined. The unit of activity is defined as the amount of enzymes that hydrolyzes 1 µmol substrate in 1 min.

Data analysis

All the treatments were repeated three times. The data was analyzed by one-way analysis of variance (ANOVA) using descriptive and equal variance assumed design. The data were statistically analyzed by using software SPSS (16.0).

RESULTS

Aphidicial activities of the filtrate and conidia

The overall percent mortalities of all the fungal isolates were lower for conidial treatment as compared to filtrate and the mean percent mortality values at day three and six clearly reveals these differences (Table 2). Significant difference was observed among the mortality of different fungal strains recorded at different intervals of time (days) for both filtrate and conidial application (Tables 3 and 4). The Aphidicial activities of both the filtrate and conidia

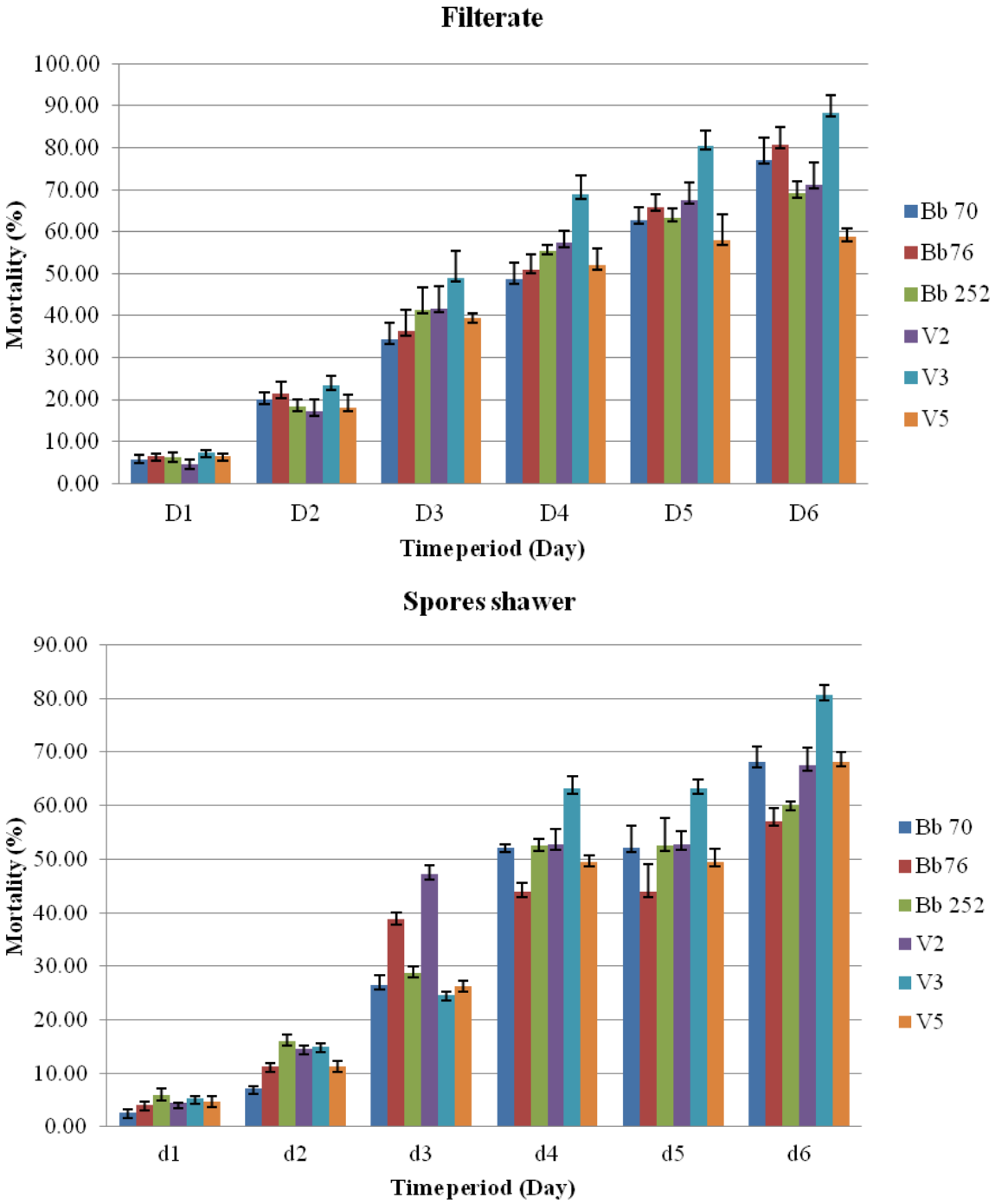

Figure 1. Filtrate and conidial activity of six different fungal isolates verses different interval of times (days).

were in exponential phase till the 6th day of application but some of the fungal strains showed high mortality rate at an earlier stage of application than the others (Figure 1). *V. lecanii* 3 was found to be the most virulent strain with maximum mortality rate in both filtrate and conidial applications as compared to the other strains tested. The *Verticillium lecanii* 3 has shown maximum mortality rate of 49.06 (3rd day), 88. 36 (6th day) and 24%. 56 (3rd day) and 80.70% (6th day) for filtrate and conidial treatment, respectively (Table 2 and Figure 1). *B. bassiana* 70 and *B. bassiana* 76 had maximum percent mortality in filtrate application. The filtrate treatment mortalities for the strain *B. bassiana* 70 were 34.17 (3rd day) and 77.15% (6th day) whereas for *B. bassiana* 76 the filtrate mortalities were

36.17 (3rd day) and 80.85% (6th day).

Among the three isolates (*V. lecanii* 3, *B. bassiana* 70 and *B. bassiana* 76), the isolate *V. lecanii* 3 has highest percent mortalities both in filtrate treatment (49.05%) at 3rd day and in conidial treatment (80.70%), as well as in filtrate treatment (88.36%) at 6th day of inoculation. On the basis of these facts *Verticillium lecanii* 3 (V3) was considered as the most virulent strain among all the six isolates. The reason for virulence of conidia or toxicity of the filtrate of the fungal isolates was farther evaluated by studying the cuticle degrading enzymes of the entomopathogenic fungal isolates.

Enzymatic activity of the filtrate

Extracellular hydrolytic fungal enzymes (chitinolytic, proteolytic and lipolitic) activities are important for infection of the host and degradation of host cuticle. These enzymatic activities were investigated for all the six fungal isolates (U/ml). The graphical representation of the all the three enzyme activities clearly reveals the differences between the fungal isolates versus interval of time (days) (Figure 2).

The enzymatic activities of different fungal strains recorded at different intervals of time (days) showed significant differences (Figure 2). Significant differences were observed for chitinase activities of the filtrate among the six fungal isolates at each of the six days (Table 6) and among interval of time (days) for all the six isolates (Table 5). The fungal strain *V. lecanii* 3 (V3) showed maximum chitinase activity (0.35 U/ml) at day six while *V. lecanii* 5 (V5) and *B. bassiana* 252 (Bb252) had maximum activities of 0.33 (day five) and 0.28 (day four), respectively (Figure 2, chitinase activity). Some of the strains like Bb252 have high chitinase activity (0.23 U/ml at 3rd day) in the earlier days but did not achieve the highest value among all isolates and decline soon. This may be due to the stability of the secreted enzyme or the amount of the secreted enzyme. Similarly, there is no regularity among all the activities for their highest values, for example, in our results, the Bb70 had highest activity (0.15 U/ml) value at 3rd day of inoculation and then a regular decrease in the activities was observed and in Bb76 and V5, the activities increased gradually till the 5th day (0.13 U/ml and 0.31 U/ml) of inoculation and then declined slowly. While in both V2 and V3 the activities remained in log phase (in production of chitinase) till the sixth day (0.22 and 0.35 U/ml) showing stable activities which favors the fungal entrance to the haemocoel. Among the six isolates, *V. lecanii* 3 had highest chitinase activity at 6th day which correlates with the results of conidial and filtrate treatments where the percent mortalities of *V. lecanii* 3 at 6th day were highest (80.70 and 88.36) in all the six isolates. This shows that the isolate *V. lecanii* 3 (V3) has stable and high efficiency of chitinase production which participates in the degradation of host cuticle for longer time than the other strains.

Significant differences were observed for protease activities of the filtrate among the six fungal isolates at each day (Table 6) and among time interval (days) for each fungus (Table 5). The isolate *B. bassiana* 70 has maximum activities of 0.493 and 0.64 U/ml at day three and four of inoculation, respectively (Figure 2, Protease). Among the rest of isolates, *B. bassiana* 76 was found to be in log phase (in production of protease) till the 6th days with the maximum activity 0.60 U/ml at 6th day (Figure 2, protease activity). Bb70 and V2 achieved the highest activities at 4th day of inoculation and Bb252 and V3 at the 5th day while the Bb76 and V5 remain in log phase (in production of protease) till the six day of inoculation (Figure 2, protease activity). The protease activities were higher both at 3rd and 6th day for *B. bassiana* 70 and *B. bassiana* 76 which correlates with the percent mortality results of the filtrate. Similarly, the V5 remains in log phase (in production of protease) till the 6th day but lower in activity than the other isolates.

Significant differences were observed for lipase activities of the filtrate among the six fungal isolates (Table 6) and among interval of days for each fungus (Table 5). Highest lipase activity (0.73 U/ml), among all isolates was observed at 4th day of incubation for isolate *V. lecanii* 3 while *V. lecanii* 5 had maximum activity (0.53 U/ml) at 3rd day (Figure 2, Lipase activity). Similarly, V2 had also a high lipase activity of 0.34 U/ml at 3rd day, showing that the *V. lecanii* specie has high lipase production capacity than the *B. bassiana* species (Figure 2, Lipase activity). Isolates Bb70 and V2 show maximum activities at 3rd day and isolates Bb76 and V3 at 4th day while Bb252 and V5 had maximum activities at 5th day of incubation. Also, V3 had highest activity at 4th day of incubation and *V. lecanii* 3 was virulent or toxic both in conidial and filtrate application, respectively. Similarly, *B. bassiana* 70 and *B. bassiana* 76 had high toxicity in filtrate treatment as well as have high protease activities. The virulence of fungal isolate *B. bassiana* 70 and *B. bassiana* 76 is assumed to be due to high protease enzyme production in the filtrate. The isolate *V. lecanii* 3 has highest chitinase and lipase activities but the activity of lipase is much higher than that of chitinase hence it is assumed that lipase play the vital role in the pathogenicity of *V. lecanii* 3.

DISCUSSION

Our study has focused on the virulence or toxicity level of six entomopathogenic fungal isolates, its effective application and the role of individual enzyme in the cuticle degradation of the target pest. The efficacy of filtrate for percent mortality of the aphids is much higher than that of conidial application (Figure 1). Kim et al. (2009)

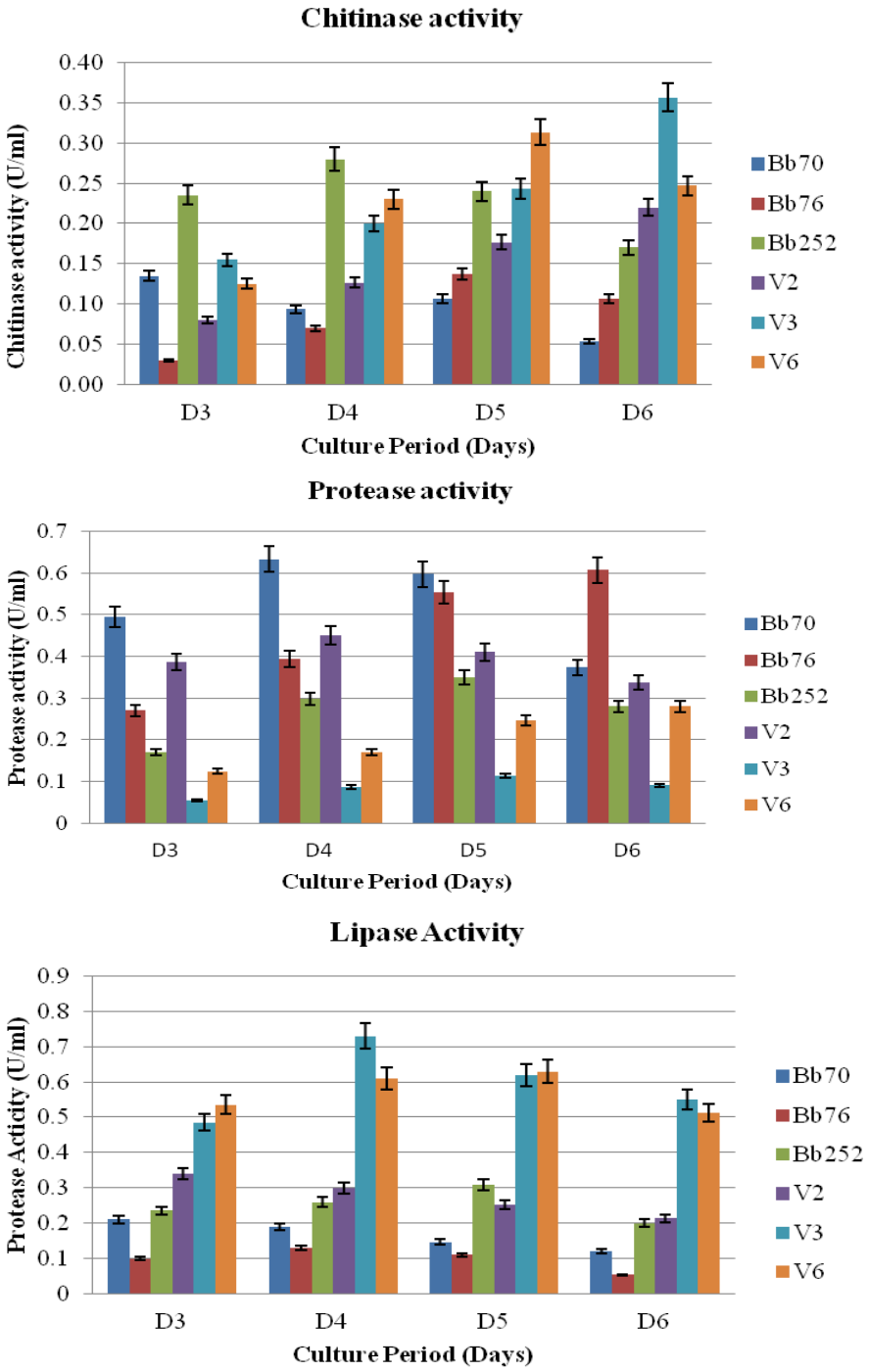

Figure 2. Enzymatic activity U/ml (chitinase, protease and lipase) of six fungal strains under different interval of times.

demonstrated that degradation and hemocoel deformation were observed in the supernatant treated aphid cuticles when compared to control (0.1 % tween 80) and the population reduction of aphid was directly proportion to the concentration and dose of supernatant. The reason for maximum activity was due to the cuticle degrading enzymes and metabolites already secreted into the filtrate while the germination of conidia on the surface of cuticle and the related enzyme secretion requires longer period of time. Similarly, other characteristics, such as

Table 5. Analysis of variance for the enzymatic activity (chitinase, protease and lipase) of the incubation time interval (days) for six fungal strains.

Fungal strain	Chitinase			Protease			Lipase		
	df	F	Sig.	df	F	Sig.	df	F	Sig.
Bb70	3	12.45	0.0034	3	43.79	0.000	3	16.19	0.002
Bb76	3	27.58	0.0003	3	113.12	0.000	3	13.30	0.003
Bb252	3	10.23	0.0059	3	60.50	0.000	3	35.41	0.0001
V2	3	22.02	0.0006	3	13.67	0.002	3	40.18	0.000
V3	3	41.15	0.0000	3	6.10	0.018	3	23.04	0.0005
V5	3	23.97	0.0005	3	31.03	0.000	3	42.09	0.000

Table 6. Analysis of variance for the enzymatic activity (Chitinase, Protease and Lipase) of the six fungal strains at different incubation time interval (days).

Day	Chitinase			Protease			Lipase		
	df	F	Sig.	df	F	Sig.	df	F	Sig.
D1	5	38.35	0.000	5	257.49	0.000	5	459.23	0.000
D2	5	56.97	0.000	5	162.5	0.000	5	345.85	0.000
D3	5	44.94	0.000	5	181.88	0.000	5	433.73	0.000
D4	5	64.45	0.000	5	178.79	0.000	5	258.02	0.000

conidial viability, speed of germination, hyphal growth rate, and spore production in response to environmental temperature, relative humidity and UV light also influence the efficacy of a fungal isolate as a microbial control agent (Luz and Fargues, 1998; Milner, 1997).

Bateman and Alves (2000) and Altre and Vandenberg (2001) demonstrated that the application of culture filtrate is more effective in killing insect pests as compared to conidial application especially in case of short life cycle insects having more chance to escape the conidial attachment on their cuticular surface before germination or conidial pathogenesis. Hence our results are similar to the previous work done. The filtrate has more chance to contaminate the cuticle of aphid to carry bioactive metabolites for deformation of the aphid cuticle and have no effect of any physical factors. The production of high yield of toxic enzyme is easy to obtain through filtrate optimization as compared through genetic manipulation in the fungal strain. Hence the production, transport and storage of filtrate are easy as compared to conidia which are the basic requirement of commercialization. The commercial entomopathogenic fungal products are based on the conidia having some limitation in terms of phyllospheric application that is, killing of target pest in slower time than that of chemical pesticides (Wraight et al., 2001). Of the total insecticides market, a small percentage is covered by entomopathogenic fungal products (Yatin et al., 2006), because the entomopathogenic fungal products are mainly based on conidia (Wraight et al., 2001). Hence, application of culture filtrate is important in terms of biocontrol agent. Development of entomopathogenic fungal products based on filtrate will help to compete the chemical pesticides in the market, bypassing the limitations associated with conidial based products. Vandenberg (1996) found that isolates of any individual fungal species had different pathogenicities to the same cohort of the host aphid. In our results also the percent mortalities rates varies among all the six fungal isolates tested in the bioassay. The fungal strain *V. lecanii* 3 was found to be the more effective strains with high mortalities against the aphids in filtrate and conidial application. While isolate *B. bassiana* 70 and *B. bassiana* 76 have high toxicity against the target pest in the filtrate application. Butt et al. (1992) demonstrated that virulence was not related to host and geographical origin of isolate. In our studies, the original host of *V. lecanii* 3 was white fly but it has highest pathogenicity against green peach aphid.

Improvements in the virulence of entomopathogenic fungi can be achieved by finding the reasons for virulence in different entomopathogenic fungi and understanding mechanisms of pathogenesis and then genetically modifying the targeted virulence factors. The production of degradative enzymes by entomopathogenic fungi is an important factor to determine the virulence of the isolate towards the target host. In the present study, we correlate the enzyme activities to enzyme production.

Bing-Lan et al. (2003) demonstrated that the chitinase activity in the flask cultivation was similar to the production of chitinase enzyme. In this study, we evaluate

the enzymatic activities of all the six fungal isolates and then correlate with aphidicial activities of filtrate and conidial treatments to find the most virulent isolate. Previous studies have shown that the physiological characteristics and enzyme production of fungi are related to their virulence (Feng and Johnson, 1990). The most virulent fungal isolate (in conidial treatment) *V. lecanii* 3 had high chitinase (0.35 U/ml) activities in the filtrate. The virulence of *B. bassiana* against *M. persicae* was enhanced by over expression of chitinase gene (Fang et al., 2005).

St. Leger et al. (1986) demonstrated that the over expression of protease and chitinase potentiate the virulence of pathogenic fungi against the target pest. Enzymes like chitinase and protease among the metabolites are reported to play important role in killing the insect (Campos et al., 2005). *B. bassiana* 70 and *B. bassiana* 76 have high protease activities of 0.64 and 0.60 U/ml in the filtrate making the isolates toxic in filtrate treatment. St. Leger (1996) found that proteases play an important role in cuticle penetration and the virulence mechanism for the same pest varies with different strains of the entomopathogenic fungal isolates. In our study, the virulence or toxicity of all the isolates are different from each other for the same pest.

The fungal isolate *V. lecanii* 3 showed highest lipase activity (0.73 U/ml) among all fungal isolates. Lipolitic enzymes of many pathogenic microorganisms described so far play an important role in the infection process (Schofield et al., 2005). Similarly, the lipolitic activity favors adhesion to the host cuticle (Göttlich et al., 1995) and the host infection process begins with conidial adhesion to host surface followed by cuticle penetration (Askary et al., 1999).

Silva et al. (2005) demonstrated that lipase is significant in the insect infection process of fungus *M. anisopliae*.

The fungal isolate *V. lecanii* 3 possesses highest lipase activity and chitinase activities but the lipase activity is much higher than chitinase hence it is assumed that lipase enzyme play an important role in the virulence of fungal infection or cuticle degradation as compared to chitinase and protease.

Conclusion

The fungal filtrate has shown maximum mortality rate as compared to conidial application with some advantages of being not effected by abiotic factors and the slow germination rate of conidia. Among the different fungal strain tested, *B. bassiana* 70 and *B. bassiana* 76 were found to be most effective strains with high mortality rates, especially in its early days of filtrate application limiting the aphid's population. *V. lecanii* 3 was found to have maximum percent mortality both in filtrate treatment at 3rd day and maximum percent mortality in conidial treatment at 6th day of application. The enzyme protease was found to be effective in the pathogenicity of the fungal strains *B. bassiana* 70 and *B.* bassiana 76. Similarly, *V. lecanii* 3 was found to have high activities for chitinase and lipase as compared to other fungal isolates. When the lipase and chitinase activities values for isolate *V. lecanii* 3 were compared, the lipase activity was 0.73 U/ml much higher than chitinase activity 0.35 U/ml which proves that lipase is the most important for virulence or toxicity as compared to chitinase and protease.

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