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Full Length Research Paper

Isolation and identification of *Metarhizium anisopliae* from *Chilo venosatus* (Lepidoptera: Pyralidae) cadaver

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Sugarcane stem borer, *Chilo venosatus* Walker (Lepidoptera: Pyralidae) is a significant sugarcane pest in South China. Conidia or mycelia collected from the surface of sugarcane stem borer cadavers were cultured. The colony morphology, mycelia and conidial yield were observed with three-agar culture media: potato dextrose agar medium (PDA), potato dextrose with 1% (w/v) peptone agar medium (PPDA), and oatmeal agar medium (OMA). 16 different isolates were identified as *Metarhizium anisopliae* (Metschnikoff) based on macromorphological, micromorphological, and molecular characteristics, and PPDA was the better culture medium for vegetative growth and conidial yield (10^9 conidia/ml) than PDA (10^8 conidia/ml) and OMA (10^8 conidia/ml). To confirm whether these isolates were pathogenic to *C. venosatus*, their virulence to the sugarcane stem borer was tested in the laboratory. Both HS (10 isolates) and LY (6 isolates) strains were pathogenic to *C. venosatus*. Several highly virulent strains were screened *in vitro* (the mortalities of the eight isolates HS1, HS6, HS7, LY2, LY3, LY6, HS3 and HS9 were from 96 to 100%), and tests for controlling the sugarcane stem borer were preliminarily performed *in vivo*. The results show that significant (p=0.01) reductions in adult population were caused by the strains. So, *M. anisopliae* isolated from the cadavers of *C. venosatus* Walker is a potential biocontrol agent against this pest in South China.

Keywords: *Metarhizium anisopliae*, isolation, identification, *Chilo venosatus*, culture medium, biological control.

INTRODUCTION

Sugarcane is an important tropical crop and contributes to almost two-thirds of the world's yield of sugar for hundreds of years (Menossi et al., 2008). The sugarcane stem borer, *Chilo venosatus* Walker (Lepidoptera: Pyralidae) is an important pest of sugarcane culture and many others crops (Graça, 1976; Long and Hensley, 1972). Larvae of *C. venosatus* feed within the stem of sugarcane which may result in 25% (w/w) sugarcane production loss (Pan et al., 2009). This lepidopteran pest causes considerable harvest loss in the cane-growing areas of South Africa, Swaziland, Australia, Brazil, India, Antilles, Central and South America, and South China (Gerardo et al., 1994; Katrina et al., 2000; Chillar, 1993; Daniela et al., 2008; Shang and Huang, 2010).

Due to a lack of natural antagonists and increasing resistance to chemical pesticides, the sugarcane stem borer has become a serious and devastating pest with significant economic impact (harvest losses with costs of up to several hundred million US $ per year) in South China. All important sugarcane-growing regions, such as

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Abbreviations: PDA, Potato dextrose agar medium; **PPDA,** potato dextrose with 1% (w/v) peptone agar medium; **OMA,** oatmeal agar medium.

Zhanjiang (Guangdong Province), Nanning (Guangxi Province) as well as Lingao and Danzhou (Hainan Province) have recently documented the occurrence of *C. venosatus* (Zhou et al., 2008; Pan et al., 2009; Shang and Huang, 2010; Zhang et al., 2008).

In the past, various management strategies including treatment with chemical agents, such as chlorpyrifos (Zhou et al., 2008) and sumithion (Zhang et al., 2008) as well as the application of frequency vibration-killing lamp and other physical techniques have been used to control the adverse impact of the sugarcane stem borer (Yu et al., 2009; Pan et al., 2009; Shang and Huang, 2010). Although effective in controlling the sugarcane stem borer, chemical and physical approaches could result in serious environmental pollution and indiscriminate killing of ecologically important organisms (Pan et al., 2009). Biological control agents such as *Trichogramma* (Smith, 1996; Matthias and Paul, 2002; Zhen et al., 2001), *Lixophaga diatraeae* (Nicholls et al., 2002), *Bacillus thuringiensis* (Katrina et al., 2000), *Metarhizium anisopliae* (Gao, 1996) as well as sex attractants causing mating disruption (Lin, 2007), are environmentally friendly alternatives to control the outbreak and maintenance of the sugarcane stem borer.

The entomopathogenic fungus *M. anisopliae* has been effective in controlling more than 200 species of insect pests (Pu and Li, 1996) such as *Anopheles arabiensis*, *Anoplophora glabripennis*, *Ceratitis capitata*, *Psoroptes mites*, *Locusta migratoria migratorioides*, *Rhipicephalus evertsi evertsi*, and *Phthorimaea operculella* (Dickson et al., 2010; Shanley et al., 2009; Dimbi et al., 2009; Quesada-Moraga et al., 2008; Brooks and Wall, 2005; Niassy et al., 2011; Marius et al., 2011; Sabbour, 2002). The application of *M. anisopliae* has several advantages over the conventional chemical pesticides, such as limited harm to humans, honey bees, livestock, and crops (Monique et al., 2011). However, there are no reports available if *M. anisopliae* strains isolated from different insect pests can effectively control *C. venosatus* in South China. In addition, exploratory surveys for the isolation of highly virulent *M. anisopliae* strains have been suggested. Our laboratory already had isolated *M. anisopliae* from natural infections of coconut hispid beetle *Brontispa longissima* (Gestro) (Coleoptera: Chrysomelidae), and preliminary studied on against this pest in Hainan island, China (Zhan et al., 2007). In this study, cadavers of *C. venosatus* were recovered from sugarcane, and mycelia and conidia were collected from the cuticle surfaces. Subsequently, fungi were isolated on selective medium and their identification, virulence to *C. venosatus* larvae *in vitro*, and effects of application *in vivo* were tested.

MATERIALS AND METHODS

Collection of sugarcane stem borer

Sugarcane stem borer larvae were collected from seriously infected regions, including the fields near Wenfengyuan of Xuwen, Zhanjiang (Guangdong Province, five isolates), Xianhu of Wuming, Nanning (Guangxi Province, two isolates), Bohou of Lingao, and Wangwu of Danzhou (Hainan Province, nine isolates) of China. Stems of sugarcane infected with *C. venosatus* were cut, and dead and healthy larvae were collected. Cadavers were transferred to sterilized Petri dishes and healthy larvae were transferred to sugarcane seedlings, which were planted in plastic boxes for further rearing. Cadavers were used to isolate the entomopathogenic fungi, and the living insects were used to evaluate the insecticide potential of isolated fungi against *C. venosatus*.

Preparation of culture media

Three different culture media were prepared: potato dextrose agar (PDA), PDA with 1% (w/v) peptone (PPDA), and oatmeal agar medium (OMA). The dodine oatmeal agar medium (DOA) was prepared as the selective medium by adding elements (mainly contained 200 $\mu g ml^{-1}$ dodine (Beilharz et al., 1982; Fernandes et al., 2010), 100 $\mu g ml^{-1}$ penicillin, 50 $\mu g ml^{-1}$ streptomycin (Chase et al., 1986), and 200 $\mu g ml^{-1}$ chloromycetin) in OMA for screening entomopathogenic fungi (Beilharz et al., 1982; Chase et al., 1986; Liu et al., 2007; Du et al., 2008).

Isolation and identification of *M. anisopliae*

Cadavers of the sugarcane stem borer larvae were collected from the field. Sterile needles were used to isolate mycelia or conidia from the cuticles of cadavers and were transferred into a 1.5 ml Eppendorf tube containing sterile distilled water. Conidial suspension (5×10^5 conidia/ml) was prepared and 100 µl were transferred homogeneously spread with drigalski to a Petri dish containing 15 ml of DOA and incubated at 28°C. After three days, the dishes were checked daily until the eighth day. Fungal colonies appearing on each Petri dish were transferred to Eppendorf test tubes containing PPDA and incubated at the same conditions as earlier stated.Each isolate was isolated and observed with a light microscope. A single conidium on the surface of water-agar medium was collected with a sterile capillary tube and transferred onto PPDA to isolate the culture. Identification of the isolates were performed at a species level according to Pu and Li (1996), Tulloch (1976), Roddam and Rath (1997), Zimmerman (1993), and Driver et al. (2000). *M. anisopliae* DNA was isolated by using the method described by Pfeifer and Khachatourians (1993). Primer sequences (forward (TW81): GTTTCCGTAGGTGAACCTGC and reverse (AB21): ATATGCTTAAGTTCAGCGGGT) were synthesized by SBS Genetech Co., Ltd. (Curran et al., 1994). The PCR reaction mix (50.0 µl final volume) consisted of 37.3 µl of ddH_2O, 5.0 µl of 10×PCR Buffer (plus 20 mmol/L $MgCl_2$), 4.0 µl of dNTP mixture (50 mmol/L), 1.0 µL of each primer (250 nM final concentration), 0.7 µl of Taq DNA polymerase (2 U/µl), and 1.0 µl of genomic DNA (10 ng/µl). A blank (no template control) was also incorporated in each assay. The thermo cycling program consisted of one hold at 94°C for 4 min, followed by 33 cycles of 30 s at 94°C and 30 s at 55°C centigrade and 60 s at 72°C. After completion of these cycles, melting-curve data were then collected to verify PCR specificity, contamination and the absence of primer dimers. In addition, the internal transcribed space of the strains was analyzed by comparing the sequence in the region of ITS1-5.8S-ITS2 with Vector NTI 9.0.

Tests on biological characters of isolates

Tests on colony macromorphological aspects on different culture medium

All isolates were cultured in Petri dishes containing PDA, PPDA, and

OMA. Colony color and mycelia texture were evaluated daily (starting from the 5th day after incubation) and documented by photography. The sizes of 50 conidia of each strain were measured with a Leica microscope and average values were compared for all strains.

Tests on colony growth rate and conidia yield on different culture media

All isolates were cultured on solid PDA, PPDA, and OMA. Conidia were harvested at the 12th day after culture by surface-scraping. Homogenous spore suspensions were made by placing harvested spores in 20 ml of sterile distilled water in glass bottles containing 0.1% (v/v) Tween-80 and agitated on a vortex mixer for 30 s. Spore concentrations were determined using a hemocytometer. The procedure was replicated three times for each strain.

Tests on virulence to *C. venosatus*

All isolates were cultured and maintained on PPDA. Conidia were harvested and spore suspensions (1.0×10^5, 1.0×10^6, 1.0×10^7, and 1.0×10^8 conidia/ml) were prepared in sterile water with 0.1% (v/v) Tween-80. In addition, three and four instars larvae of *C. venosatus* were reared for one week, then, 30 larvae were selected for each treatment. Each 30 larvae were sprayed (used hand-held sprayers) with 5 ml of conidial suspension and then placed into sugarcane seedlings, which were planted in plastic boxes. The boxes were placed into an insect-rearing cage and incubated at ambient temperature (25 to 32°C). Each treatment was replicated three times. The control was sprayed with 5 ml of sterile water containing 0.1% (v/v) Tween-80. The commercial formulation (BioCane granule, Australia, 1.0×10^8 conidia/ml) suspension was also sprayed with 5 ml. After the 15th day, dead larvae were selected, and mortalities were calculated for every isolate. Abbott (1925) was then used for correction. Subsequently, all dead sugarcane stem borers were transferred to Petri dishes to evaluate mycosis growth on the surface. The procedure was replicated three times for each isolate.

Preliminary applications on sugarcane stem borers *in vivo*

Preliminary tests on sugarcane stem borer *in vivo* application was performed at the Bohou farm of Lingao, where sugarcane was seriously infected by *C. venosatus*. Each treatment was 130 m long by 25 m wide with a total area of 3250 m^2. The conidial suspension which was prepared with the wettable powder of *M. anisopliae* (the highly virulent isolates HS1, HS6, HS7, LY2, LY3, LY6, HS3 and HS9), were used to control *C. venosatus in vivo*, which was mixed with 43% (w/w) additives (consisted of 25% (w/w) wetting agents, 18% (w/w) dispersant, 16% (w/w) suspending agents and 41% (w/w) stabilizing agents), and adjusted to $\geq1.0\times10^7$ conidia/ml. The first control (Con 1) was BioCane granule (Australia). The second control (Con 2) was also brushed with only water with 43% (w/w) additives. After three months, the suspensions (15 ml/m^2) were sprayed on the stem of each sugarcane twice with an interval of three months, especially at the bell site part of sugarcane and the eclosion hole part of sugarcane. The adult sugarcane stem borer was caught in water tray traps (20 to 30×7 to 10 cm) baited with synthetic sex pheromone with an interval of two months. Finally, the sugarcane was harvested and the fresh weight compared. The trials were replicated three times.

Statistical analysis

To analyze for significant (p=0.01) difference between the results of repeated experiments, the data from each experiment were pooled and evaluated by one-way analysis of variance (ANOVA) (SPSS 14.0 for Windows). Abbott (1925) was then used for correction.

RESULTS

Strains isolation

16 filamentous fungal isolates from cadavers were screened on DOA on different Petri dishes. The isolate from each Petri dish was regarded as a strain. All of these strains were purified and transferred to PPDA for further identification. As a result, all isolates were identified as *M. anisopliae*.

According to the macromorphological and molecular characteristics, we found two distinct strains based on the differences in colony morphology and molecular characteristics, and were thus recorded as HS (10 isolates) and LY (six isolates) isolates. After four days of incubation, the colonies of all HS isolates on PPDA were nearly completely covered with mycelium, but few mycelium were present on the LY isolates under the same conditions. However, there were no significant differences in conidial shape and size of the two kinds of isolates: cylindrical with obtuse ends, slightly narrowing in the center, and the conidial width (1.5 to 3 µm) and length (4 to 8 µm). Also, the structure of conceptacles was not different from those described by Pu Zhelong (Pu and Li, 1996). Based on nucleic acid sequence analysis, both isolates, HS and LY, were *M. anisopliae* var. *anisopliae* (Figure 1) (Curran et al., 1994).

Screening of optimal culture medium for isolates with colony growth and conidia yield

The results show that PPDA was the optimal culture medium for colony growth and conidia yield for both *M. anisopliae* HS and LY strains. The results are summarized in Table 1. For three to seven days, colony growth rate on PPDA was faster than on the other media. The average conidial yield of *M. anisopliae* HS1 isolate was 1.02×10^9 conidia/ml on PPDA, which was significantly (p=0.01) more than 3.81×10^8 conidia/ml on PDA and 1.96×10^8 conidia/ml on OMA, other isolates had the same results.

Pathogenicity to *C. venosatus* and application effects *in vivo*

Both HS (10 isolates) and LY (six isolates) strains were pathogenic to *C. venosatus* (Figures 2a, b, c and d). Using 1.0×10^7 conidia/ml, the larvae mortality ranged from 60 to 100%. HS1, HS6, HS7, LY2, LY3, LY6, HS3 and HS9 isolates were the most pathogenic to *C. venosatus* and were significantly (p=0.01) higher than the two control treatments (Figure 2e). The mortality of the first control

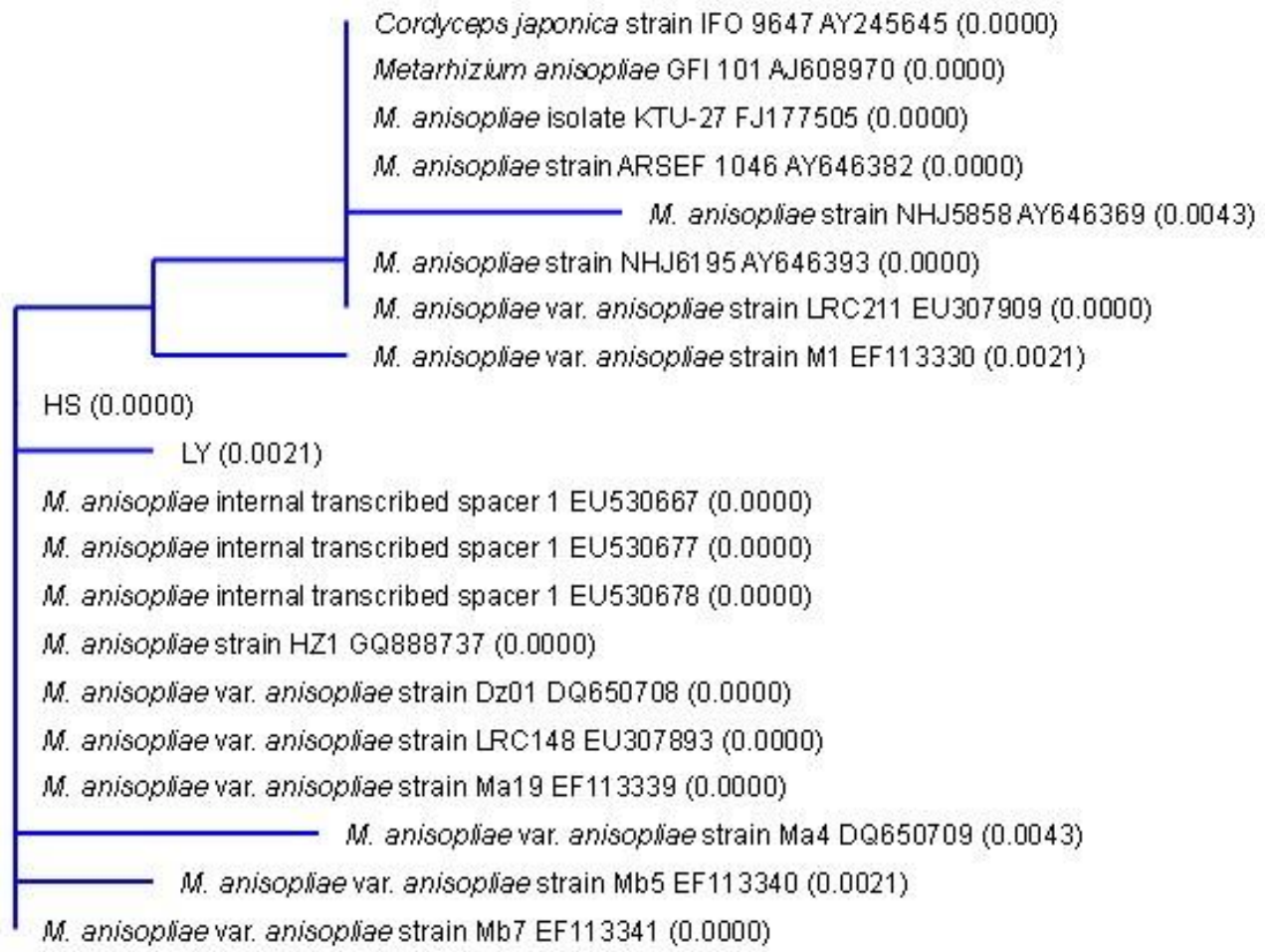

Figure 1. Nucleic acid sequence phylogenetic tree of new isolated strains and reported *Metarhizium anisopliae* strains. The codes following the names are the GenBank Accession numbers. The GenBank Accession number of HS strain sequence (10 isolates) was HM118821, and the GenBank Accession number of LY strain sequence (six isolates) was HM055582. The analysis, assembling, and similarity of nucleic acid sequence of the region of ITS1-5.8S-ITS2 search were used with Vector NTI 9.0 software.

(Con 1) was only 28%, and few mycelia growth was found on cadavers. In moist conditions, conidia and mycelia were observed on the surface of cadavers and identified as the same fungi species used in the initial inoculation. The high-virulence isolates HS1, HS6, HS7, LY2, LY3, LY6, HS3 and HS9 were also significantly (p=0.01) higher than the commercial formulation of *M. anisopliae* (the second control, Con 2). The results show that the strains isolated from endomopathogenic fungi in *C. venosatus* were pathogenic and that eight of the strains were highly virulent to *C. venosatus*.

Then, the highly virulent isolates HS1, HS6, HS7, LY2, LY3, LY6, HS3 and HS9 were used to control *C. venosatus in vivo*. The averages of the eight sugarcane treatments after 300 days was significantly (p=0.01) higher than the two control treatments (Figure 3). The sugarcane fresh weight after harvesting the eight treatments using isolates HS1, HS6, HS7, LY2, LY3, LY6, HS3 and HS9 were from 2.35×10^4 to 2.80×10^4 kg; the first control (Con 1) was 1.65×10^4 kg, and the second control (Con 2) was 2.16×10^4 kg. The average population of the adult sugarcane stem borer taken before application varied from 59.3 to 29.4 after 120 days and to 17.0 after 300 days, and cadavers with mycosis were present at more than 75%. Few changes occurred at the first control (Con 1), in which the adult sugarcane stem borer population varied from 53.4 to 57.0 and to 54.1 at the same time, and few cadavers were found. The averages of adult sugarcane stem borers population of the second control (Con 2) varied from 56.3 to 37.4 and to 25.0 (Figure 4).

DISCUSSION

In this study, we reported 16 isolates of *M. anisopliae*, isolated from different fields in South China, which had high pathogenicity against *C. venosatus*. Strains of *M.*

Table 1. Yield of conidia of 16 *M. anisopliae* isolates on different culture media (PPDA, PDA, OMA).

Isolate	HS1	HS2	HS3	HS4	HS5	HS6	HS7	HS8	HS9	HS10	LY1	LY2	LY3	LY4	LY5	LY6
Yield (PPDA) (10^9 conidia/ml)	1.02[a]*	0.83[a]	0.95[a]	0.82[a]	0.76[a]	1.10[a]	1.06[a]	0.82[a]	0.71[a]	0.86[a]	0.62[a]	0.78[a]	0.76[a]	0.52[a]	0.59[a]	0.72[a]
Yield (PDA) (10^8 conidia/ml)	3.81b[c]	3.59[bc]	3.79[bc]	3.74[bc]	3.61[bc]	4.11[bc]	3.89[bc]	3.71[bc]	3.63[bc]	3.59[bc]	2.89[bc]	3.12[bc]	3.04[bc]	2.88[bc]	2.91[bc]	2.97[bc]
Yield (OMA) (10^8 conidia/ml)	1.96[c]	1.52[c]	1.77[c]	1.49[c]	1.41[c]	2.23[c]	1.99[c]	1.66[c]	1.56[c]	1.45[c]	1.02[c]	1.35[c]	1.26[c]	0.96[c]	1.01[c]	1.16[c]

*The same capital letters indicate no significant difference for the mean value (Tukey–Kramer test, F=1.25, p=0.01). Abbott (1925) was then used for mortality correction.

anisopliae have been isolated from soils (Roddam and Rath, 1997; Tarasco et al., 1997) and insects (Pedro and Candido, 1997; Poprawiski and Yule, 1991) from different regions of the world. Some strains have been used to control sugarcane pests, such as Acrididae, Dynastidae and Cercopidae (Zhang et al., 2006).

This is the first study to report that strains of *M. anisopliae* were isolated from *C. venosatus* in South China. All isolated strains were *M. anisopliae* var. *anisopliae*, according to macro-morphological and molecular characteristics analyses. The fungi have a broad-spectrum insecticidal activity (Wekesa et al., 2005; Alonso-Díaz et al., 2007; Zhang et al., 2006).

Our study reveals that PPDA is a better culture medium for colony growth and conidia yield than PDA and OMA. Before this study, PDA was commonly used as the medium to culture *M. anisopliae* (Liu et al., 2007; Du et al., 2008). The improved DOA is an effective selective medium for screening *M. anisopliae* from both soils and insects.

The results show that the isolates were pathogenic to *C. venosatus*, based on the symptoms after inoculating the sugarcane stem borer with the isolates. In pathogenic tests, the mortality in treatments was significantly (p=0.01) higher than the controls (the first control was treated with chemical BioCane granule and the second control was treated with water with 43% (w/w) additives). In addition, fungal outgrowth was found on the surface of dead sugarcane stem borer in humid conditions but not in the blank control (treated with water with 43% (w/w) additives). Eight highly pathogenic strains of HS1, HS6, HS7, LY2, LY3, LY6, HS3 and HS9 were applied *in vivo*, which caused a significant (p=0.01) reduction in the adult population.Our studies show that *M. anisopliae* has potential as a biocontrol agent against the sugarcane pests. Recently, the effective strategies on *C. venosatus* control performed in Guangdong and Hainan Provinces and others, in 1990 to 2010, involved rearing of natural antagonists of *Trichogramma*, *L. diatraeae*, and spraying insecticides. However, the biological control effect of *Trichogramma* and *L*. *diatraeae* were easily affected by environmental elements (Zhang, 1997; Talekar and Shelton, 1993; David et al., 1991). It was reported that the transgenic sugarcane lines expressing high levels of synthetic *Cry1Ac* proteins were highly resistant to sugarcane stem borer attack (Weng et al., 2006). Biological control agents for the corn borer, such as the parasitic fungus *Beauveria bassiana* and the release of the parasitic wasp *Trichogramma ostriniae,* were used in cornfields (Cherry et al., 1999; Hoffmann et al., 2002). In contrast, *M. anisopliae* isolated from local sugarcane stem borer had better environmental resistance. As an important part of an integrated pest management (IPM) strategy, *M. anisopliae* could be widely used. *M. anisopliae* can be considered promising for biological control of sugarcane stem borer by reducing the pest population and lessening the dependence on chemical control.

Conclusion

In conclusion, we have isolated 16 *M. anisopliae* isolates, in which eight have high pathogenicity against *C. venosatus*. Furthermore, all of these isolates could be used for controlling sugarcane stem borer. Biological studies, including the isolation and identification of *M. anisopliae* and the illustration of cultural process, are required for a better understanding of the pathogenic activities and roles. The role of *M. anisopliae* against *C. venosatus* has explicit evidence in natural conditions.

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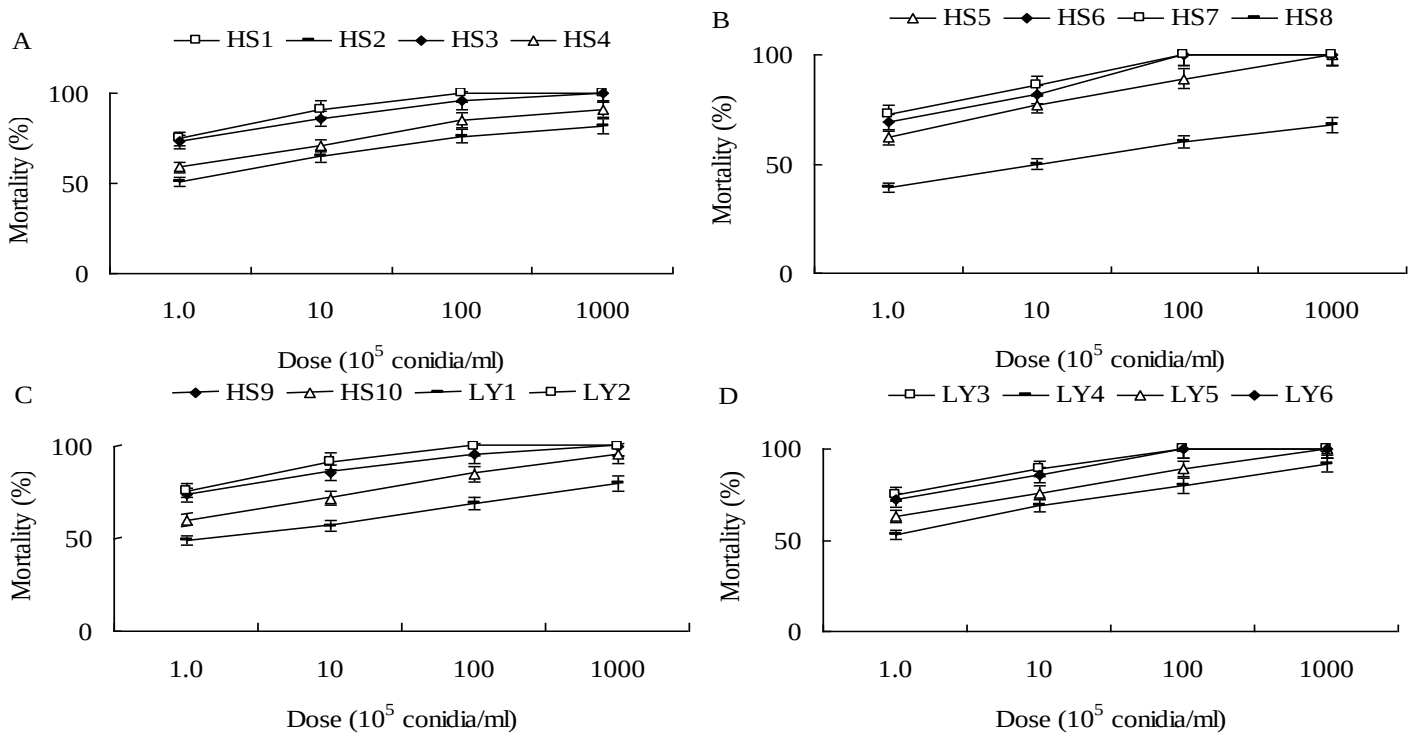
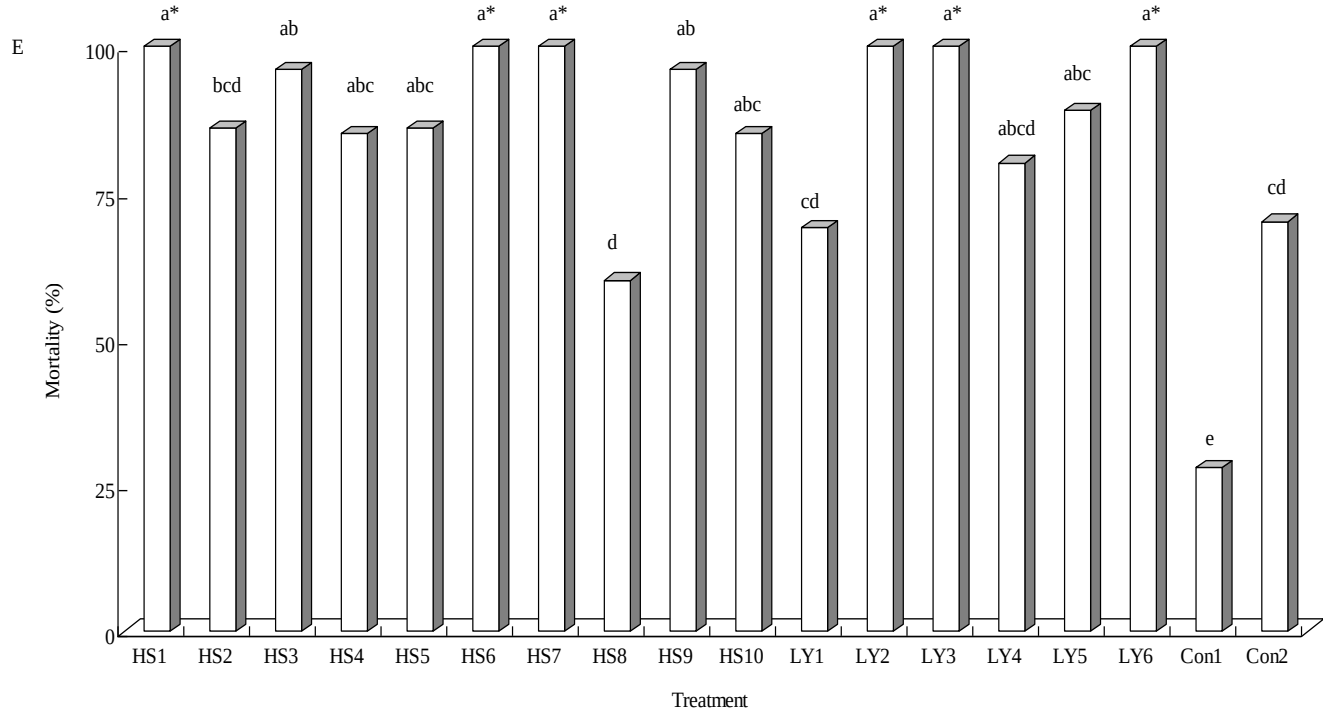


Figure 2. Comparing mortalities of *C. venosatus* larvae infected by different doses of the 16 *M. anisopliae* isolates at 15th day after inoculation. 1.0×10^5 conidia/ml showed 39 to 75% mortality, 1.0×10^6 conidia/ml showed 50 to 92% mortality, 1.0×10^7 conidia/ml showed 60 to 100% mortality, 1.0×10^8 conidia/ml showed 68 to 100% mortality, respectively (A, B, C and D). At 1.0×10^7 conidia/ml, there were eight highly pathogenic *M. anisopliae* isolates to *C. venosatus* larvae (E). The first control (Con 1) was sprayed with 5 ml of sterile water containing 0.1% (v/v) Tween-80. The second control (Con 2) was BioCane granule (Australia), used for controlling underground pests of sugarcane such as *Apogonia* sp. (Coleoptera: Scarabaeidae). *The same capital letters indicate no significant difference for the mean value (Tukey–Kramer test, F=4.68, p=0.01). Abbott (1925) was then used for correction.



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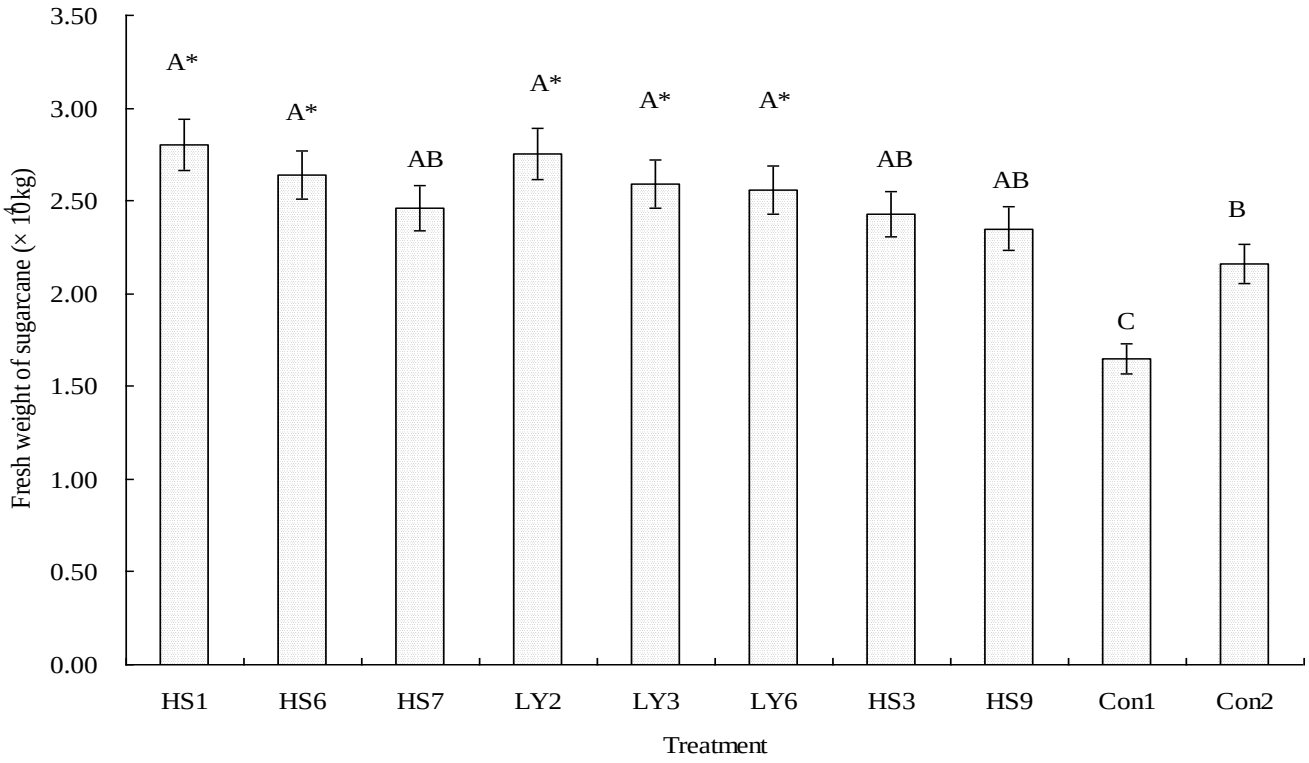

Figure 3. Comparing sugarcane fresh weight after harvest with different treatments. The first control (Con 1) was also brushed with only water with 43% (w/w) additives. The second control (Con 2) represented BioCane granule (Australia). *The same capital letters indicate no significant difference for the mean value (Tukey–Kramer test, F = 3.71, p = 0.01). Abbott (1925) was then used for correction.

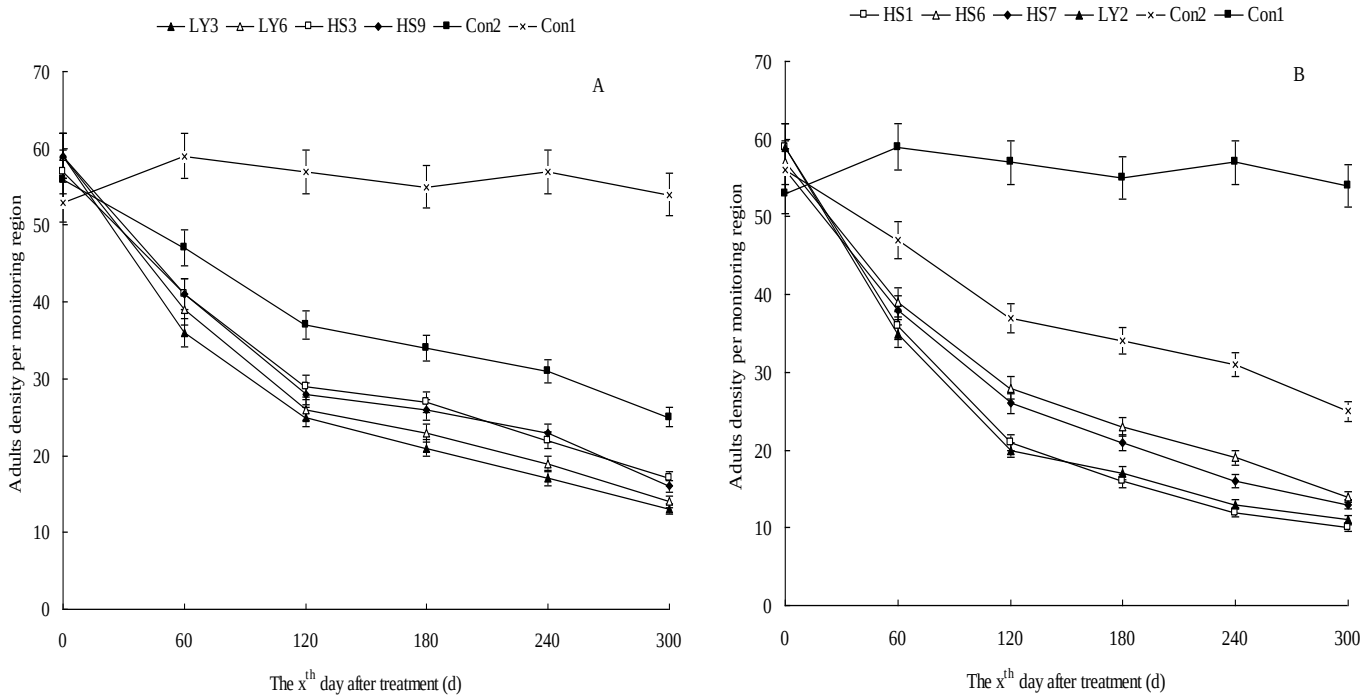

Figure 4. Comparing effects of *M. anisopliae* to the density of *C. venosatus* adults. By statistical analysis, the density of *C. venosatus* adults were closely related to the days after treatment. The first control (Con 1) was also brushed with only water with 43% (w/w) additives. The second control (Con 2) was BioCane granule (Australia).

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