Pakistan J. Zool., vol. 44 (2), pp. 537-544, 2012.

Catalase Production Influences Germination, Stress Tolerance and Virulence of *Beauveria bassiana* Conidia

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Abstract.- Catalases are the most important enzymatic systems used to degrade hydrogen peroxide (H_2O_2) into water and oxygen, thereby lowering intracellular hydrogen peroxide levels. Entomopathogenic fungi display increased catalase activity during germination and growth, which is necessary to counteract the hyperoxidant state produced by oxidative metabolism. We studied the influence of five different hydrocarbons on catalase production by *B. bassiana* in order to determine the importance of catalase induction in fungal germination, stress tolerance and virulence. Conidia produced by colonies grown on different carbon sources showed higher rates of catalase production compared to the control and the catalase activity of conidia produced on n-octacosane was four times catalase activity than the activity of the control. This increase in catalase activity was accompanied by a higher level of resistance to exogenous hydrogen peroxide and a reduction in the germination time. The higher expression of catalase also affected the virulence of *B. bassiana*. Conidia from *n*-octacosane treatment proved most aggressive showing lowest ST_{50} value (5.49±0.34 days), and those from control treatment were the least virulent having ST_{50} value of 8.42±0.75 days. Our study has helped to identify that over expression of the catalase improves the pathogenic ability of *B. bassiana* against *Plutella xylostella.*

Keywords: Anti-oxidizing enzymes, Lepidoptera, microbial control, biocontrol program, Hypomycetes.

INTRODUCTION

Biological control agents, such as insect pathogenic fungi, offer an environment friendly alternative to chemical pesticides. However, their use has been limited by poor efficacy (St Leger *et al*., 1996). Detailed knowledge about the mechanisms of fungal pathogenesis is needed for mycoinsecticide improvement. The fungal infection is dependent upon numerous biological events which are initiated by the adhesion of fungal spores to the insect cuticle, spore germination, and hyphal growth. In each of these stages, the fungus faces environmental and nutritional challenges in addition to the defense mechanisms deployed by the host (Roberts and St Leger, 2004). Fungal germination is marked by a significant increase in oxygen consumption which starts a few hours before germ tube emergence and reaches its highest level during exponential growth (Braga *et al*., 1999). Therefore, it is possible that the amount of hydrogen peroxide increases during germination and growth. Hydrogen peroxide formed during aerobic metabolism is capable of producing other reactive oxygen species which can damage many cellular components (Kawasaki and Aguirre, 2001). Catalases are the most important enzymatic systems used to degrade H_2O_2 into water and oxygen, thereby lowering intracellular hydrogen peroxide levels (Wang *et al.,* 2007). These enzymes and superoxide dismutase are the cell's primary defense mechanisms against hydrogen peroxide. In fact, entomopathogenic fungi displays increased catalase activity during germination and growth, and this may be necessary to counteract the hyperoxidant state produced by oxidative metabolism (Miller *et al*., 2004). In *B. bassiana*, the peroxisomal catalase could be involved in insect hydrocarbon catabolism which proceeds through complete β-oxidation (Pedrini *et al*., 2006; Crespo *et al*., 2008). Thus, the catalase genes in fungi participate in the processes of cellular differentiation, detoxification, and catabolism.

Like all microorganisms, entomopathogenic fungi have specific biological characteristics that influence their activity in the environment (Parker *et al*., 2003). To select a fungal pathogen for insect control, it is necessary to study simple characteristics that are required to kill the target insects under both field and greenhouse conditions. According to Moore and Prior (1993), useful characteristics

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0030-9923/2012/0002-0537 $ 8.00/0
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include good mass-production features (*e.g.*, high sporulation on artificial media), high virulence against the target organisms, and the ability to survive in the environment in which the pest occurs. Fungal isolates with rapid germination and hyphal growth rates have an advantage as biological control agents because host infection can potentially occur quickly (Hajek and St Leger, 1994; Varela and Morales, 1996). The production of blastospores that germinate more rapidly has the potential to increase infectivity. Lane *et al.* (1991) evaluated the influence of carbon- and nitrogen-limited media on the production and quality of blastospores of the deuteromycete *B. bassiana*. These authors noted that the germination rate and survival of *B. bassiana* blastospores following storage was associated with appropriate concentrations of nitrogen and carbon in the culture medium. The carbon-nitrogen ratio in the basal medium influences the production of different hydrolytic enzymes involved in penetration of the host integument and subsequent infection. In *B. bassiana*, the specific activity of peroxisomal catalases was increased when glucose was replaced by an insect-like hydrocarbon (Pedrini *et al.*, 2006). Therefore, isolate virulence or specificity could depend in part on the ability to produce a battery of catalases with specificity for the range of different carbon compounds in the cuticle of a particular insect, in response to particular host cues at the correct stage in penetration.

The present work is an attempt to explore the potential for this by comparing the influence of different carbon compounds on catalase production by *B. bassiana,* which have shown considerable pathogenic ability in previous studies and is part of our recent biocontrol program for diamondback moth. Additionally, germination, H_2O_2 sensitivity and virulence were investigated as a function of culture conditions.

MATERIALS AND METHODS

Fungal strain and culture conditions

Beauveria bassiana isolate, B005 originally isolated from soil, deposited to the collection at Engineering Research Center of Biological Control, South China Agricultural University was used during these studies.

The fungus was grown in minimal media (MM) consisting of 0.2% NH_4NO_3, 2% dextrose, 0.3% KH_2PO_4, and 2% 50× salt stock solution (25 g $MgSO_4$, 0.09 g $ZnSO_4$, 0.05 g $FeSO_4$, 0.015 g $MnSO_4$, and 0.02 g $CuSO_4$). Cultures were grown in solid MM containing 2% agar supplemented with carbon compounds (chitin, chitosan, hexadecane, n-ocatcosane, n-tetradecane). Each of the hydrocarbons, hexadecane, n-ocatcosane, n-tetradecane, (2.5ml of a 10% (w/v) hexane solution), was layered onto the surface of the media and evaporated, respectively. One percent chitin or chitosan (obtained from crabshell) was added to the medium whereas the addition of 1% glucose served as a control. The conidia were collected by scraping the colony after 10 days of inoculation and were washed three times by centrifugation and suspended in sterile 0.1% Triton X-100 solution. After this the hyphae in suspended solution were removed by filtration through a sheet of sterile muslin cloth.

Preparation of cell free extract

The cell-free extract was prepared as described earlier by Kayali and Tarhan (Kayali and Tarhan, 2006). Briefly, conidia harvested by filtration, were washed in distilled H_2O, and then in cold 50 mM potassium buffer (pH 7.8), followed by suspension in 50 mM potassium buffer (pH 7.8). The cell suspension was disrupted by homogenization. The temperature during treatment was maintained at 4–6°C by chilling in an ice-salt bath. Cell-free extracts were clarified at 8,000 g for 15 min at 4°C. The supernatants were assayed immediately for total protein and catalase activity.

Protein and enzyme assay

Protein concentration was quantified by the Lowry method using bovine serum albumin as the standard (Lowry *et al.*, 1951). Catalase activity was assayed by the method described by Beers and Sizer (1952), in which the decomposition of H_2O_2 was analyzed spectrophotometrically at 240 nm. One unit of catalase activity was defined as the amount of enzyme that decomposes 1 mmol H_2O_2/min at an initial H_2O_2 concentration of 30 mM at pH 7.0 and 25ºC.

SDS-PAGE was carried out with 12% polyacrylamide running gels (Lammeli, 1970). After

electrophoresis the gels were stained by using silver nitrate.

Assay of sensitivity to H_2O_2

The sensitivity of conidia to H_2O_2 was estimated using the method previously described by Paris *et al*. (2003). Conidia (1×10^6 $ml^{-1)}$ were incubated at 28°C for 30 min with various H_2O_2 concentrations (5, 10, 15, 25, and 50 mM). The H_2O_2 solution was discarded by centrifugation, and conidia were suspended in the original water volume. A total of 500 conidia from each treatment were plated on Sabouraud dextrose (SD) agar medium (in triplicate) and incubated at 28°C for 72 h. The numbers of viable colonies were counted and their survival rates were determined.

Conidia germination

To determine the rate of conidial germination, the conidia produced on different carbon sources were harvested with deionized water containing 0.03% Tween 80 and sieved using filter paper (Whatman No. 2; Science Kit & Boreal Laboratories, New York, NY, USA) into sterile vials. Conidia were counted using a compound microscope and a hemocytometer (0.0625 m^2; Fuchs-Rosenthal Merck Euro Lab, Darmstadt, Germany) to calibrate a suspension of 1×10^8 conidia ml^{-1}. The fungal suspensions were gently streaked over SD agar medium and incubated at 28°C for 12 h. Conidia germination was observed at every 2-h interval. Populations of at least 200 cells were counted under the microscope, and the percentage of germlings and ungerminated conidia was determined. A germling was defined as a cell with a germ tube equal in length to the width of the conidia.

Insect bioassay

A conidial suspension of 1×10^8 conidia ml^{-1} of *B. bassiana* was prepared from the conidia produced by colonies grown on each medium as described above. Leaves having second *Plutella xylostella* instars were dipped into each conidial suspension for 20 s, and then removed to air dry before being transferred to 20-cm diameter clean glass Petri dishes. A piece of filter paper (20 cm in diameter) was placed at the bottom of the dish with a few drops of water for maintenance of moisture. Leaf disks were replaced every 2 days except during the pupal stage. Control leaves were also dipped into 0.02% solution of Tween-80 prepared with distilled water. Each treatment and control contained four leaves with 10 *P. xylostella* per leaf assayed at one time, using randomized groups of insects from a single batch.

The insects were placed in an air-conditioned room at 26±2°C and > 95% R.H. and monitored daily until adult emergence, and the deaths of *P. xylostella* were recorded at 12-h interval. To survey the infective mortalities, cadavers were taken out and cultured separately at 26±2°C and > 95% R.H. If spores of *B. bassiana* were recovered from a cadaver, the cadaver was regarded as having died from infection by *B. bassiana.*

Chemical and reagents

Hexadecane, *n*-ocatcosane, *n*-tetracosane were purchased from Sigma (St Louis, MO, USA). All other chemicals were obtained from Guangzhou Jinhuadu chemicals and reagents, Guangzhou, China. Chitin and chitosan obtanined from crab shell was purchased from Sinopharm chemicals, Shanghai, China

Experimental design and statistical analysis

Each study was conducted three times with freshly prepared fungal suspension. Data regarding the catalase activity and fungal germination were analyzed by Analysis of variance (ANOVA) and treatment means were compared by using Tukey's studentized range test for mean comparisons at 5% level of significance. The data regarding germination rate was analyzed through repeated measures ANOVA. The median lethal dose (LD_{50}) of H_2O_2which is a quantitative index for the tolerances of the *B. bassiana* spores grown on different carbon compounds was calculated by probit analysis. Median survival time (ST_{50}) estimates of *P. xylostella* were obtained by using standard Kaplan-Meier survivorship analysis. All statistical analysis was performed using SAS 8.01 (2000).

RESULTS

Effect of different hydrocarbons on catalase induction by B. bassiana

Catalase activities of *B.bassiana* conidia were significantly affected by different carbon compounds ($F_{5,\ 12}$ = 24.72; P <0.0001). *n*-octacosane proved to be the most active inducer of catalase activity among all the treatments with mean activity of 263.8 ± 13.71 U/mg. The lowest catalase activity (54.54 ± 4.37 U/mg) was observed in control. Catalase activity observed for the cell free extracts from supernatants having hexadecane and chitin were statistically different from each other having mean values of 116.2± 7.42 and 215.37 ± 9.50 U/mg, respectively (Fig. 1).

SDS-PAGE was used to obtain the catalase profile of crude cell free extracts of conidia produced on different carbon compounds. The analysis of samples (obtained from the conidia grown on different carbon compounds) showed two distinct regions of bands. These zones were more prominent for the samples obtained from the conidia grown on n-octacosane and hexadecane showing comparatively higher induction of the enzyme when compared to the other treatments. The samples obtained chitin and chitosan treatments showed a clear bands in the first zone where as the band were not clearly visible in the second zone (Fig. 2).

Sensitivity of B. bassiana *conidia produced on different hydrocarbons to H_2O_2*

We analyzed the sensitivity of conidia to H_2O_2 exposure in *B. bassiana* by examining the ability of the conidia to germinate and form colonies. Statistical analysis showed that significant differences exsisted among treatment in term of LD_{50} values ($F_{5,\ 12}$ = 51.34, P<0.001). The results indicated that viability of conidia grown on basal medium having n-octacosane against different concentrations of H_2O_2 was superior to that of the other treatments (Fig.3A). The hydrogen peroxide lethal dose 50 (LD_{50}) was 170% higher in n-octacosane treatment (LD_{50} = 22.63 mM) than in control (LD_{50} = 8.11 mM). This result suggests that overexpression of catalase improved the ability of conidia against exogenous hydrogen peroxide (Fig. 3B).

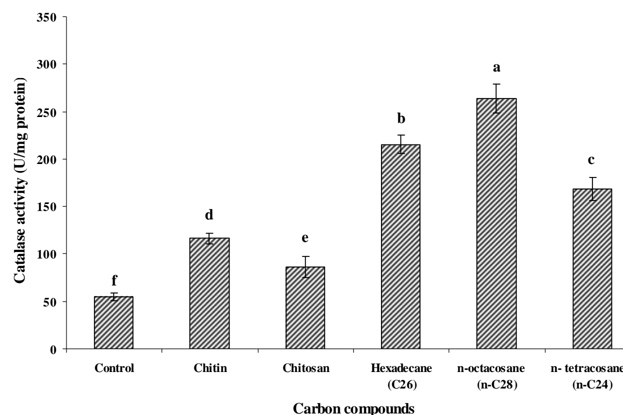

Fig. 1. Specific catalase activity of *B. bassiana* induced by different carbon compounds. Legends with different letters are significantly different from each other (Tukey's, P<0.05).Bars represent standard error of means (Based on three independent replicates).

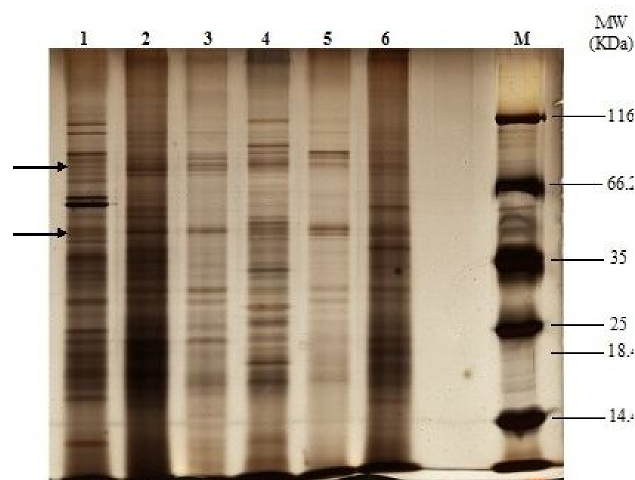

Fig. 2. SDS-PAGE profile of crude catalase produced by *B. bassiana* conidia grown on different carbon compounds. Lane 1, control; 2, chitosan; 3, n-tetracosane; 4, hexadecane; 5, chitin; 6, n-octacosane; M, molecular weight marker.

Germination of B. bassiana *conidia produced on different hydrocarbons*

The data shown in Figure 4 explains the efficiency of germination in *B. bassiana* when different carbon compounds were added to basal medium for catalase expression. The analysis of repeated measures ANOVA resulted in significantly different interaction effect between different

treatments and time intervals for germination rate ($F_{20,57}$ = 31.52, P<0.0001). The results showed that conidia grown on basal medium having n-octacosane germinated faster than the other treatments showing 95.67% germination after 48 hrs whereas almost similar rates of germination were observed for conidia obtained from n-tetracosane and hexadecane treatments (Fig. 4). This result suggests that over expression of catalase diminishes oxidative stress and accelerates the germination process.

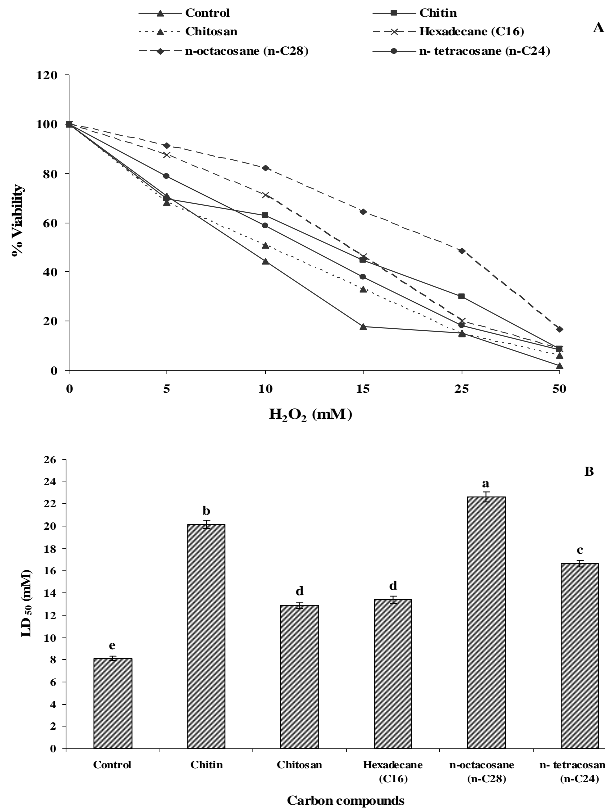

Fig. 3. Effect of different hydrogen peroxide concentrations on the survival of *B. bassiana* conidia produced on different carbon compounds (A) % viability of *B. bassiana* conidia (b) LD_{50} of H_2O_2. Legends with different letters are significantly different from each other (Tukey's, P<0.05). Bars represent standard error of means (Based on three independent replicates)

Virulence of B. bassiana *conidia produced on different hydrocarbons*

We assessed the biological activity of the conidia produced on the basal medium having different carbon compounds by inoculating 2nd instar *P. xylostella* with these conidia. There were significant differences in virulence to *P. xylostella* second instars associated with the type of carbon compounds used in growth medium from which the conidia had been obtained ($F_{5,12}$ = 21.46, P = 0.001). Conidia from n-octacosane treatment proved to the most virulent showing lowest ST_{50} value (2.11 ± 0.13 days), and those from control treatment were the least virulent having ST_{50} value of 4.1 ± 0.32 days (Fig. 5).

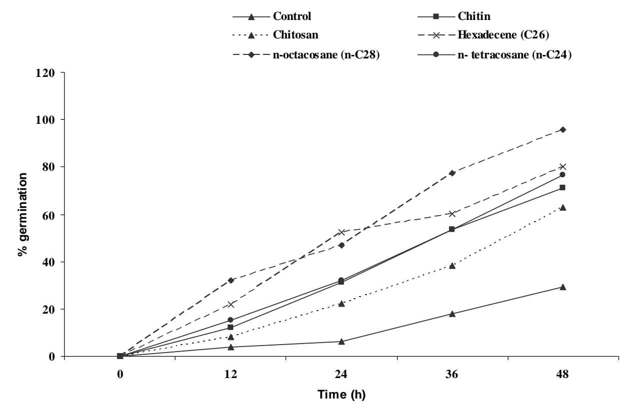

Fig. 4. Germination of *B. bassiana* conidia produced on different carbon compounds.

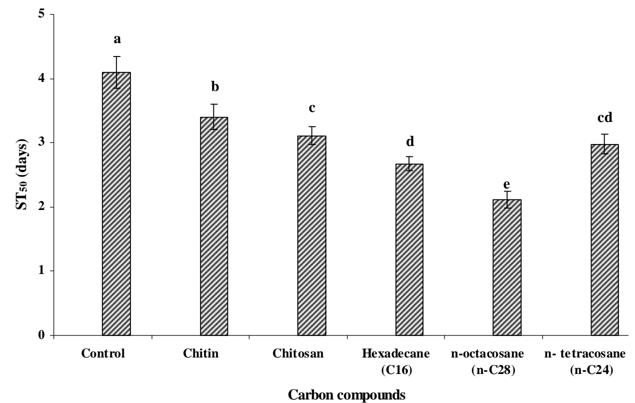

Fig. 5 Virulence of *B. bassiana* conidia produced on different carbon compounds. Legends with different letters are significantly different from each other (Tukey's, P<0.05). Bars represent standard error of means (Based on three independent replicates).

DISCUSSION

The present work was undertaken to investigate the effect of certain nutritional factors on catalase production by *B. bassiana* in relation to its effect on the germination, H_2O_2 tolerance and virulence which are considered as important factors for the selection of a fungal pathogen in any biological control program.

The studies on the catalase activities of *B. bassiana*, revealed very interesting features of the cells growing on hydrocarbons. The catalase activities of the carbon compounds grown conidia were much higher than those of the glucose, chitosan, or chitin grown conidia of *B. bassiana* used in this experiment. Although the physiological role of catalase in the hydrocarbon-grown cells remains to be elucidated, the observation mentioned above suggested that there would be a certain relationship between the metabolism of carbon compounds and the high catalase activity. The enzyme activity of conidia grown on different compounds varied markedly at the end of the growth period (Fig. 1). On the other hand, the enzyme activity of the glucose-grown cells stayed at a significantly lower level when compared to other treatments (Fig. 1). This phenomenon may be explained by the concept of "glucose repression" since the glucose-grown cells showed the maximal activity (55.98±4.20 U/mg) at the exponential phase. These results strongly suggested that the catalase activity and the concomitant increase of microbodies might be essential for the metabolism of hydrocarbons. The difference in the inducibility of catalase between hydrocarbons and other compounds may indicate that catalase will play an important role in the initial oxidation of hydrocarbons rather than in the β-oxidation of fatty acids (Teranishi *et al.*, 1974a). Although it has not yet been clear whether these phenomena were due to "catabolite repression" or inhibition of the incorporation of hydrocarbons by glucose, the former is more probable because the morphological change in *Candida tropicalis* depending upon hydrocarbons was observed when grown on a hydrocarbon medium supplemented with glucose (Breidenbach *et al.*, 1968). Cyclic AMP, which was applied toward protoplasts of *Saccharomyces cerevisiae* to eliminate the glucose effect, was not effective on the derepression of the catalase induction in the intact cells of *C. tropicalis*. However, it is the problem whether cyclic AMP was incorporated into the intact yeast cells or not (Teranishi *et al.*, 1974b).

A hyperoxidant state is defined as an unstable, transient state in which ROS surpass the antioxidant capacity of the cell. A hyperoxidant state develops when morphogenetic transitions start and during the germination of conidia (Michan *et al.*, 2003). According to this hypothesis, during the germination of fungal conidia, a high level of oxidative metabolism occurs and a hyperoxidant state ensues. Hydrogen peroxide is a reactive oxygen species that presumably occurs as a metabolite in all aerobic organisms. Hydrogen peroxide is universally cytotoxic at high concentrations, mainly, due to strong oxidant, hydroxyl radical radical (Apel and Hirt 2004). To our knowledge, there are few reports regarding the effects of catalase over expression on fungal sensitivity to H_2O_2. In *Schizosaccharomyces pombe*, a catalase gene mutant that completely lacks catalase activity is more sensitive to hydrogen peroxide, whereas transformed cells that over express catalase activity are more resistant to hydrogen peroxide when compared to the wild type (Mutoh *et al.*, 1999). In *S. cerevisiae*, catalase over expression reduced the levels of ROS and increases specific growth rates in the presence of high lactic acid concentrations (Abbott *et al.*, 2009). As anticipated, hydrogen peroxide suppressed the fungal spore viability in a dose dependent manner. However, the viability of conidia produced by n-ocatcosane medium was least effected when compared to the other treatments (Fig. 3). Broadly speaking, it is possible that hydrogen peroxide may have inhibitory effects but these effects were diminished or abolished by the catalase induction in different treatments. Our results also showed that the higher rates of catalase production in n-octacosane treatments helped the conidia in removing the excessive amount of H_2O_2 generated and allowing the cells to germinate faster than other treatments. Similar results were also observed by Hernandez *et al.* (Morales-Hernandez *et al.*, 2010) who observed that over expression of cat1 could be responsible for the germination of *M. anisopliae* mutant 13–19% faster than the wild type. The transgenic strains overexpressing the cat1 gene have twice the catalase activity of the wild-type strain, in accord with the

extra copy of the gene which is detected in both transgenic samples tested. This higher catalase activity produced a higher level of resistance to exogenous hydrogen peroxide and a reduction in the germination time. An excessive amount of H_2O_2 is harmful to the cells, so the quick and efficient removal of hydrogen peroxide is essential for an aerobic living organism (Zamocky *et al.*, 2008). With twice the amount of catalase activity, this removal process may be more efficient, allowing the cells to grow in higher concentrations of H_2O_2.

The most aggressive inocula of *B. bassiana* isolates against *P. xylostella* were produced on n-ocatcosane medium that produced the lowest ST_{50} values when compared to the other treatments as it also had the highest rates of catalase production. The catalase production emerges, therefore, as a significant virulence determinant in successful fungal infection. These results are similar to what has been found in other entomopathogenic fungi studied. Morales-Hernandez *et al.* (2010) observed that over expression of cat1 in *M. anisopliae* improved the fungal virulence against *P. xylostella* where *M. anisopliae* grew and spread faster in the soft tissue of the insect, resulting in a significant decrease (25%) in the time of death for the insect and requiring 14 times less than the usual dose to kill 50% of the population. These results are important because overexpression of the protease Pr1 reduces (by the same level) the time of death (St. Leger *et al.* 1996). The improvement for entomopathogenic fungi as a biopesticide is even greater if we consider that insects got sick and stopped eating much earlier than those who were infected with the wild-type fungus.

The germination of fungal spores on insect cuticle requires a sequential action of appropriate hydrolytic enzymes (Samuels and Paterson, 1995). These enzymes could facilitate the early stages of fungal infection. Moreover, some of these catabolic enzymes may be important in the invasion of fungi into the haemocoel or body cavity. During these studies, the effects of catalase activity on germination and virulence were also observed as a function of culture conditions and this revealed some interesting results. Conidial germination and virulence was directly related to the rates of catalase production under different growth conditions. A possible reason for such an enzymatic activity pattern can be the repression of hydrogen peroxide, as previously suggested for *M. anisopliae* (Morales-Hernandez *et al.*, 2010). Our study has helped to identify that over expression of the catalase improves the pathogenic ability of *B. bassiana* against *Spodoptera exigua*. We conclude that catalase production is important during germination and growth for maintaining control over the ROS produced by germination, mycelial growth, and possibly during the invasion process. However, further work is needed to isolate and study of each catalase gene, determination of the kinetic parameters for the protein and the effect of catalase inhibitors will help us understand the relationship between endogenous reserves and the desired attributes of virulence of fungal pathogens.

ACKNOWLEDGEMENT

This research was funded by grants from "863" program (2011AA10A204-5), the National Natural Science Foundation of China (31170391) and China Postdoctoral Science Foundation (20100480760).

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(Received 22 September 2011, revised 13 December 2011)