海南椰心叶甲病原菌金龟子绿僵菌的分离、鉴定及其生防潜力

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摘要 椰心叶甲 [*Brontispa longissima* (Gestro)] 是椰子的重要害虫, 近年来, 该虫在海南岛发生普遍, 椰子受害严重。由于椰心叶甲受到自然界中某些致病微生物的侵袭, 在受害的椰子树心叶上常可发现椰心叶甲僵虫, 并发现大部分僵虫表面长出了霉菌, 本研究的目的在于从椰心叶甲僵虫表面的霉菌中分离出绿僵菌, 并对分离菌株进行鉴定和致病性测定。从僵虫表面刮下孢子或菌丝体, 置于绿僵菌选择性培养基 (DOA) 上培养, 挑出真菌菌落, 经纯化后, 进行生物学特性、菌落生长速率及产孢量的测定, 并从 PPDA、OMA、V8A 和 PDA 中筛选菌落生长及产孢最适培养基, 同时对所分离的菌株进行对椰心叶甲的致病性测定。结果表明, 所有分离菌株均鉴定为金龟子绿僵菌 [*Metarhizium anisopliae* (Metschnikoff)], PPDA 是菌落生长及产孢的最适培养基, 大多数菌株对椰心叶甲有较强的致病力。选取强毒菌株 MA4 在田间进行防治效果的初步测定, 结果表明, 该菌株能显著降低椰心叶甲成虫的虫口密度。这些金龟子绿僵菌菌株是首次从海南的椰心叶甲僵虫中分离到的昆虫病原真菌, 该菌对海南的椰心叶甲具有很好的生防潜能。

关键词 金龟子绿僵菌; 分离; 鉴定; 生物防治; 椰心叶甲

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Isolation and identification of *Metarhizium anisopliae* from natural infections of Coconut hispid beetle *Brontispa longissima* (Gestro) (Coleoptera: Chrysomelidae), and preliminary studies on against this pest in Hainan island, China

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Abstract: Coconut hispid beetles, *Brontispa longissima* (Gestro) (Coleoptera: Chrysomelidae) have been prevalent all over Hainan Island in China, and millions of coconut palms were attacked by this pest. The objective of this work was to isolate and identify strains of entomopathogenic fungi from natural infections of *B. longissima* collected from coconut palms. Conidia or mycelia collected from the surface of beetle cadavers were cultured and the colonies were screened on dodine oatmeal agar medium (DOA). The colony morphology, mycelia growth rate and conidial production were further studied on various agar media: PPDA, OMA, V8A and PDA. In order to confirm whether these isolates were pathogenic to *B. longissima*, their virulence to the beetles were also tested in laboratory. The results showed two different kinds of strains MA and MB were all identified as *Metarhizium anisopliae* (Metschnikoff) base on the macromorphological and micromorphological characteristics, and PPDA was the optimal culture medium for vegetative growth and conidial production. Several high virulent strains were screened *in vitro*, and application on controlling coconut hispid beetles was carried out preliminarily *in vivo*. The results showed a significant reduction in adult population was caused by the isolate. It was proved that *M. anisopliae* isolated from the natural infected cadavers of *B. longissima* have great potential as a means of biocontrol against this serious pest.

Key Words: *Metarhizium anisopliae*; isolation; identification; biological control; *Brontispa longissima*

Brontispa longissima (Gestro) was originally described from the Am Islands (Maluku Province) [1]. It was native to Indonesia, and also to Papua New Guinea, including the Bismarck Archipelago. It has now spread widely in Australia, Pacific Islands and Asia such as Singapore, Vietnam, Nauru, Thailand and Maldives [1].

From 2002, this beetle has spread to Hainan Island. Because of the absence of natural antagonists, it has become a serious and devastating pest on the island. At the beginning, it was prevalent in several important coconut-growing regions such as Haikou, Wenchang and Sanya. In recent years, it has broken out to cover 17 counties and cities, almost 2 million coconut palms were attacked and resulting in heavy losses. Thus, emergency operations were carried out assisted by local governments. It has been proved effectively by introduction and enhancement of parasitoids- *Tetrastichus brontispae*, *Asecodes hispinarum* and spraying of entomopathogenic fungi [1]. Chemical control was also recommended by using relatively safe pesticides to eliminate the pest before biological control.

The entomopathogenic fungus, *Metarhizium* has been proved effectively in controlling more than 200 species of insect pests [2]. However, there are no reports about *Metarhizium* strains isolated from other insect pests whether can be used to control *B. longissima* effectively in Hainan. The exploratory surveys for parasitoids in the original home of this pest and the isolation of highly virulent strains have been suggested. In this study, a number of host cadavers of *B. longissima* were found on the tree, and mycelia and conidia were collected from the cuticle surfaces. Fungi were isolated on the selective medium, and their identification, virulence to *B. longissima in vitro* and effects of application *in vivo* were further tested.

1 Materials and methods

1.1 Collection of natural infectious and healthy beetles

Coconut hispid beetles (*B. longissima*) were collected from seriously infected regions including Haikou and Wenchang of Hainan Island. Foliar buds of coconut palms infected with beetles were cut down and a number of larvae and adults were picked out. Cadavers were collected and transferred in sterilized Petri dishes, and the healthy ones were transferred into glass bottles for further rearing. Cadavers were used to isolate entomopathogenic fungi, and the living insects were used to perform pathogenic tests for isolated fungus.

1.2 Preparation of culture media

Four different media were prepared: PDA (potato dextrose agar), PPDA (PDA with 2% peptone), V8A

(0% V8 vegetable juice agar)[5], and OMA (oatmeal agar) medium. DOA medium was prepared as the selective medium by adding selective elements for screening entomopathogenic fungi.

The selective elements of DOA medium mainly contained Dodine 200μg/ml, Penicillin 100μg/ml, Streptomycin 50μg/ml [4], Carbendazim 0.8μg/ml and Metalaxyl 0.5μg/ml.

1.3 Isolation and identification of *Metarhizium anisopliae*

Cadavers of coconut hispid beetles including larvae and adults were collected from Wenchang and Haikou. Mycelia or conidia were isolated with sterile needles from cuticle of cadavers and transferred into a 1.5 ml Eppendorf tube containing sterile distilled water. Conidial suspension (5×10^4 conidia/ml) was prepared and 100μl of it were transferred and incubated on a Petri dish containing 15ml DOA medium at temperature of (28 ± 2)℃. Three days later, daily checks were performed up to the tenth day. Discs of the fungal colonies appearing on each Petri dish was then transferred to test tubes containing PPDA medium, and incubated under the same conditions described above.

Purification of every strain was carried out under an inverted Olympus microscope. A single conidium on the surface of water agar medium was picked up with a sterile capillary tube and transferred onto PPDA medium for obtaining pure culture. Identification of the isolates were performed at a species level according to the papers or books such as published by Pu zhelong [2], Hawksworth [3], Roddam *et al* [6], and Zimmerman [7]. The strains were identified by evaluation of the macromorphological characteristics of the colonies ,such as diameter, color, mycelia texture and other fearures, as well as the micromorphological characteristics, such as the aspects of hypha, conidiophore, conidial fructification and conidium, which were detected under biological microscope.

1.4 Tests on biological characters of isolated strains

1.4.1 Tests on colony growth rate and conidia production on different culture medium

All strains were cultured in Petri dishes containing PDA, PPDA, V8A and OMA medium. After five days of incubation, the daily colony diameter was measured by crisscross method up to the tenth day. The average of the increases in colony diameter per day was considered as the growth rate.

Conidia were harvested from 15 days old cultures by surface scraping. Homogenous spore suspensions were made by placing harvested spores in 20 ml sterile distilled water in glass bottles containing 0.1% Tween80, and then were agitated on a vortex for 30 Sec. Spore concentrations were determined using a haemocytometer. The procedure was replicated three times for each strain.

1.4.2 Tests on colony macromorphological aspects

All isolates were cultured on the optimal medium (PPDA) according to above results. After 4 days, daily checks of different colony color and mycelia texture were carried out and documented by photography. The sizes of 50 conidia of each strain were measured under a Leica microscope and average values were compared for all strains.

1.5 Tests on virulence to *B. longissima* and preliminary applying on adults *in vivo*

All isolates were cultured and maintained on PPDA medium. Conidia were harvested and its suspensions ($\times 10^8$ conidia ml^{-1}) were prepared. Twenty adults or larvae of *B. longissima* reared for 1 week were sprayed with 3 ml conidial suspension respectively and then placed into glass bottles and fed by fresh coconut leaf buds (replaced once every 3 days). The glass bottle was capped with a plastic lid with several tiny punctures to allow aeration for insects living and incubated at ambient temperatures of 25 – 30℃. Each isolate was repeated 3 times on adults or larvae. The control was only sprayed with 3 ml of sterile water containing 0.1% Tween-80. After rearing for 9 days, dead beetles was put down and mortalities were calculated for every strain. All dead beetles were transferred to Petri dishes in humid ambient to see whether have mycosis growth on the surface.

Preliminary effect tests of application on adults *in vivo* were carried out in Sanjiang farm of Haikou, where

coconut palms were infected seriously by *B. longissima*. The conidial suspension were prepared by mixing 80% plant oil and 20% paraffine, and adjusted to the conidia concentration of $\geqslant 1 \times 10^9$ conidia ml^{-1}. The suspensions (20ml per-tree) were brushed with brush on the surface of bud leaves of 10 coconut palms two times after an interval of 20 days. The trials were repricated three times in the same experiment area. Controls were also brushed only by 80% plant oil and 20% paraffine.

2 Results

2.1 Strains isolation

From a number of cadavers (Fig. 1), 21 filamentous fungal colonies were isolated on DOA medium from different Petri dish (Fig. 2). The isolate from different Petri dish was regarded as a strain, respectively. All of these strains were purified and transferred to PPDA medium for further identification. As a result, all isolates were identified as *Metarhizium anisopliae*, and no any other species such as *Beauveria* spp or others were detected.

According to the macromorphological characteristics, we found there were two distinct strains based on the differences in colony morphology and acervuli, and were thus recorded as MA (13 strains) and MB (8 strains) isolates. After 5 days of incubation, The colony of all MA strains on PPDA medium were almost entirely covered with acervuli but few acervuli in all MB strains under the same conditions (Fig. 3). However, there was no difference in conidial shape between the two kinds of isolates: cylindrical with obtuse ends, slightly narrowing in the center (Fig. 4), and the conidial width (2.5 – 3μm) and length (6 – 8μm) as well as the structure of conceptacles were no different from those described by Pu Zhelong.

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

The results showed PPDA was the optimal culture medium for colony growth and conidia production for both MA and MB strains. The results were summarized in Fig. 5 (only show testing results of MA4 and MB4, the others were as same as the two strains). Colony growth rate on PPDA media was faster than the others, especially during 1 to 3 days. Otherwise, there was no significantly difference in colony growth rate on other media. The average conidial production of MA4 (others were as the same as this strain) strain was 2.62×10^9 conidia ml^{-1} on PPDA media, which was significantly more than 2.68×10^8 conidia ml^{-1} on OMA, 1.96×10^8 conidia ml^{-1} on PDA and 1.85×10^8 conidia ml^{-1} on V8. However, the last three media showed no significant differences in conidial production (Fig. 6).

2.3 Pathogenicity to *B. longissima* and application effects *in vivo*

All strains both MA (13 strains) and MB (8 strains) were showed pathogenic to *B. longissima* according to the testing results. The mortality ranged from 52% to 100% either in adults or larvae, showing significantly higher than the control. Isolates of MA2, MA3, MA4,

Table 1　Pathogenicity of MA and MB strains against *B. longissima* on the fifth day after inoculation

Isolate	Mortality of adults* (%)	Isolate	Mortality of larvaeI* (%)
MA4	100 a	MA1	100 a
MA9	100 a	MA2	100 a
MB2	100 a	MA3	100 a
MB5	100 a	MA9	100 a
MB6	100 a	MB2	100 a
MB7	100 a	MB4	100 a
MA2	97 ab	MB6	100 a
MB4	97 ab	MB7	100 a
MA1	92 abc	MA4	98 ab
MA3	92 abc	MB5	98 ab
MA8	90 abcd	MA8	97 ab
MA6	78 bcde	MB3	95 ab
MA10	77 cde	MA10	88 abc
MA5	75 cde	MA6	85 abc
MB8	75 cde	MA11	85 abc
MA11	70 def	MB8	85 abc
MB3	70 def	MA12	80 abcd
MA12	67 ef	MA0	78 bcd
MA0	65 ef	MA7	72 cd
MA7	65 ef	MA5	70 cd
MB1	52 ef	MB1	62 d
CK	20 g	CK	30 e

* Means followed by the same small letters are not significantly difference ($P = 0.05$)

接彩图 1 – 4

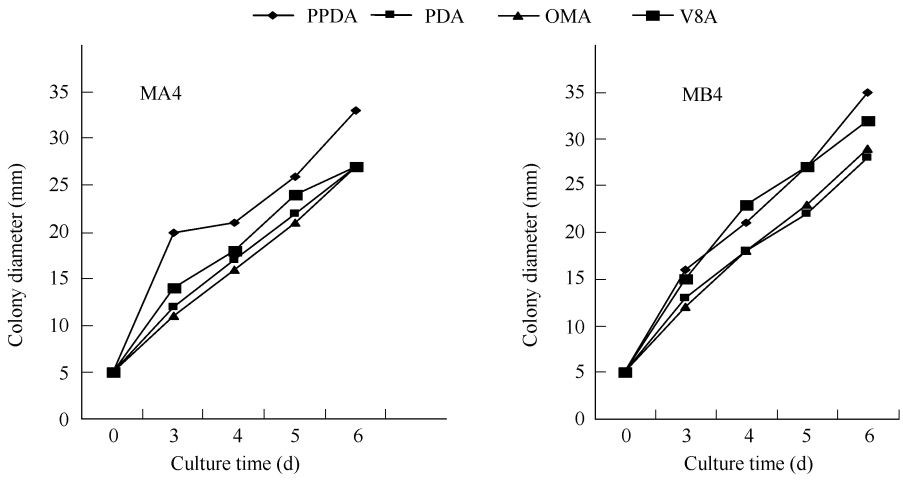

Fig. 5　Colony growth rate of MA4 and MB4 strains on different culture medium

MA8, MA9, MB2, MB4, MB5, MB6 and MB7, were high pathogenic to *B. longissima* (Table 1) among of all strains. In moist condition, conidia and mycelia were observed on the surface of cadavers and identified as the same fungi species used in the initial inoculation. The mortality of the control was only 20% and 30% in adults and larvae, respectively, and few mycelia growth were found on cadavers. The results showed that the strains isolated from natural occurring endomopathogenic fungi in *B. longissima* were pathogenic to *B. longissima* and most of strains were highly virulent to the beetles.

In this study, the high virulent strain of MA4 were employed to apply on controlling *B. longissima in vivo*. The averages of adult beetles population taken before application varied from 55.2 to 19.0 after 90 days and to 11.8 after 120 days, and cadavers with mycosis were found at more than 90% trees, while few changes occurred at controls, which the adult beetles population density taken varied from 45.2 to 48.0 and to 46.7 at the same time detection (Fig. 7), and few cadavers were found.

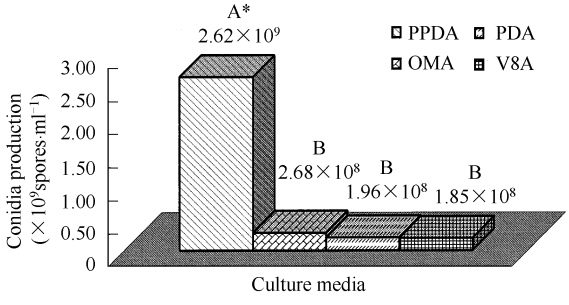

Fig. 6　Conidia production of MA4 strain on different culture media. * Means above the same capital letters are not significantly difference ($P=0.01$)

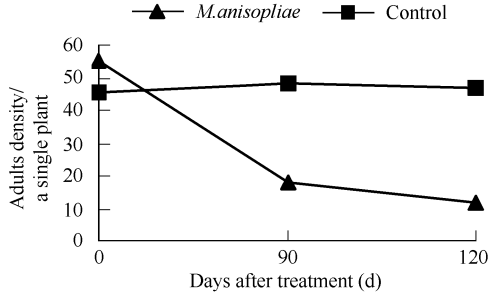

Fig. 7　Effect of *Metarhizium anisopliae application* on adults of *B. longissima* density

3　Discussion

From the cadavers of *B. longissima* collected from Haikou and Wenchang of Hainan, 21 filamentous fungi were isolated and identified as *Metarhizium anisopliae*. PPDA was the optimal culture medium for colony growth and conidia production among PPDA, PDA, OMA and V8A.

M. anisopliae has already been isolated naturally from soils [6,8,9] and insects [10~12] in different regions of the world. And *M. anisopliae* had ever been applied to against the coconut leaf hispid [3,14]. However, it was the first

time that strains of *M. anisopliae* were isolated from *B. longissima* in Hainan Island of China. Our studies proved that these isolates were pathogenic to *B. longissima* based on the symptoms after inoculation of the beetles with these isolates. In pathogenic tests, the mortality in treatments was significantly higher than the control. In addition, fungal outgrowth was found on the surface of dead beetles in humid condition, but not in the control. A high pathogenic strain of MA4 were employed to apply *in vivo*, with which caused significantly reduction in the adult population. The results showed the great potential of *M. anisopliae* as a means of biological against the coconut leaf hispid. Recently, the effective strategies on *B. longissima* control were performed in Hainan, involved rearing of natural antagonists of *T. brontispae*, *A. hispinarum* and spraying insecticides. As an important part of integrated pest management (IPM) strategy, *M. anisopliae* could be widely used due to few harmful side effects caused by it. *M. anisopliae* can be considered as promising for biocontrol on coconut hispid beetles by reducing the pest population and lessening the dependence on chemical control.

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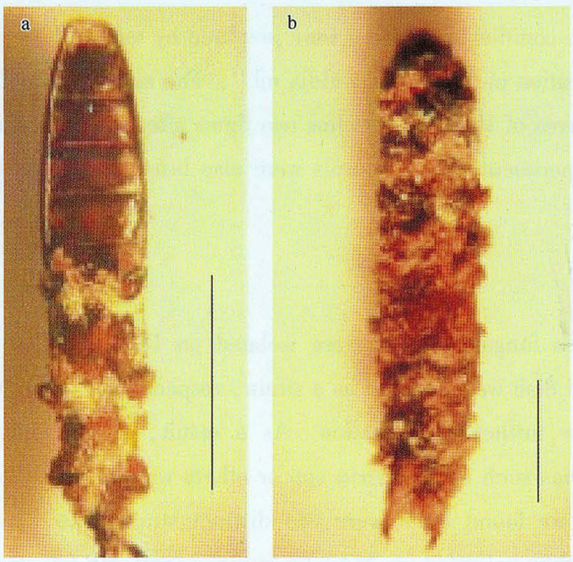

Fig. 1 Cadavers of *B.longissima* adult and larva infected by fungi. a, scale bar:3mm; b, scale bar:2mm

Fig. 2 Colony outgrowth in the selective DOA media on 90mm Petri dish

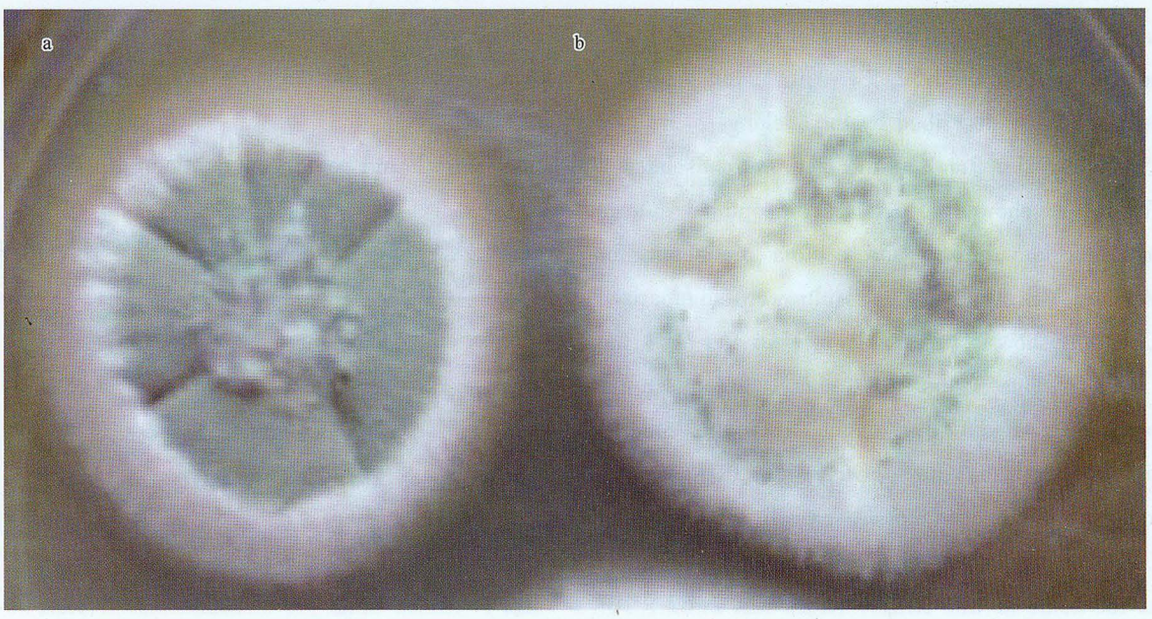

Fig. 3 Colony aspects of MA(a) and MB(b) strains of *Metarhizium* spp. after cultured 5 days in PPDA culture media on 90mm Petri dish

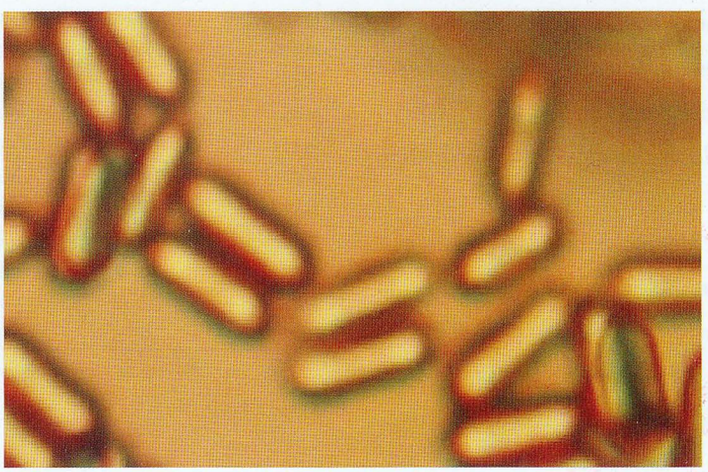

Fig.4 Spores aspects of *Metarhizium* spp. under Objective lens 40X