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Effects of a Pathogenic Beauveria bassiana (Hypocreales: Cordycipitaceae) Strain on Detoxifying and Protective Enzyme Activities in Xylotrechus rusticus (Coleoptera: Cerambycidae) larvae

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Effects of a pathogenic *Beauveria bassiana* (Hypocreales: Cordycipitaceae) strain on detoxifying and protective enzyme activities in *Xylotrechus rusticus* (Coleoptera: Cerambycidae) larvae

Jun-Nan Ding[1,3], Hui-Hui Zhang[2], and De-Fu Chi[3,*]

Abstract

In recent years, biological methods have been used widely for controlling stem-boring pests in forests. In this study, the larvae of *Xylotrechus rusticus* (L.) (Coleoptera: Cerambycidae) were infected with 4 strains of 2 species of *Beauveria* (Hypocreales: Cordycipitaceae). Larval detoxifying and protective enzyme activities were measured at different times after infection. The results showed significant differences in the pathogenicity of the 4 strains. The most virulent strain, *Beauveria bassiana* (Bals.-Criv) Vuill. strain BbCC01, caused 93.3% mortality, with an estimated LT50 of 4.69 d. The detoxifying and protective enzyme activities in larvae infected with strain BbCC01 changed significantly. To resist the infection, the activities of the detoxifying enzymes (carboxylesterase, glutathione S-transferase, and acetylcholinesterase) first increased and then slowly decreased. The activities of the protective enzymes (catalase, superoxide dismutase, and peroxidase) showed a similar trend. The enzyme activities peaked at 72 to 96 h post-infection. In this study, the *B. bassiana* strain BbCC01 proved to exhibit high virulence towards *X. rusticus* larvae. This research provides important information regarding the biological control of *X. rusticus* and elucidates the patterns of detoxifying and protective enzyme activities in the susceptible larvae.

Key Words: forest pest; detoxifying enzyme; protective enzyme; quarantine pest; high virulence

Resumen

En los últimos años, los métodos biológicos se han utilizado ampliamente para controlar plagas barrenadoras de tallos en bosques. En este estudio, las larvas de *Xylotrechus rusticus* (L.) (Coleoptera: Cerambycidae) fueron infectadas con 4 cepas de 2 especies de *Beauveria* (Hypocreales: Cordycipitaceae). Se midieron la actividad de enzimas de desintoxificación y de protección en las larvas en diferentes momentos después de la infección. Los resultados mostraron diferencias significativas en la patogenicidad de las 4 cepas. La cepa más virulenta, *Beauveria bassiana* (Bals.-Criv) Vuill. cepa BbCC01, causó 93.3% de mortalidad, con un estimado TL50 de 4.69 d. Las actividades de desintoxicación y de protección de las enzimas en las larvas infectadas con la cepa BbCC01 cambiaron significativamente. Para resistir a la infección, las actividades de las enzimas detoxificantes (carboxilesterasa, glutatión S-transferasa, y la acetilcolinesterasa) primero aumentaron y luego disminuyeron lentamente. Las actividades de las enzimas protectoras (catalasa, superóxido dismutasa, y peroxidasa) mostraron una tendencia similar. Las actividades enzimáticas alcanzaron un máximo de 72 a 96 horas después de la infección. En este estudio, la cepa *B. bassiana* BbCC01 resultó exhibir una alta virulencia hacia larvas de *X. rusticus*. Esta investigación provee información importante sobre el control biológico de *X. rusticus* y aclara los patrones de actividad de las enzimas desintoxicantes y de protección en las larvas susceptibles.

Palabras Clave: plagas forestales; enzima desintoxicante; enzima protectora; plagas de cuarentena; alta virulencia

The genus *Xylotrechus* (Coleoptera: Cerambycidae) has a Holarctic range with about 100 species in the Palaearctic, 23 species in Canada and the USA and a total of 26 species in the New World (Linsely 1964). Some *Xylotrechus* species are pests in North America (NAPIS Pest Tracker 2007), and there is much concern that *Xylotrechus rusticus* (L.), which is included among "the most unwanted invasive plant pests," could enter North America via wood packing (Indiana Cooperative Agricultural Pest Survey 2013).

Xylotrechus rusticus is a stem-boring pest of Salicaceae (Malpighiales) trees (Xu 1979) and is mainly distributed in Russia (Caucasus and Siberia), Iran, Turkey, the Czech Republic, Japan, and North Korea, where it impacts poplar, willow, birch, oak, linden, maple, and elm trees (Xiao 1983). In Russia, *X. rusticus* was found damaging aspen and birch trunks following forest fires. In Central Europe, *X. rusticus* was found in deteriorating woods. It seems that *X. rusticus* in Europe tends to infest mostly weak or dead trees and does not cause severe damage (Cai et al. 2000). In 2003, *X. rusticus* was included in the National List of the Quarantine Pests by the National Bureau of Forestry, China (Zhou et al. 2006). The pest has been confirmed in northeastern China and has shown great potential threat and dispersal tendency in recent years. Controlling the dispersal of *X. rusticus* is very difficult due to unique biological characteristics of this insect (Hu et al. 1998). In the past, control of this pest relied

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mainly on chemical pesticides. However, the extensive use of chemical pesticides can cause serious environmental damage (Wang et al. 2002). Finding a safe, pollution-free, effective, and sustainable prevention method for *X. rusticus* is currently a high priority.

Entomogenous fungi are fungi with the ability to parasitize insects, eventually leading to the death of the insect host. These include *Beauveria bassiana* (Bals.-Criv) Vuill., *Beauveria brongniartii* (Sacc.) Petch, *Isaria farinosa* (Holmsk.) Dicks. (Hypocreales: Cordycipitaceae), and *Metarhizium anisopliae* (Metschn.) Sorokin (Hypocreales: Clavicipitaceae) (Shah & Pell 2003).

In China, *B. bassiana* has been used since the Tang and Song dynasties and has been reported to control more than 60 species of insect pests (Fan 2004). Infection with *B. bassiana* affects the normal physiological and metabolic activities in the insects (Dresner 1949; Genthner et al. 1994; Tolleson et al. 1996). It causes damage to the free amino acids in the hemolymph, decreases enzyme levels of intracellular lactate dehydrogenase, malate dehydrogenase, and esterase, and interferes with a variety of important metabolic enzymes, such as carboxylesterase and glutathione S-transferase (Zhang & Zhao 1992; Jordão et al. 1996; Wu et al. 1997). Acetylcholine esterase is a direct target of organophosphate and carbamate insecticides. This class of enzymes plays an important role in the detoxification of exogenous compounds (Gillespie et al. 2000).

The process by which *B. bassiana* infects the insects can be divided into the following 5 steps: 1) conidia attachment to the host body and germination; 2) formation of infection structures; 3) penetration into the host body cavity and invasion of hemolymph; 4) mycelium growth within the host, production of toxins against the insect's immune system, and insect death; and 5) after a period of parasitic growth, pathogenic fungal growth from within the host body, conidia formation on the surface of the insect's body, and initiation of a new infection cycle (Latge et al. 1987; Clarkson & Chamley 1996; Wang & You 1999). Following *B. bassiana* infection, the insect initiates a non-specific stress reaction that activates the anti-oxidation defense system, which consists primarily of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD). The main functions of these enzymes are to clear the free radicals from the body, defend against harmful substances damaging cell membranes and organelles, and aid the body in adapting to changing environmental conditions or to resist the interference by exogenous substances. This is an important component of innate insect immunity (Hajek & St Leger 1994; Li & Gong 1998).

In 2003, Liu & Bauer (2008) used the *B. bassiana* GHA strain for the control of emerald ash borer (*Agrilus planipennis* Fairmaire; Coleoptera: Buprestidae) adults. At a concentration of 10^{15} spores per mL, the adult infection rate reached 83%, suggesting effective biological control. Goble et al. (2014) conducted bioassay experiments using 2 Asian strains of *Beauveria* and *Metarhizium* (NBL851 and F52, respectively), and compared them with 2 *Beauveria* strains isolated in North America (ARSEF6215 and ARSEF10279) for infection of Asian longhorn beetles, *Anoplophora glabripennis* (Motschulsky) (Coleoptera: Cerambycidae). Their results showed that the Asian strains were more virulent than the North American strains against *A. glabripennis* suggesting high host specificity of the tested pathogens.

In recent years, there has been progress using *B. bassiana* to control the pine sawyer beetle (Liu et al. 2005), the Asian longhorned beetle (Xu et al. 2003), the mulberry borer (Su et al. 2007), and other stem-boring insects in China. However, there are no reports on the use of *B. bassiana* to control *X. rusticus*, a beetle that has caused serious damage to poplar in the "Three-North shelterbelt." To our knowledge, this is the first study involving direct infection of *X. rusticus* larvae with *B. bassiana* to examine the external morphology of larvae post-infection, identify the median lethal time (LT50) and median lethal concentration (LC50), select a virulent *B. bassiana* strain against *X. rusticus* larvae, and determine the detoxification and protection enzyme activities in the infected larvae. This study helps to elucidate host physiological reactions to infection with *B. bassiana*.

Materials and Methods

ARTIFICIAL DIET FOR *X. RUSTICUS* LARVAE

Large branches of poplar were collected, washed, peeled, and crushed into powder. Then 50.16 g of powder was chilled to 4 °C and added to 500 mL buffer (1.2 g agar, 4.8 g sucrose, 0.07 g $CaCO_3$, 0.07 g NaCl, and 1.47 g KH_2PO_4 in 42 mL distilled water). The mixture was sealed and autoclaved at 0.1 MPa and 121 °C for 30 min. After the mixture was cooled to 50 °C, 0.12 g vitamin C and 0.11 g erythromycin were added under stirring with a glass rod. The prepared diet was refrigerated at 4 °C for 1 d and warmed to room temperature before use in culturing the larvae.

XYLOTRECHUS RUSTICUS LARVAE

The study was conducted from May to Jul 2013 at the Key Laboratory of Forest Pest Biology, Northeast Forestry University, State Forestry Bureau (Harbin, Heilongjiang Province, China). *Xylotrechus rusticus* larvae were collected from the town of Tang Fang, Bin County, Harbin, China. A poplar tree infested with *X. rusticus* larvae was cut into 30 cm long wood blocks and brought to the laboratory to extract larvae. The collected larvae were placed on the above-mentioned diet and held in an incubator (25 °C, 75 ± 10% RH, darkness) for 15 d. The dead and sick larvae were removed. Healthy, uniformly sized 5th instars were used for the bioassays.

BEAUVERIA STRAINS

The *B. bassiana* strains BbAC830 and BbCC01 and *B. brongniartii* strains BbCF327 and BbAC749 (Table 1) were obtained from the Key Laboratory of Forest Pest Biology, Northeast Forestry University, National Forestry Bureau, China. All strains were cultured with improved Martin culture medium (200 g/L potato extract, 20 g/L glucose, 3 g/L KH_2PO_4, 1.5 g/L $MgSO_4 \cdot 7H_2O$, 8 mg/L vitamin B1, and 20 g/L agar, pH 6).

REAGENTS AND INSTRUMENTS

The reagents α-naphthyl acetic acid (α-NA), eserine, fast blue B salt, sodium dodecyl sulfate (SDS), disodium salt of ethylenediamine-

Table 1. General information on the strains of *Beauveria*.

Strain	Host	Geographic origin
Beauveria bassiana strain BbAC830	*Dendrolimus superans* Butler (Lepidoptera: Lasiocampidae)	Shenyang, China
Beauveria bassiana strain BbCC01	*Xylotrechus rusticus* (L.) (Coleoptera: Cerambycidae)	Harbin, China
Beauveria brongniartii strain BbAC749	*Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae)	Sichuan, China
Beauveria brongniartii strain BbCF327	*Holotrichia diomphalia* (Bates) (Coleoptera: Melolonthidae)	Shandong, China

tetraacetic acid (EDTA-Na$_2$), dithiothreitol (DTT), reduced glutathione (GSH), nitrobenzene (CDNB), riboflavin (vitamin B2), N-phenylthiourea, polyvinyl pyrrolidone (PVP), nitro blue tetrazolium (NBT), and methionine (Met) were purchased from Sigma Corporation (Shanghai, China). P-nitrophenyl phosphate disodium salt hexahydrate (PNPP), sodium cacodylate trihydrate, and hexadecylpyridinium chloride monohydrate were purchased from Aladdin Company (Harbin, China). Phenylmethyl sulfonyl fluoride (PMSF) was purchased from Biotopped Company (Harbin, China). Alpha-Napthyl acetate was purchased from Shanghai Yuanye Biotech Co. Ltd. (Shanghai, China). Na$_2$HPO$_4$, NaH$_2$PO$_4$, and H$_2$O$_2$ were purchased from Tianjin Yongda Chemical Reagent Co., Ltd (Tianjin, China). All chemicals were analytical grade.

The instruments used in the study were sourced as follows: DHP-9080 water-thermostatic incubator (Taicang Lab Equipment Co., Taicang, China), THZ-D desktop thermostat oscillator (Jintan Shenglan Instrument Manufacturing Co. Ltd., Jintan, China), 78HW-1 thermostatic heating magnetic stirrers (Jintan Yitong Electronics Co. Ltd., Jintan, China), UV-721 spectrophotometer (Xinmao Instrument Co. Ltd., Shanghai, China), electronic balance (Explorer Pro, Shanghai, China), high-speed refrigerated centrifuge (Kendro Laboratory Products Co., Xian, China), HPG-400BX light incubator (Harbin Donglian Electronic Technology Development Co. Ltd., Harbin, China), TH-500BQ Ultrasonic Cleaning Device (Jining Tianhua Ultrasonic Electronic Equipment Ltd., Jining China), ice-making machine (SANYO Electric Co., Beijing, China), and micro-pipettes (Dragon Medical Equipment Co. Ltd., Shanghai, China).

EXPERIMENTAL METHODS

Pathogenicity of 4 *Beauveria* Strains and their LT50 Values against *X. rusticus* Larvae

Conidia from the 4 strains were isolated and scraped into 0.1% Tween-80 sterile solutions to make suspensions at 10^8 spores per mL. The *X. rusticus* larvae were immersed in spore suspension for 30 s and then incubated at 26 °C and 75 ± 10% RH in darkness for 10 d. The control group was treated with 0.1% Tween-80 sterile water. Ten larvae were used in each group, and experiments were repeated in triplicate. The post-infection larval mortality was observed daily. The infection and mortality rates were recorded, and the LT50 values were calculated to select the strain with the highest virulence for further experiments. The mortality rates were corrected as follows. Corrected mortality rate (%) = (C$_1$ -C)/(1 -C) × 100%, where C$_1$ is the cumulative mortality rate of the treated larvae and C is the mortality rate in the control.

LC50 of BbCC01, the High-Virulence Strain

By means of the above described methods, strain BbCC01 was selected as a high-virulence strain. Using sterile 0.1% Tween-80 solutions, 5 concentrations were prepared based on counts in a hemacytometer, i.e., 1 × 10^5, 1 × 10^6, 1 × 10^7, 1 × 10^8, and 7.2 × 10^8 spores per mL. The 0.1% Tween-80 sterile solution served as the control. Larvae were treated and held for 8 d as described above. Each experiment was repeated 3 times with 20 larvae per replicate. Probability analysis was used to determine the toxicity regression equations and the LC50 values.

Induction of External Color Change of *X. rusticus* Larvae by Infection

The strain BbCC01 was used at 1 × 10^8 spores per mL to infect the larvae. Firstly, the *X. rusticus* larvae were immersed in the BbCC01 spore suspension and cultured. Subsequently, the change of color on the surfaces of the larvae was observed to be persistent, and the color change was recorded by an Olympus DP27 digital camera.

Preparation of Enzyme Solution

After the body weights of larvae (*n* = 10) were measured, their heads were removed and their bodies were submerged in 50 mM phosphate buffered saline (PBS, pH 7.0) to create a 5 g/mL larval suspension. The suspension was ground using a glass homogenizer in an ice bath, homogenized by ultrasonic disruption for 10 min, and centrifuged at 4,000 *g* at 4 °C for 15 min. The supernatant was collected and stored at –80 °C. The enzyme solutions were thawed prior to analysis.

Protective Enzyme Activities in *X. rusticus* Larvae

Catalase (CAT) Activity. The CAT activity was measured according to methods described by Chance & Maehly (1995). In summary, 4 mL H$_2$O$_2$ (0.3%) solution was added to 2.6 mL of 50 mM PBS (pH 7.0), and the OD$_{240}$ was determined using a UV-721 spectrophotometer. Twenty microliters of the enzyme solution was added to 3 mL substrate solution and incubated at 25 °C. The OD$_{240}$ was measured immediately and every 30 s, 3 times. One unit of enzyme activity was defined as a 10-fold reduction in OD$_{240}$ according to Equation 1.

$$\text{CAT activity units } (\Delta OD/\text{min}) = (\Delta A \times V) / (0.1 \times T \times V_E) \quad (1)$$

Where ΔOD is OD$_{240}$ reduction per min; *V* is the total reaction volume (mL); *T* is the last reading time (min) after hydrogen peroxide was added; and V_E is the enzyme solution volume (mL).

Superoxide Dismutase (SOD) Activity. The SOD activity was measured according to methods reported by Beauchamp & Fridovich (1971). Here, 50 µL enzyme solution (1% PVP, l0.04% phenyl thiourea, and 10 mM EDTA, at a 10:3:3 volume ratio) was added to 3 mL reaction solution (13 mM methyl-methionine, 0.1 mM EDTA, 7.5 µM NBT, and 50 mM PBS, pH 7.0) and an additional 0.6 mL of 4 µM riboflavin. The tubes containing no enzyme solution were designated as the maximum-light tubes. After 10 min exposure to light at 4,000 lx, the solution was immediately protected from light, and the OD$_{560}$ absorption was measured by UV spectrophotometry. The tubes with no light exposure were used as controls. The reactions were measured 3 times. The SOD enzyme activity was then calculated according to Equation 2. One unit of enzyme activity was defined as the amount of enzyme needed to reach 50% inhibition. The enzyme activity was expressed as enzyme units per mg of protein (U/mg).

$$\text{SOD activity } (\Delta OD/\text{min}) = (A \times V \times 60) / (T \times V_E \times 50\%) \quad (2)$$

Where *A* is the maximum-light tube OD$_{560}$ – reaction tube OD$_{560}$; *V* is the total reaction volume (mL); V_E is the volume of the enzyme solution (mL); and *T* is the reaction time (min).

Peroxidase (POD) Activity. The POD activity was measured according to methods described by Li (2000) and calculated using Equation 3. In short, reagents were added to test tubes in the order 2.91 mL of 1.0 mM PBS (pH 7.0), 0.05 mL of 20 mM guaiacol, 0.02 mL enzyme solution, and 0.02 mL of 40 mM H$_2$O$_2$. The mixture was incubated in a 35 °C water bath for 5 min. Next, 0.02 mL of 20% TCA was added to terminate the reaction, and the OD$_{470}$ was measured.

$$\text{POD activity } (U/\text{g FW}) = (\Delta A_{470} \times V_t) / (W \times V_s \times 0.01 \times t) \quad (3)$$

Where ΔA_{470} is the absorbance change in reaction time; *W* is the fresh weight of sample (g); *t* is the reaction time (min); V_t is the total volume of enzyme solution (mL); and V_s is the enzyme solution volume used in the measurement (mL).

Detoxification Enzyme Activities in *X. rusticus* Larvae

Carboxylesterase (CarE) Activity. The CarE activity was measured according to methods reported by Van Asperen (1962). Briefly, the following reagents were added in the order 0.1 mL diluted enzyme solution (or 0.04 M PBS [pH7.0] as the blank control) and 2 mL of 3×10^{-4} mM α-naphthol–physostigmine (1:1) acetone solution. The solution was incubated in a 35 °C water bath for 10 min, 1 mL color reagent (1% fast blue B salt, 5% SDS at a 2:5 volume ratio) was added to terminate the reaction, and the OD_{600} was measured. A standard curve was prepared with a dilution series of α-naphthol, and the CarE activity calculated with Equation 4. The experiment was repeated 3 times.

$$\text{CarE activity unit (nM/min)} = (\Delta OD_{600} \times V) / (\varepsilon \times L) \tag{4}$$

Where ΔOD_{600} is the change in absorbance value per min; V is the enzymatic reaction volume (mL); ε is the extinction coefficient of the products (13.6 L/mM/cm); and L is the length of the optical path (1 cm).

Based on the α-naphthol standard curve, the amount of α-naphthol generated by CarE activity was calculated. Combined with the reaction time and protein content, the hydrolysis of α-acetic acid naphthalene per min per mg of protein was calculated. The CarE activity was expressed as μM/min/mg.

Glutathione S-Transferase (GST) Activity. The GST activity was measured using methods described by Booth et al. (1961) and calculated according to Equation 5. In brief, reagents were added as follows: 2.3 mL of 0.1 M PBS (pH 7.0), 0.5 mL reduced glutathione (GSH), 0.1 mL enzyme solution, and 0.1 mL of 1 mM CDNB substrate. Inactivated enzyme solution was used as a blank control. At 25 °C constant temperature, the 3 min absorption curve was measured with a UV-721 spectrophotometer at a wavelength of 340 nm to calculate the initial reaction velocity value (OD_{340}/min). Next, 0.3 mL trichloroacetic acid was added to terminate the reaction. The enzyme solution was added after termination and was used as a blank. The reaction mixture was centrifuged at 1,500 *g* for 5 min. The supernatant was used to measure the absorption at 340 nm.

$$\text{GST activity unit (nM/min)} = (\Delta OD_{340} \times V) / (\varepsilon \times L) \tag{5}$$

Where ΔOD_{340} is the change in absorbance per min; V is the total enzymatic reaction volume (mL); ε is the extinction coefficient of the products (9.6 L/mM/cm); and L is the optical path length (1 cm).

Acetylcholine Esterase (AchE) Activity. The AchE activity was measured according to methods reported by Gao (1987) and calculated with Equation 6. Briefly, larvae were mixed with 0.1 M PBS (pH 7.5), homogenized on ice, and centrifuged at 4,000 *g* and 4 °C for 15 min. After filtration, the supernatant was used as an enzyme stock solution, placed on ice, and diluted as necessary. Then, 0.1 mL of 0.01 M thioiodide acetylcholine was added to 2 test tubes with plugs. In one of the tubes, 0.1 ml of the enzyme solution was added, and the tube was incubated for 15 min in a 30 °C water bath. Next, 3.6 mL of 0.125 M DTNB chromogenic reagent was added to the tube. In the other tube, 0.1 ml of enzyme solution was added, and the OD_{412} was measured immediately and expressed in nM/min.

$$\text{AchE specific activity} = (\Delta OD_{412} \times V) / (\varepsilon \times L \times V_E) \text{ (mmol/min)} \tag{6}$$

Where ΔOD_{412} is the change in absorbance per min; V is the total enzymatic reaction volume (mL); V_E is the enzyme reaction volume (mL); ε is the extinction coefficient of the products (13.6 L/mM/cm); and L is the optical path length (1 cm).

Protein Concentration

The Coomassie brilliant blue staining (Bradford) method was used to determine protein concentrations (Perry et al. 1996). Seven different concentrations of the bovine serum albumin, namely 0, 0.02, 0.04, 0.06, 0.08, 0.10, and 0.12 mg/mL, were used to construct the standard curve. First, 0.3 mL diluted sample (1:20 in distilled water) and 5.7 mL Bradford reagent (0.1 mg/mL Coomassie brilliant blue G-250, 5% alcohol, and 14% phosphoric acid) were mixed and kept at room temperature for 5 min, and the absorption at 595 nm was determined. Each reaction was repeated 3 times. According to the standard curve, the protein concentration of each sample was calculated.

STATISTICAL ANALYSES

DPS and Excel software were used to process and analyze the experimental data and to calculate LT50 and LC50 values and 95% confidence intervals. SPSS17.0 software was used to conduct analyses of variance and the Duncan test for the separation of means; these results were subjected to multiple mean comparisons. Each datum was expressed as the mean ± standard error (SE) from 3 replicates.

Results

EXTERNAL CHARACTERISTICS OF *X. RUSTICUS* LARVAE AFTER INFECTION WITH *BEAUVERIA*

The external characteristics of the *X. rusticus* larvae following infection with *Beauveria* are shown in Fig. 1. There were no obvious differences in symptoms of larvae induced by the various *Beauveria* strains. At the early stage of infection, there was no apparent difference in characteristics between the control and the infected larvae (Fig. 1A). As the infection progressed, the larvae reduced their activity, became paralyzed, and finally died, with white mycelium growing on the surface of the carcass (Fig. 1B). At this point, the mycelia of *Beauveria* were attached to the exoskeleton, especially in areas of intersegmental membranes (Fig. 1C). Following incubation under moist conditions, an uneven change in larval body color was observed (Fig. 1D).

MEDIUM LETHAL TIME (LT50) AND MEDIUM LETHAL CONCENTRATION (LC50) OF *BEAUVERIA* STRAINS IN *X. RUSTICUS* LARVAE

The lethal effects of the 4 strains of *Beauveria* infecting *X. rusticus* larvae are shown in Table 2. All 4 strains were infectious, with strain BbCC01 causing the highest (85.7%) and strain BbAC830 causing the lowest infection rate (63.3%). Furthermore, strain BbCC01 had the shortest LT50 of 4.69 d. The LT50 for strain BbAC830 was 6.62 d, or 1.41 times that of strain BbCC01. Based on the above results, strain BbCC01 was selected for further experiments.

Mortality rates of the larvae were significantly different 8 d after infection with various concentrations of strain BbCC01 (Table 3). At a concentration of 1×10^5 spores per mL, the larval mortality rate was 21.1%. At a concentration of 7.2×10^8 spores per mL, the mortality rate reached 100%. According to the dose regression equation, the LC50 of strain BbCC01 was 4.77×10^6 spores per mL.

EFFECTS OF INFECTION WITH STRAIN BBCC01 ON DETOXIFICATION ENZYME ACTIVITIES IN *X. RUSTICUS* LARVAE

As shown in Fig. 2A, the CarE activity in infected larvae first increased and then decreased. In the first 24 h, it did not change significantly compared with the control. The CarE activity increased signifi-
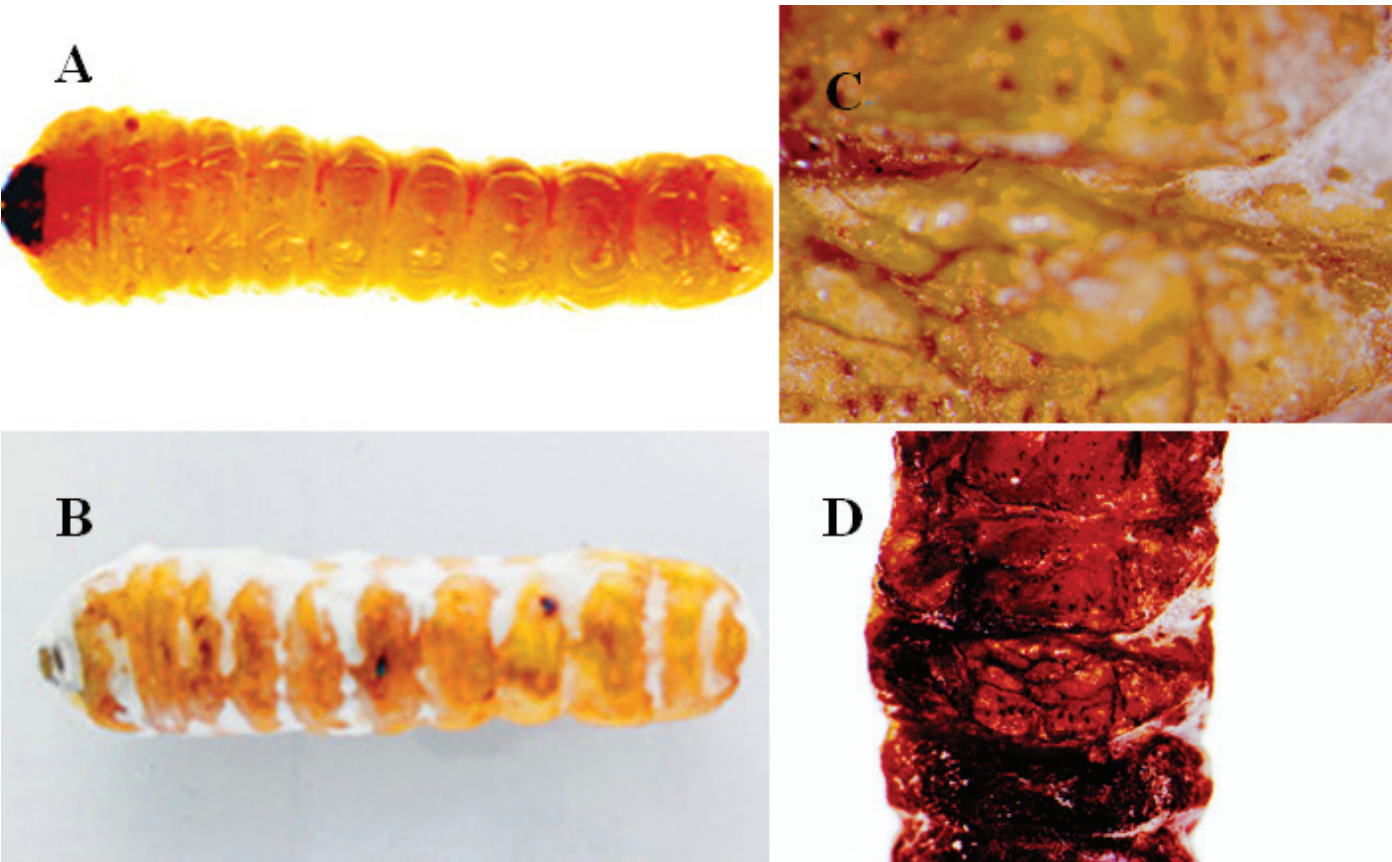
Fig. 1. Symptoms of *Xylotrechus rusticus* larvae following infection by *Beauveria bassiana*. A. Control larvae. B. Mycelium covering the larval body at 120 h post-infection. C. Mycelium on intersegmental membranes. D. Blackish-brown discolored infected larval cuticle.

cantly after 48 h and reached a maximum value, 1.81 times as high as that in the control (*P* < 0.05), at 96 h post-infection. From 120 to 192 h post-infection, the CarE activity decreased continuously and reached the lowest level at 192 h post-infection, about 0.544 times as high as that in the control.

With an increasing infection time, the GST enzyme activity first increased and then decreased (Fig. 2B), reaching a maximum level at 96 h post-infection (*P* < 0.01). It declined from 120 to144 h post-infection but remained higher than in the control. At 168 h post-infection, the GST activity was reduced below the control and reached the lowest level at 192 h post-infection, 0.5 times as high as that in the control.

The AchE enzyme activity in infected larvae first decreased, increased, and then decreased again, whereas that in control larvae remained stable until it declined at 192 h (Fig. 2C). At 24 h, the AchE activity was not different between infected and control larvae. At 48 h post-infection, the AchE activity decreased and then increased significantly to 1.513 times that of the control (*P* < 0.05) at 72 h post-infection. From 96 to 144 h post-infection, the AchE activity decreased

and remained low until 192 h post-infection, with a value 0.467 times that of the control.

EFFECTS OF INFECTION WITH STRAIN BBCC01 ON PROTECTIVE ENZYME ACTIVITIES IN *X. RUSTICUS* LARVAE

Figure 3A shows that following the infection of larvae with strain BbCC01, the CAT enzyme activity first increased and then decreased. From 24 to 96 h, the CAT activity in infected larvae was clearly higher than that in control larvae, reaching a maximum level 2.474 times that of the control (*P* < 0.05) at 96 h post-infection. As the infection progressed, the CAT activity declined until it reached the lowest level at 192 h post-infection, at 0.42 times that of the control.

The POD activity in control larvae was relatively stable over time, whereas that in infected larvae significantly increased from 24 to 96 h post-infection and significantly decreased from 120 to 192 h post-infection (Fig. 3B). At 96 h post-infection, the POD activity reached a maximum level, at 1.944 times that of the control (*P* < 0.05). At 120 h post-infection, the POD activity was 1.696 times that of the control,
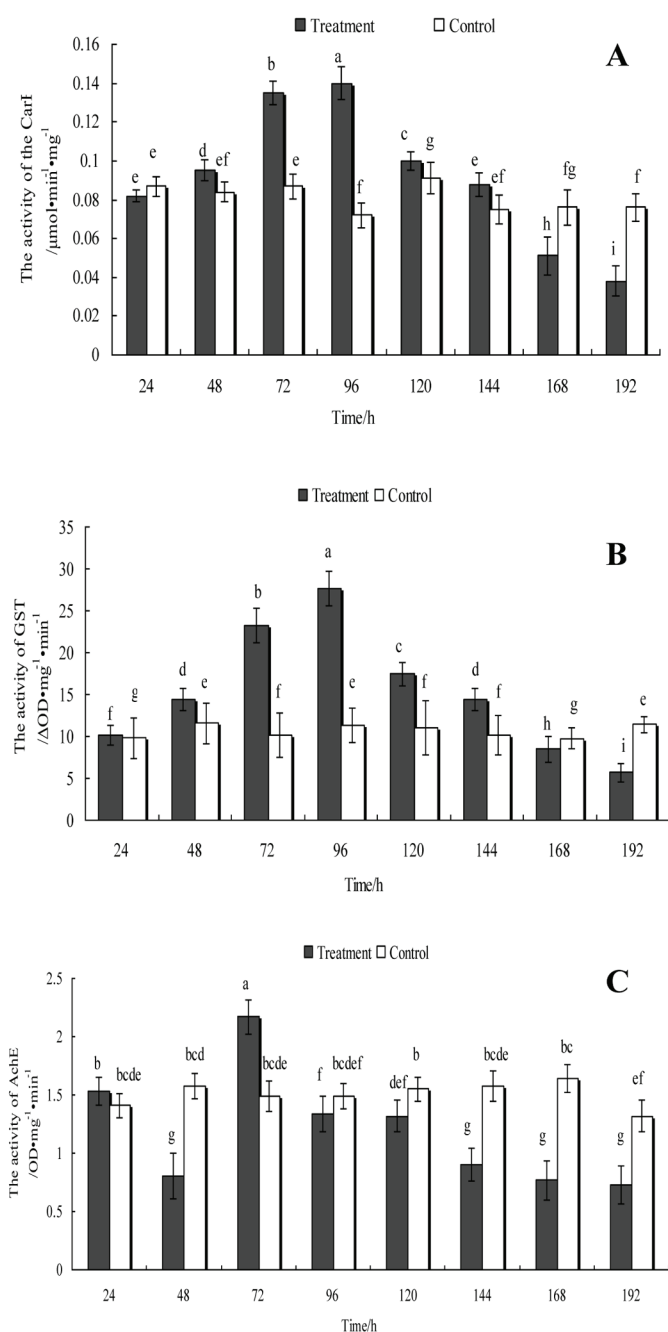
Table 2. Pathogenicity of 4 *Beauveria* strains infecting *Xylotrechus rusticus* larvae (10 d post-infection).

Strain	Number of tested larvae	Cumulative mortality (%)	Corrected mortality (%)	Infection (%)	LT50 (d)
BbAC830	30	70.00	67.86	63.33	6.62
BbAC749	30	83.33	82.13	66.67	5.67
BbCC01	30	93.33	92.86	85.71	4.69
BbCF327	30	76.67	75.00	73.91	6.23
Control	30	5.67	n/a	n/a	n/a

n/a = not applicable

Table 3. LC50 of *Beauveria bassiana* strain BbCC01 infecting *Xylotrechus rusticus* larvae (8 d post-infection).

Spore concentration (mL⁻¹)	Number of tested larvae	Cumulative mortality (%)	Corrected mortality (%)	Dose regression equation	LC50 (spores per mL)
1×10^5	60	21.05	20.90	$y = 1.1494 + 0.6102x$	4.77×10^6
1×10^6	60	31.58	31.43	($R^2 = 0.9198$)	
1×10^7	60	68.42	68.28		
1×10^8	60	94.74	94.6		
7.2×10^8	60	100	100		

Fig. 2. Effects of infection by *Beauveria bassiana* strain BbCC01 on detoxifying enzyme activity in *Xylotrechus rusticus* larvae over time. A. Carboxylesterase (CarE). B. Glutathione S-transferase (GST). C. Acetylesterase (AchE). Data are expressed as mean ± SE (*n* = 3). Different letters indicate significant differences between means (*P* < 0.05).

and although it continued to decline, it did not sink below the activity measured in control larvae.

The SOD activity in infected larvae increased significantly to its maximum from 24 to 72 h post-infection (*P* < 0.05), then dropped at 96 h post-infection to a level 0.94 times that of the control (Fig. 3C). After another increase at 120 h post-infection, the SOD activity declined continuously to reach its lowest level at 192 h, at 0.81 times that of the control.

Figure 4 shows that following infection with strain BbCC01, the protein content in *X. rusticus* larvae first increased, then decreased, whereas that in control larvae remained unchanged. At 72 h post-infection, the protein content reached the maximum level, at 4.346 times that of the control (*P* < 0.05). From 96 to 192 h post-infection, the protein content decreased continuously and reached the lowest level at 192 h, at 1.174 times that of the control.

Discussion

Previous reports have shown that following invasion of the host body, the *B. bassiana* mycelium continues to grow after overcoming the immune system, invading organs and filling the body cavity. After infection, the hemocytes of the host lose their phagocytic function, and normal cell circulation is hindered, causing physical hunger and mechanical damage to the cells (Pekrul & Grula 1979; Rajendran & Gopalan 1999). Meanwhile, during mycelial growth, toxins and metabolites secreted by the mycelium including beauvericin and oxalic acid salts cause physicochemical changes in the hemolymph, interfere with the normal metabolic functions in the host, cause morphological changes, and eventually lead to host death due to an inability to maintain normal life processes (Broome et al.1976; Mohamed et al.1978; Cheung & Grula 1982; Dombrink-Kurtzman 2003).

Detoxification enzymes (such as CarE, GST, and AchE) in insects can effectively metabolize the exogenous toxic compounds (Zhang et al. 2001). They play important roles in maintaining the normal physiological activities in the body (Kontogiannatos et al. 2011). In this study, the activities of detoxifying enzymes were determined in *X. rusticus* larvae at different times after infection with *B. bassiana.* The results showed that in the early stage of infection with strain BbCC01, CarE activity in the larvae significantly increased and then decreased with increasing infection time. This might be due to a host response initiated by the infection (Li 2007), in which CarE activity was remarkably enhanced to metabolize the exogenous lipids, reaching the maximum value at 96 h. The enhanced CarE activity would also accelerate the breakdown of the larvae's fat bodies. Eventually, the defensive capabilities of the larvae would be lost. While the mycelia within the larvae continued to grow, the CarE activity began to decline and sank below that in control larvae at 168 h post-infection.

GST serves a variety of physiological and metabolic functions (Su et al. 2007). The enzyme is able to catalyze harmful or polar compounds with glutathione and can discharge various potential toxic compounds
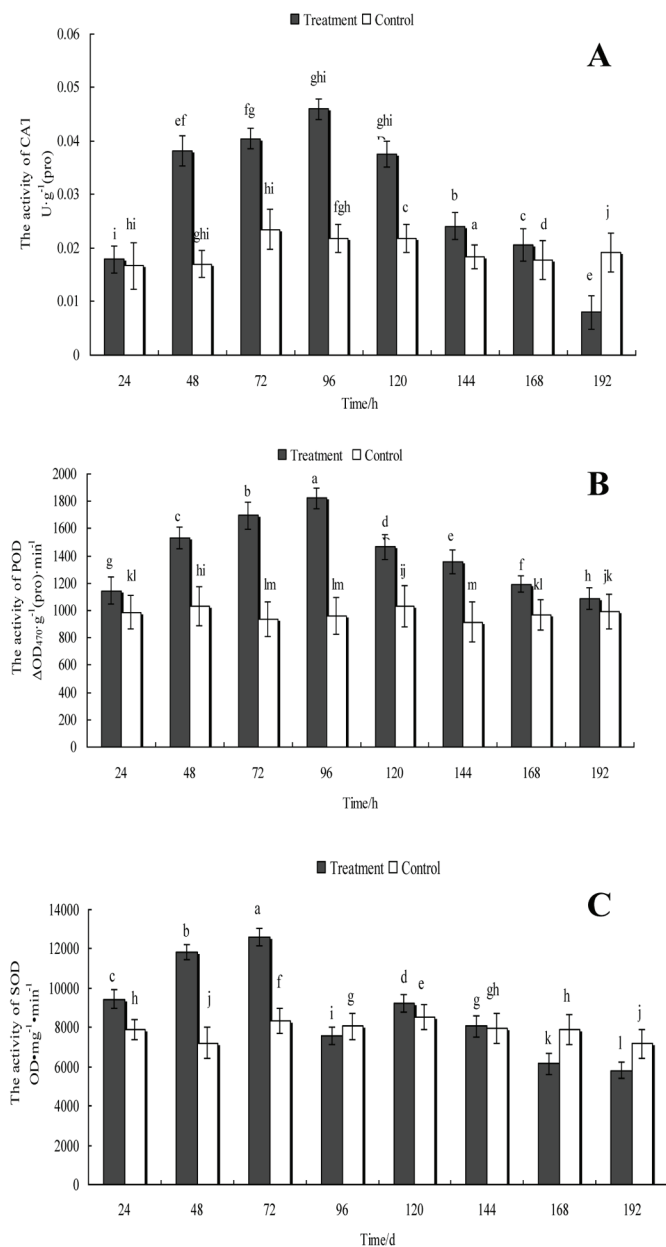
Fig. 4. Change of the protein content in *Xylotrechus rusticus* larvae infected with *Beauveria bassiana* strain BbCC01. Data are expressed as mean ± SE (*n* = 3). Different letters indicate significant differences between means (*P* < 0.05).

Fig. 3. Effects of infection by *Beauveria bassiana* strain BbCC01 on protective enzyme activity in *Xylotrechus rusticus* larvae over time. A. Catalase (CAT). B. Peroxidase (POD). C. Superoxide dismutase (SOD). Data are expressed as mean ± SE (*n* = 3). Different letters indicate significant differences between means (*P* < 0.05).

and some carcinogens from the body in a non-enzymatic fashion (Ding 2007). The function of GST is to conjugate endogenous glutathione with toxic pro-electron material to form a nucleophilic center that protects substances such as proteins and nucleic acids and acts as a means of excreting toxic substances. In this study, we found that 48 h following infection, GST activity increased significantly to ward off the invasion and maintain the normal physiological functions in the insect. At 96 h post-infection, the GST activity reached its maximum level. However, at 120 h post-infection, GST activity continuously declined due to the damage caused by the mycelium growth.

AchE is an important insect hydrolase for maintaining normal functions of the nervous system through rapid hydrolysis of the neurotransmitter acetylcholine into choli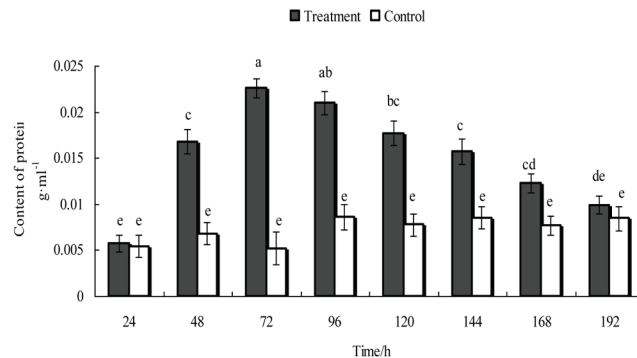ne and acetic acid to stop nerve impulse transmission. Organophosphate and carbamate pesticides function primarily by binding to AchE and inhibiting its catalytic activity (Niu et al. 2005; Machado et al. 2012). When AchE is suppressed, acetylcholine cannot be decomposed in a timely manner and is accumulated in the synaptic cleft, resulting in neural hyperactivity, convulsions, poisoning, and death. In our results, the AchE activity in 24 to 48 h infected larvae was suppressed to a certain extent. This may be because, following infection, the *B. bassiana* spores germinate, penetrate the larval body, and release toxin in the body. At 48 to 72 h post-infection, the immune regulatory functions in the larvae showed a stress reaction, and AchE activity was elevated. At 72 to 96 h post-infection, a large number of spores had germinated and invaded the body, gradually reducing the effectiveness of the host's defense system until the AchE activity was suppressed. AchE activity in 96 to 192 h infected larvae was lower than that in control larvae and directly impacted the larvae's nervous system.

Under stress conditions, the insect produces large quantities of reactive oxygen species (ROS), such as superoxide anion (O_2^-) and the hydroxyl radical (HO^-). These compounds are cytotoxic and help to kill pathogens and parasites. However, excessive reactive free oxygen radicals can cause damage to the organism itself (Li et al. 2006). There are free radical scavenging systems, such as the protective enzyme systems in the insect's body, including SOD, CAT, and POD. All 3 enzymes work coordinatedly to maintain the organism in a state of dynamic equilibrium, keeping free radicals in the cell at low levels to prevent the cells from damage (Gao et al. 1995). Studies have shown that, after infection by fungi, the insect's protection systems are activated to ward off infection and to maintain the normal physiological activities (Song et al. 2002; Zhang et al. 2003).

At 24 to 72 h after the larvae were infected, the SOD activity increased, suggesting that O_2 consumption increased and there was a substantial increase in the free radical HO^-. This, in turn, activated SOD to clear the excess free radicals from the body. At 72 h post-infection, the SOD activity reached its maximum level. At 96 to 192 h post-infection, the SOD activity decreased significantly, indicating that with progressing infection, the larvae were no longer able to synthesize SOD and eventually died. This finding is in line with previous research results (Gao et al. 1995; Song et al. 2002; Zhang et al. 2003).

At the early stage of infection, the larvae could prompt an immune defense response, increasing CAT activity to adapt to the effects of the toxins. Meanwhile, in vivo metabolism produced and accumulated a large amount of H_2O_2. This led to increased CAT activity. In the late stages following infection, due to the damage to larval tissues and organs, CAT synthesis and activity could no longer be sustained. At 192 h post-infection, the enzyme activity was significantly lower than in the control.

After infection, the larvae produced toxic oxidation substances that induced the synthesis of POD by the immune system to maintain normal physiological functions. However, at 96 h post-infection, possibly due to proliferation of the mycelium or the toxins it produced, POD enzyme synthesis was affected, and POD enzyme activity decreased. In general, at 72 and 96 h post-infection, SOD activity reached a maximum, whereas CAT and POD activities peaked at 96 h, indicating that SOD was activated first, followed by the CAT and POD enzyme systems. POD enzyme activity was higher than in the control at all time points, indicating that POD may play a more important role than SOD and CAT in the immune reaction against *B. bassiana* infection in *X. rusticus* larvae. The results also showed that, at 72 to 92 h post-infection, the activities for SOD, CAT, and POD had reached their maxima. The 3 enzymes work together to maintain the free radicals at a low level to prevent damage. The overall activity trends were consistent with previous research (Gao et al. 1995; Liochev & Fridovich 2002, 2007; Song et al. 2002; Zhang et al. 2003; Zhang 2005; Li et al. 2007).

Our results showed that the protein contents in infected larvae were significantly greater than in control larvae. Between 24 and 72 h post-infection, the invasion by *B. bassiana* activated the defense system in the larvae. Immune response–related proteins and enzymes were produced. Furthermore, due to the proliferation of *B. bassiana* in the larval body, the organ functions in the larvae were blocked and their normal physiological activities were affected resulting in the inhibition of protein synthesis and protein transportation. Tissues and organs were damaged and dissociated. These processes released more protein causing further increase in the protein content. From 96 to 192 h post-infection, the protein content continuously declined until the death of the larvae.

In this study, a highly pathogenic *B. bassiana* strain was selected to infect *X. rusticus* larvae. The results showed that the mortality rate caused by *B. bassiana* strain BbCC01 reached 93.3%, and the LT50 and LC50 were 4.69 d and 4.92×10^7 spores per mL, respectively. The virulence of strain BbCC01 was higher than that of the other 3 *Beauveria* strains from different hosts. Thus, strain BbCC01 could have potential as a biological insecticide to control *X. rusticus* larvae. For the first time, this study also showed the changing patterns of the detoxifying and protective enzyme activities and of the protein content in the hemolymph, following infection of *X. rusticus* larvae with a highly pathogenic *B. bassiana* strain. For all enzymes tested, the activities showed an overall increasing and then decreasing trend, except for AchE, whose activity first decreased, then increased, and finally decreased. In line with these results, the protein content in the hemolymph showed an overall increasing and then decreasing trend as well. These results suggest that *B. bassiana* overcame the host's immune system, inhibiting the activities of various protective and detoxifying enzymes and resulting in host death. This study elucidates the pathogenesis of *B. bassiana* and its interaction with *X. rusticus*. Moreover, the results suggest that *B. bassiana* is a potential biological control agent of *X. rusticus*, which may be used against susceptible pests in the forests throughout the world.

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