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The isolation and identification of pathogenic fungus from the diseased *Tessaratoma papillosa* Drury (Hemiptera: Tessaratomidae) and their pathogenicity

Xiang Meng Correspond., 1, Junjie Hu 2, Gecheng Ouyang 1

1 Guangdong Institute of Applied Biological Resources, Guangzhou, Guangdong, China

2 College of Life Science, Guangzhou University, Guangzhou, Guangdong, China

Corresponding Author: Xiang Meng
Email address: mengxiangxs@126.com

Background. Litchi stink-bug, *Tessaratoma papillosa* Drury (Hemiptera: Tessaratomidae) is a major pest on litchi and longan in Southern China. It is urgent to develop valid biological agent for control the pest and improve IPM strategy on orchard farming. Entomopathogenic fungi was regarded as avital ecological factor pressing pest populations in field, however, there were fewer searches conducted on entomopathogenic fungi against litchi stink-bug. Methods & Results. In this study, two pathogenic fungus were isolated from the adult diseased *T. papillosa* by normal methods and rDNA-ITS homogeneous analysis, they are identified as *Paecilomyces lilacinus* and *Beauveria bassiana*. Laboratory tests showed that the two entomopathogenic fungi both had a good lethal effect to young nymph and old nymph of *T. papillosa*. The toxicity determination showed that the LC50 value for *Beauveria bassiana* was higher than *P. lilacinus*. Conclusion. These pathogenic fungus did not have the risk of pollution or residue, and they can be a alternative option for integrated pest management approache.
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Xiang Meng 1, Junjie Hu 2 and Gecheng Ouyang 1

1 Guangdong Key Laboratory of Animal Conservation and Resource Utilization, Guangdong Public Laboratory of Wild Animal Conservation and Utilization, Guangdong Institute of Applied Biological Resources, Guangzhou, Guangdong Province, China.

2 College of Life Science, Guangzhou University, Guangzhou, Guangdong Province, China.

**Correspondence**

Gecheng Ouyang 1

Email addresses: 18922369378@189.cn; mengxiangxs@126.com
Abstract:

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Conclusion. These pathogenic fungus did not have the risk of pollution or residue, and they can be a alternative option for integrated pest management approache.

Keywords: *Tessaratoma papillosa* Drury; entomopathogenic fungi; bioactivity; biological control
Introduction

Litchi stink-bug, *Tessaratoma papillosa* Drury (Hemiptera: Tessaratomidae) is a major pest on litchi and longan in South East Asia and Southern China. The nymphs and adults feed on the tender branchlets, spica and young fruit juice for almost a year, which may be slow growth, wilt, die, and even cause flowers and fruits falling (Waite & Hwang 2002). They had seriously affected the production of litchi and longan. When this insect is disturbed, it may release large quantities of disagreeable acid fluid from its scent gland (Zhao *et al*., 2012) which causes young leaves wilting and induces brown spots on old leaves and fruits, reducing their edible value and economical value (Bootam & Leksawasdi 1994). Furthermore, it can cause the human eye or skin hurt and inflamed directly (Liu 1965), and also indirectly spread plant viruses, such as witches’ broom disease (Chen *et al* 2001). It is not only sucking the gramineous plants, but also the virus between crops Chemical pesticide was used in the traditional *T. papillosa* control ways. However, A huge variety of pesticides not only did not kill the pests, but also resulted in fruit pesticide residues and pest resistance. As a further consequence, It had seriously impact on human health and safety. How to improve the *T. papillosa* harmless control has become urgent problems of litchi green fruit production (Xu 2005).

The egg parasitoid Anastatus spp is mainly used in biological control method of *T. papillosa* at present (Zou 2008), But there are few reports about other biological control. As an alternative to chemical control or as part of IPM programs, there are many available microbial
insecticides resources to utilize for biological control of insect pests. Being a kind of pathogens of agricultural pests, entomopathogenic fungi is a key factor to control insect population density in ecosystem. Many entomopathogenic fungi, such as *Metarhizium anisopliae*, *Beauveria bassiana*, *Paecilomyces fumosoroseus*, *Verticillium lecanii*, *Aschersonia montagne* has been developed for the fungus pesticide products, and applied in large biological control of important pests in agriculture and forest. However there are few reports about entomopathogenic fungi of litchi pests control. In this study, we isolated two pathogenic fungus from the adult diseased *T. papillosa* by normal methods and rDNA-ITS, and investigate its bioactivities to *T. papillosa*. We expect to have a certain application valuable bio-control strain of *T. papillosa* control.

**Materials and methods**

**Test strain**

The strains were isolated from the adult of *T. papillosa* in litchi orchard, located in Huangwei village, Conghua city, Guangdong Province, China. Good growth and spore production traits of strains were selected as test strains. And they were purified and routine identified according to methods based on the phenotypic properties of the pathogenic fungus morphology (Pu *et al* 1996, Liang *et al* 2005, Luangsa-ard *et al* 2005).

**Sequencing and phylogenetic analysis of strain ITS**

Total DNA was extracted according to the manufacturer’s specifications (Omega, E.Z.N.A. Fungal DNA Kit). The DNA specimens were amplified with universal primers ITS1: 5’-TCCGTAGGTGAACCTGCGC-3’, ITS4: 5’-TCCTCCGCTTATTGATATGC-3’and ITS5: 5’-
GGAAGTAAAAGTCGTAACAAGG-3’. For the PCR, PCRs were conducted in 25 μL volume containing 0.5 μL Taq DNA polymerase, 2 μL dNTPs, 2.5 μL 10× buffer, 1 μL of each primer, 1 μL of total DNA, 17 μL ddH₂O (TaKaRa, Japan). Amplification was performed in an Eppendorf Mastercycler ep (Eppendorf, Hamburg, Germany), with PCR-negative controls containing ddH₂O instead of template DNA. The amplification profile was as follows: one denaturation step at 94°C for 5 min, then 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min; followed by a final extension at 72°C for 5 min. To check the results, 5 μL of PCR products were run in a 1.0% agarose gel stained with GoldView in 0.5× TBE buffer [45 mmol/L Tris-Cl, 1 mmol/L EDTA] (Sangon, China), and the amplification products were analyzed by comparison with a DNA size marker (TaKaRa, Japan) run in parallel on the same gel. This band was excised from the agarose gel, purified using a DNA gel extraction kit (Omega, USA), cloned into the pMD18-T vector (Takara, Japan), and sequenced completely in both directions (Invitrogen, Guangzhou, China).

The sequencing results were analyzed by “BLAST” tools on the National Center for Biotechnology Information (NCBI) website. Then the strains with high homology were contrasted with the test strain. The multiple sequence alignments of deduced amino acid sequences were performed using ClustalW (Thompson et al 2002) multiple-alignment software (http://www.ebi.ac.uk/clustalw/index.html). The phylogenetic tree was constructed using the MEGA 4 software (Tamura et al 2007).

Pathogenicity test of strain

Preliminary screening of the test strains pathogenicity in T. apapillosa
The purified strains were inoculated and cultivated on petri dish plates for 5 days, the received conidium were used for bioactivity assay in young nymph, old nymph and adult of *T. apapillosa* respectively. Put these insects into $1 \times 10^8$ conidium/mL spore suspension of the selected strains for 10s respectively, then took them out and dried them, introduced them inside a petri dish (17 cm in diameter and 3.0 cm high). 10 insects per arena, provided with water supplied on soaked cotton, The petri-dishes were then sealed with a plastic wrap to prevent insects from escaping and placed in a climatic chamber at 26 ± 1°C, 14: 10 (L: D) photoperiod, and 60–80% RH. The negative controls consisted of arenas with the same densities of *T. apapillosa* but without any test strains treatment. Each assay was replicated three times for each petri dish insects’ density. We observe infection situation at regular intervals every day, and the dead *T. apapillosa* were selected and single cultivated. The dead *T. apapillosa* caused by the two tested strains were calculated the cumulative mortality respectively, if the culture of entomopathogenic fungi was agrees with the test strains (Hu et al. 2007). The corrected mortality was calculated according to the formula of Abbott.

Corrected mortality (%) = [(Mortality of treatment group - Mortality of control group) ×100/ (100- Mortality of control group)]

Toxicity determination of the test strains

The high virulent strains from preliminary screening test were used for further toxicity determination of young nymph, old nymph and adult *T. apapillosa* respectively. The conidial suspension of test strains were diluted into 5 concentration gradient respectively by sterile water ($1 \times 10^8, 5 \times 10^7, 2.5 \times 10^7, 1.25 \times 10^7, 6.25 \times 10^6$ conidium/mL), and with sterile water as
blank control. 10 insects were treated for each concentration, and each assay was replicated three times. Corrected mortality were calculated, and the LC\textsubscript{50} was determined by DPS9.50.

**Results**

*The characteristic of infected T. apapillosa and the results of routine identification*

We separated and purified two strains (Ta-01, Ta-02) from the adult of *T. apapillosa* in litchi orchard (Fig. 1). The artificial infection test in adult of *T. apapillosa* showed that both strains can cause the infected insects became moved slowly and a slight spasm phenomenon after 48~72 hrs. Ta-01 strain infected *T. apapillosa* can cause some white hypha grown from antenna and intersegmental membranes of dead *T. apapillosa* after they had cultured 7 days. And Ta-02 strain can cause infected *T. apapillosa* stopped their activity and clung tightly to branch of litchi until they became stiffness, died and grown gray mycelium and conidium in metamere and intersegmental membranes of dead *T. apapillosa* after 7 days or longer. The performances in routine identification (Fang *et al* 2001, Li *et al* 2004, Lu *et al* 2007) show that the two strains are *Paecilomyces* spp and *Beauveria* spp according to reference, respectively.

*Sequencing of ITS and Phylogenetic analysis*

The results of rDNA-ITS sequencing of two strains showed that 559 bp and 451 bp of special DNA fragments was sequenced (Fig. 2). The yielded sequence was compared with the sequences of 18SrDNA accessed in GenBank by BLAST (Fig. 3). Phylogenetic analysis indicated the obtained sequence Ta-01 shares high homology with *Paecilomyces lilacinus* (99%), they formed a cluster; and the obtained sequence Ta-02 formed a cluster with *Beauveria bassiana*, the homologies was 99%. Together with the routine identification and molecular
identification, Ta-01 and Ta-02 was proved to be *P. lilacinus* and *B. bassiana*.

**Pathogenicity analysis of strain**

In this study, *P. lilacinus* and *B. bassiana* were isolated and pathogenicity analysed. The bioassay results showed that, the two strains both had good pathogenicity in *T. apapillosa*. And the higher pathogenicity strain is *B. bassiana*, the corrected mortality rate of infected young nymph, old nymph and adult *T. apapillosa* within 10d were 88.89±11.11%, 75.56±7.70%, 65.56±5.09% respectively, and they had a greater difference between infected young nymph and adult *T. apapillosa* by *B. bassiana* (P>0.05). However, the corrected mortality of infected young nymph, old nymph and adult *T. apapillosa* by *P. lilacinus* were 78.15±11.67 %, 68.89±10.18%, 63.33±5.77%, and they were no significant difference (P>0.05) (Table 1). The toxicity determination of the two strains showed the LC$_{50}$ value for *B. bassiana* infected young nymph and old nymph of *T. apapillosa* within 10 d were 1.43×10$^7$ and 1.36×10$^7$, whereas they were higher than *P. lilacinus* infected different of *T. apapillosa* (young nymph: 1.92×10$^7$; high-instar nymph: 1.95×10$^7$; adult: 2.54×10$^7$ (Table 2).

**Discussion**

Research biological control technology of major pest of litchi tree is a basic safeguard to produce pollution-free litchi. *T. apapillosa* Drury is a major pest on litchi and longan in Southern China. Previous studies pay more attention on utilizing part of natural resources for its control. Especially using the parasitic wasp for controlling *T. apapillosa* and other important litchi pests has made substantial progress (Liu et al 1995, Han et al 1999, Chi et al 2006). The azadirachtin tree injection should also be tested against *T. apapillosa* and other pests of litchi (Marie Joy
Entomopathogenic fungi was regarded as a vital ecological factor pressing pest populations in field, however, there were few researches conducted on entomopathogenic fungi against *T. apapillosa*. In our studies, we identified primarily entomopathogenic fungi which we sampled from adult diseased *T. papillosa* in litchi orchard to be *B. bassiana* and *P. lilacinus*. and we confirmed that they also play an important role in the control of *T. apapillosa*.

At present, the approaches of fungal systematics in addition to the traditional morphological identification, the determination of nucleic acid sequence is also needed. One of effective classification identification methods is rDNA-ITS sequencing by PCR. Ta-01 formed a cluster with *Paecilomyces* spp in NCBI database, and shares 99% high homology with *P. lilacinus* (KF766523.1); Ta-02 displayed more similar morphological characters in color and size of *Beauveria* spp, and showed a close relationship supported by a 99% bootstrap value with *B. bassiana* (JQ991615.1).

This study observed that the *T. apapillosa* infected with *B. bassiana* and *P. lilacinus* were initially infected from antenna, metamere and inter-segmental membranes. The results of our pathogenicity analysis of *B. bassiana* and *P. lilacinus* showed that the two strains both had good pathogenicity in *T. apapillosa*, and have a significantly different. The higher pathogenicity strain is *B. bassiana*, and there are differences mortality rate and death speed with different age of *T. apapillosa*. The toxicity determination of the two strains showed the LC50 value for *B. bassiana* was higher than *P. lilacinus*. These strains showed the potential to control *T. apapillosa*. They can be developed into commercial preparations for *T. apapillosa* biological control in litchi orchards when they are trained to have good conidial production levels in fermenter.
Entomopathogenic fungi are one of vital microorganisms to control pests and have potentials in biological control. They have the advantages of non-pollution, safety, without pesticide resistance, and they have a longer controlling duration during the host reproductive dispersal in pest populations and epidemic caused. However, in the long-term use process of biopesticide, entomopathogenic fungi can result in biological control effect present a slowly insecticidal rate and a unstable phenomenon because of strain degradation, variation, rejuvenation and contamination. The culture and store of \textit{B. bassiana} and \textit{P. lilacinus}; the correlation of \textit{B. bassiana} and \textit{P. lilacinus} virulence with different age of \textit{T. apapillosa}, ambient temperature and humidity, and other factors; and the field application and control effect of \textit{B. bassiana} and \textit{P. lilacinus} for \textit{T. apapillosa} control remains to be further research.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

• Xiang Meng conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures and tables.

• Xiang Meng and Junjie Hu performed the experiments, analyzed the data, contributed reagents/materials/analysis tools.

• Gecheng Ouyang conceived and designed the experiments, wrote the paper, reviewed drafts of the paper.

Data Availability

The raw data has been supplied as Supplemental Dataset files.
Supplemental Information

Supplemental information for this article can be found online at

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Table 1  Virulence of the two strains against the different stages of *T. apapillosa* 10 d after treatment (Mean±SD)

<table>
<thead>
<tr>
<th>Species</th>
<th>Insect stages</th>
<th>Sample(N)</th>
<th>Corrected mortality(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Paecilomyces lilacinus</em></td>
<td>young nymph</td>
<td>10</td>
<td>78.15±11.67 ab</td>
</tr>
<tr>
<td></td>
<td>old nymph</td>
<td>10</td>
<td>68.89±10.18 b</td>
</tr>
<tr>
<td></td>
<td>adult</td>
<td>10</td>
<td>63.33±5.77 b</td>
</tr>
<tr>
<td><em>Beauveria bassiana</em></td>
<td>young nymph</td>
<td>10</td>
<td>88.89±11.11 a</td>
</tr>
</tbody>
</table>
old nymph 10 75.56±7.70 ab

adult 10 65.56±5.09 b

Note: Different small letters following the data within a column indicate significant difference at 0.05 level.

Table 2  Equations of LC₅₀ values of the two strains against the different stages of *T. apapillosa* 10 d

<table>
<thead>
<tr>
<th>Species</th>
<th>Insect stages</th>
<th>Toxicity regression equation</th>
<th>Correlation coefficient</th>
<th>Conidium /mL</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Paecilomyces lilacinus</em></td>
<td>young nymph</td>
<td>Y=3.4458+1.2116x</td>
<td>0.9825</td>
<td>1.92×10⁷</td>
</tr>
<tr>
<td></td>
<td>old nymph</td>
<td>Y=3.8768+0.8712x</td>
<td>0.9494</td>
<td>1.95×10⁷</td>
</tr>
<tr>
<td></td>
<td>adult</td>
<td>Y=3.9036+0.7801x</td>
<td>0.9398</td>
<td>2.54×10⁷</td>
</tr>
<tr>
<td><em>Beauveria bassiana</em></td>
<td>young nymph</td>
<td>Y=3.4326+1.3552x</td>
<td>0.9679</td>
<td>1.43×10⁷</td>
</tr>
<tr>
<td></td>
<td>old nymph</td>
<td>Y=3.9315+0.9437x</td>
<td>0.9725</td>
<td>1.36×10⁷</td>
</tr>
<tr>
<td></td>
<td>adult</td>
<td>Y=4.0263+0.7394x</td>
<td>0.9532</td>
<td>2.07×10⁷</td>
</tr>
</tbody>
</table>

Figure 1. Two pathogenic fungus from the diseased adult of *T. apapillosa* in litchi orchard.

A: A fungus isolate Ta-01; B: A fungus isolate Ta-02. Series 1: Adult of *T. apapillosa* invaded by fungi; Series 2: Colony of the strain on PDA; Series 3: Conidioma shape and conidia.
**Figure 2.** The amplification product of rDNA-ITS gene of strain Ta-01 and Ta-02. M: DNA marker; 1: Ta-01; 2: Ta-02.

**Figure 3.** Phylogenetic based on the 28SrDNA sequence of strain Ta-01 and Ta-02 strains. MEGA 4 was used to construct the phylogenetic tree. Bootstrap analyses from 1000 replications are shown by each branch.