



### **Full Length Article**

## **Exploring the Potential of a Strain of Entomopathogenic *Pestalotiopsis* spp. in Controlling *Aphrophora flavipes***

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### **Abstract**

*Aphrophora flavipes* is a main pest of *Pinus densiflora*, *P. tabuliformis* and *Larix* spp. The use of insecticide is still the most widely practiced and important management method in the control of *A. flavipes*. A promising alternative control method is the use of entomopathogenic fungi. In this study, normal tissue separation methods were used to isolate a new entomopathogenic fungal strain, named as SMC01 from the naturally infected *A. flavipes* collected from QinHuangdao City, Hebei Province, China. Based on morphological characteristics and ITS sequence analysis, SMC01 was identified as *Pestalotiopsis* spp. To determine its pathogenicity to *A. flavipes*, conidial suspension was sprayed on branches of *P. tabuliformis* infected with *A. flavipes*. To determine the potential harm to *P. tabuliformis*, the same spore suspension was sprayed on the healthy branches of *P. tabuliformis* and the growth was observed and recorded continuously. The results showed that SMC01 had a high toxicity to *A. flavipes*, 7 days after treatment, the adjusted mortality was 84%, which was significantly higher than that of control. There was no pathogenicity to *P. tabuliformis*. All the above results revealed the strain SMC01 is a potential candidate for controlling *A. flavipes*. © 2019 Friends Science Publishers

**Keywords:** *Aphrophora flavipes*; *Pestalotiopsis* spp; Isolation; Identification; Control

### **Introduction**

*Aphrophora flavipes*, known as pine spittlebug, mainly damages *Pinus densiflora*, *P. tabuliformis* and *Larix* spp. It sucks the sap of shoots' base part in nymph stage, at the same time, ejects white foam to shadow the body. The growth and development of current shoots are influenced because of the harmed shoots, more seriously, shoots are likely to bend and droop, even wither. The adults supplement the nutrients by sucking the shoot's sap. Besides the direct damage, as an important vector, *A. flavipes* often spread plant diseases which can bring devastating disasters (Fang *et al.*, 2016; Song *et al.*, 2016).

To date, insecticide use is still the most widely practiced and important management method in the control of *A. flavipes*. But it is well-known that insecticides have some disadvantages such as causing the pollution of the environment, killing a large number of natural enemies, inducing the resistance of insect to insecticide. Finding alternatives to chemical pesticides has always been a hot topic in related fields. A promising alternative for pest control is using entomopathogenic fungi as biological insecticides (Khosravi *et al.*, 2014; Pedrini, 2018). About 750 to 800 entomopathogenic fungi species of more than

100 genera are pathogenic to insects worldwide, and new taxa are constantly being discovered (Lv *et al.*, 2014; Butt *et al.*, 2016). There have been extensive researches on numerous species of entomopathogenic fungi used as microbial agents to control insect pests in agriculture and forestry. But to our knowledge, there are no published studies on using entomopathogenic fungi as biological control of *A. flavipes*. In 2015, *A. flavipes* nymph presumed to be infected with entomopathogenic fungi were collected in pine forest of Lianfengshan garden, Qinhuangdao city, China. In this study, we, for the first time, isolated and cultured entomopathogenic fungi from naturally dead *A. flavipes*, as well as performed morphological taxonomic and molecular systematic identification. Its pathogenicity to *P. tabuliformis* and potential use for the biocontrol of *A. flavipes* were investigated. The results can provide the theoretical basis for the biocontrol of *A. flavipes*.

### **Materials and Methods**

#### **Material**

The cadavers of *A. flavipes* used to isolate entomopathogenic fungi were collected from Lianfengshan garden, Qinhuangdao city, China in November, 2015. The

*A. flavipes* which harmed *P. tabuliformis* in the Lianfengshan garden were used to test the virulence of *Pestalotiopsis* sp. We selected 1-year-old branches 30 cm in length with about 100 *A. flavipes* to do the test. The healthy *P. tabuliformis* used to test the potential virulence of *Pestalotiopsis* spp. were also selected from Lianfengshan garden. We selected 1-year-old branches 30 cm in length to do the test.

### Isolation and Culture of Entomopathogenic Fungi

The cadavers of *A. flavipes* were placed in paper bags. Isolation of entomopathogenic fungi from cadavers was carried out by the methods outlined by Li Huiping (Li *et al.*, 2011). The cadavers were surface sterilized with 1% sodium hypochlorite for 1 min and washed with sterile water twice. After the remaining water was absorbed with sterile filter paper, the cadavers were transferred to PDA plates and incubated at 25°C.

A single conidium culture technique was performed to obtain pure colonies of the fungi following the method outlined by Aboul-Nasr and Abdul-Rahman (2014). The pure colony was grown on PDA to mature and identified based on colony and conidial characters.

### Morphological Identification

The colony morphology was observed and recorded every day. Thirty conidia were chosen randomly to measure the size of conidium and the three median pigmented cells, number and length of apical appendages and basal appendages. At the same time, the color and shape of conidia and the characters of apical appendages were also described. The taxonomic identification based on the morphology of fungi was performed according to Ge *et al.* (2009).

### DNA Extraction, Polymerase Chain Reaction (PCR) Amplification and Sequencing of the ITS

The strain SMC01 was grown on PDA for 7 days at 25°C. The mycelia were collected and DNA was extracted following a protocol outlined by Li *et al.* (2013).

Polymerase chain reaction (PCR) amplification products were obtained using pairs of primers, universal primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'). PCR was performed with the 50 µL reaction system consisting of 33.1 µL of double distilled water, 5 µL of 10× Taq buffer, 2.4 µL MgCl<sub>2</sub> (25 mM), 1 µL of dNTP (10 mM each), 3 µL of each primer (10 µM), 0.5 µL Taq DNA polymerase (5 U/µL), 2.0 µL of DNA template. The thermal cycling program was run on an Eppendorf Mastercycler using the following conditions: an initial denaturation of 95°C for 3 min, followed by 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and extension

of 72°C for 2 min. A final extension at 72°C for 2 min was added at the end of the thermal cycling. Amplified products were visualized in 1.5% agarose gel by electrophoresis.

The PCR products were purified, cloned and finally sequenced by Sangon Biotech (Shanghai) Co., Ltd. The sequence was blasted against the Genbank nucleotide sequence database (<http://blast.ncbi.nlm.nih.gov/>) and the most similar sequences were selected to compare with reference sequences from the Genbank using ClustalX (2.0) software. Fungal species were determined by the highest similarity of ITS sequences to known strains. Phylogenetic tree of *Pestalotiopsis* spp. was established using software MEGA 4.

### Pathogenicity of Strain SMC01 to *A. flavipes*

The toxicity of isolate SMC01 was examined in Lianfengshan garden in May 2016. Water containing 0.05% Tween-80 was used as control. Conidia were harvested from 10 days old surface cultures directly by scraping and washing. Spore suspensions were made by suspending conidia in 20 mL sterile distilled water in little beaker containing 0.05% Tween-80. Beakers were agitated on a vibrant shaker for 5 min to produce a homogenous conidial suspension. The spore concentration was then adjusted to 2.37×10<sup>8</sup>/mL using Neubauer hemocytometer. Viability of the conidia was checked by a germination test prior to the experiment and assured to be > 90%.

The conidial suspension then was sprayed with Taishan ft-796 sprayer on four branches in different directions of *P. tabuliformis* infected with *A. flavipes*. 10 trees were used for replicates. Seven days after treatment, the dead and live *A. flavipes* in each tree were counted to calculate the mortality of *A. flavipes*. The mortality and adjusted mortality were calculated as follows:

$$\text{Mortality} = \frac{\text{Dead}}{\text{Total body counts}} \times 100\%$$

$$\text{Adjusted mortality} = \frac{\text{Mortality in treatment plot} - \text{Mortality in control plot}}{1 - \text{Mortality in control plot}} \times 100\%$$

Dead insects were surface sterilized and transferred into a Petri dish lined with moistened filter paper. The mortality due to fungi was confirmed by microscopic examination of hyphae and spores on the surface of the dead insects.

### Pathogenicity of Strain SMC01 to *P. tabuliformis*

Pathogenicity of strain SMC01 to *P. tabuliformis* was performed in Lianfengshan garden from June to July in 2016. 1-year-old needles were wounded by wiping slightly with gauze paper. Spore suspension of 2.37×10<sup>8</sup>/mL was sprayed on the healthy branches and wrapped by a thin plastic bag to keep moisture. 30 branches were used for treatment. In addition, 30 branches were sprayed with water as controls. After 24 h, the plastic bag was

removed. Every day we observed and recorded the symptoms of the treated branches.

## Results

### Morphological Taxonomy of isolate SMC01

From 35 cadavers of *A. flavipes*, 2 entomopathogenic fungi were isolated, one of which was identified as *Beauveria bassiana* with isolation rate of 28.6%, another as SMC01 with isolation rate of 51.4%.

The strain SMC01 was cultured on PDA in incubator of 25°C. On PDA plates, isolate SMC01 had white, fluffy and rotative colony which covered all petri dish in one week. Later, the isolate produced honey orange color into the medium and made the back of the Petri dish light orange (Fig. 1). 6 days later, the isolate developed sparse, ink-like fruiting bodies containing conidia on the colony surface (Fig. 2).

Pendant-drop method was used to observe the conidial morphology. The strain SMC01 had five-celled conidia, of which apical and basal cells were hyaline and triangle, and the three median cells were dark ranged from light brown to dark brown, among which the first one is a little light, the third one the most light, and the median one was dark. Mean length of the conidia varied from  $(17.1 \pm 0.2)$  to  $(26.2 \pm 0.3)$   $\mu\text{m}$  and mean width varied from  $(7.5 \pm 0.1)$  to  $(9.7 \pm 0.1)$   $\mu\text{m}$ . There was only one appendage attached on the basal cell whose mean length varied from  $(1.2 \pm 0.2)$  to  $(7.0 \pm 0.1)$   $\mu\text{m}$ . Numbers of apical appendages ranged from two to three and the mean length was  $(2.5 \pm 0.5)$  to  $(27 \pm 0.6)$   $\mu\text{m}$ . The germ tubes came out at the second cell when the conidia germinated (Fig. 3). Based on the morphology of colony and conidia, isolate SMC01 was tentatively identified as *Pestalotiopsis* spp.

### Taxonomic Identification of Isolate SMC01 by ITS Sequencing

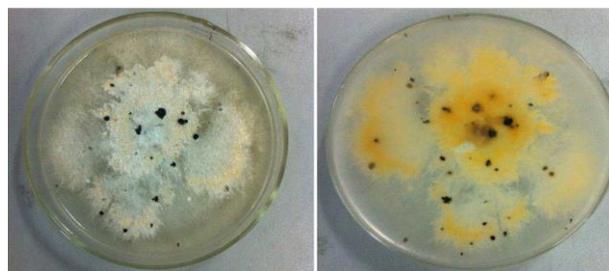
The morphological identification of SMC01 was checked by nucleotide blast analysis of the 5.8S subunit and flanking ITS4 and ITS5 of rRNA regions. The single amplicon of 700 bp was obtained. The nucleotide sequence was compared to other sequences deposited in GenBank, and it showed 98–100% identity to known fungi in the GenBank. Therefore, the result of ITS analysis supported SMC01 as one strain of *Pestalotiopsis* sp. The phylogenetic tree was established by comparing sequences of SMC01 with that of known *Pestalotiopsis* spp. in the GenBank. According to the phylogenetic tree, we found that the most similar sequences were almost on *Pestalotiopsis* spp. But it was not on the same branch with the other species (Fig. 4). This result was in accordance with the classification based on morphological characteristics. But the further bioassay is needed to determine the specific species.

**Table 1:** Mortalities of *A. flavipes* larvae treated with strain SMC01

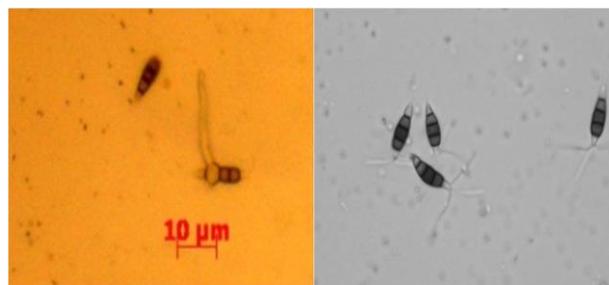
Treatment	Total number of <i>A. flavipes</i>	Mortality/%	Ajusted mortality/%
SMC01	103	85.7	84.7
Control	87	6.6	



**Fig. 1:** The colony characteristics of strain SMC01 on PDA



**Fig. 2:** The sorus characteristics of strain SMC01 on PDA



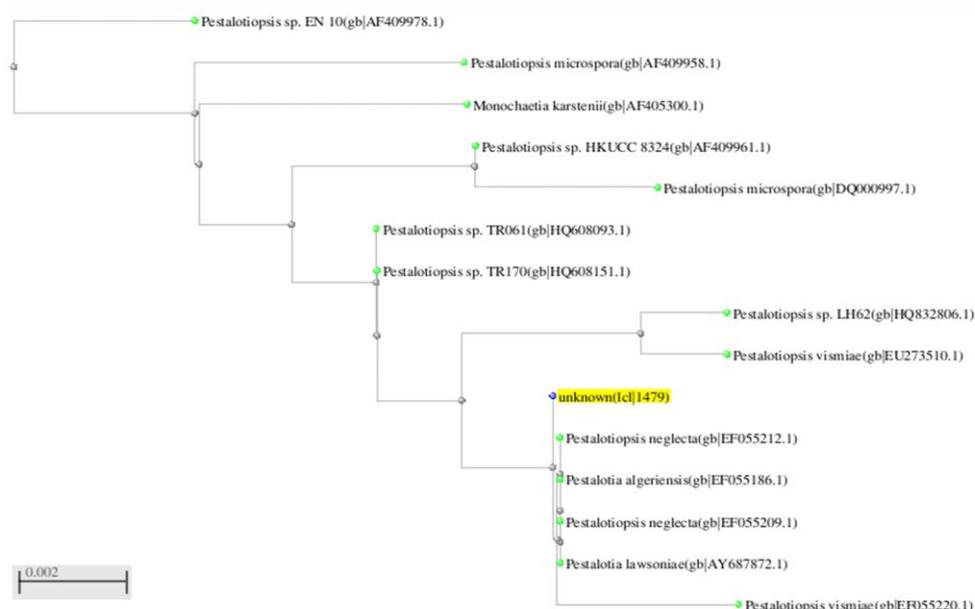
**Fig. 3:** The conidiospore characteristics of strain SMC01

### Pathogenicity of Strain SMC01 to *A. flavipes*

Seven days after the suspension was sprayed uniformly on the branches of *P. tabuliformis* with strain SMC01, the mortality of *A. flavipes* was calculated. As shown in Table 1, the adjusted mortality of *A. flavipes* was 84.7%. Compared with the natural mortality of 6.6%, SMC01 showed remarkable protection of *P. tabuliformis* by killing *A. flavipes*.

### Pathogenicity of Strain SMC01 to *P. tabuliformis*

The changes of pine branches were observed every day after treatment. And 20 days after spraying the spore suspension



**Fig. 4:** The phylogenetic tree of *Pestalotiopsis* and its related genera based on ITS sequences

on the pine branches, no disease symptoms were observed, which demonstrated that the strain SMC01 had no pathogenicity to *P. tabuliformis*.

## Discussion

*A. flavipes* was an important pest not only because of the direct damage to trees, but also the role of transmitting diseases. So far, entomopathogenic fungi infected *A. flavipes* has not been found and reported.

In this study, an entomogenous fungus was isolated from *A. flavipes*. The strain was identified as *Pestalotiopsis* sp. by morphological and ITS sequence-based molecular methods. By the pathogenicity tests, we also found that the strain had a high virulence to *A. flavipes*, but with no pathogenicity to *P. tabuliformis*, which made the strain SMC01 a potential candidate for controlling *A. flavipes*.

Currently, the most commonly studied and used pesticidal entomogenous fungi in China and worldwide are *Lagenidium giganteum*, *Beauveria bassiana*, *B. brongniartii*, *Metarhizium anisopliae*, *Paecilomyces fumosoroseus* and *Verticillium lecanii*, and so on (Liu, 2017). *Pestalotiopsis* spp. are commonly considered as plant pathogenicity causing disease in a variety of plants (Chen *et al.*, 2012; Zhao *et al.*, 2016; Shi *et al.*, 2017), and also commonly isolated as endophytes, and some species likely have endophytic and pathogenic stages in their life cycle (Tejesvi *et al.*, 2009; Watanabe *et al.*, 2010; Liu *et al.*, 2015). Some species have also been recorded as saprobes where they are recyclers of dead plant material (Maharachchikumbura *et al.*, 2011) and even rarely cause

disease in humans (Sutton, 1999) and pandas (Gu *et al.*, 2014).

About its pathogenicity to insects, to our knowledge, this was the second time to isolate entomopathogenic *Pestalotiopsis* sp. from insects. Chengqun Lv firstly isolated *Pestalotiopsis* spp. from *Hemiberlesia pitysophila* in 2011 (Lv *et al.*, 2011) and assessed its potential to control *H. pitysophila* in 2014 (Lv *et al.*, 2014). Our results make it clearer that *Pestalotiopsis* spp. can also infect insect pests as an entomopathogenic fungus.

In addition, the nymph can eject white foam when suck the sap, which is a big advantage for *Pestalotiopsis* spp. to invade and germinate in the insect body. So there is a bright prospect using *Pestalotiopsis* spp. to control *A. flavipes*. But it is necessary to study the mechanism and application method.

## Conclusion

In conclusion, a new entomopathogenic fungal strain, *Pestalotiopsis* spp., was isolated from the naturally infected *A. flavipes*. The strain had a high virulence to *A. flavipes*, and no pathogenicity to *P. tabuliformis*. So the strain SMC01 was considered a potential candidate for controlling *A. flavipes*.

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## References

- Aboul-Nasr, M.B. and M.R. Abdul-Rahman, 2014. A simple technique for single spore isolation of *Fusarium verticillioides* and *Fusarium subglutinans*. *World J. Biol. Biol. Sci.*, 2: 21–25
- Butt, T.M., C.J. Coates, I.M. Dubovskiy and N.A. Ratcliffe, 2016. Entomopathogenic fungi: new insights into host-pathogen interactions. In: *Advances in Genetics*, Vol. 94, pp: 307–346. Academic Press, Elsevier
- Chen, X.R., Y.P. Xing, T.X. Zhang, J.T. Zheng, J.Y. Xu, Z.R. Wang, Y.H. Tong and Y.R. Xu, 2012. Identification and Characterization of *Pestalotiopsis* spp. Causing Leaf Necrosis of Red Bayberry in China. *Acta Phytopathol. Sin.*, 42: 626–632
- Fang, G.F., Y.T. Zhou, Z.J. Yu and S. Li, 2016. Toxic effect of 4 insecticides against *Aphrophoridae flavipes* nymph in laboratory. *For. Pest Dis.*, 35: 39–41
- Ge, Q.X., Y.X. Chen and T. Xu, 2009. *Chinese Fungi*. Vol. 38. *Pestalotiopsis* spp. Science Press, Beijing, China
- Gu, Y., J.X. Ruan, X.P. Ma, D.S. Li, X.M. Liu, S.S. Ling and C.C. Li, 2014. *Pestalotiopsis hainanensis* strain from the body surface of *Ailuropoda melanoleuca*. *Mycosystema*, 33: 731–737
- Khosravi, R., J.J. Sendi, A. Zibae and M.A. Shokrgozar, 2014. Virulence of four *Beauveria bassiana* (Balsamo) (Asc., Hypocreales) isolates on rose sawfly, *Arge rosae* under laboratory condition. *J. King Saud Univ.-Sci.*, 27: 49–53
- Li, H.P., X.P. Wei and S.H. Yue, 2013. RAPD analysis on degenerate strains of *Beauveria bassiana* used in biocontrol of *Apriona germari*. *Sci. Sericult.*, 39: 226–230
- Li, H.P., D.Z. Huang and Z.G. Wang, 2011. Potential of *Beauveria bassiana* for biologic control of *Apriona germari*. *Front. Agric. Chin.*, 5: 666–670
- Liu, C.L., 2017. Application of Entomopathogenic fungi in biological control of agricultural and forestry pests. *Hei Long Jiang Agric. Sci.*, 2017: 68–73
- Liu, H., Y.G. Liu, S.J. Li, W. Shuai and Y.Y. Liu, 2015. Diversity traits of endophytic *Pestalotiopsis* from pine trees in Sichuan and its control effect. *J. Northwest Agric. For. Univ. (Nat. Sci. ed.)*, 43: 41–48
- Lv, C.Q., B.L. Huang, H. Ren, W.L. Li, H. Rong and L.J. Guo, 2014. Virulence of *Pestalotiopsis disseminata* GH10 strain to *Hemiberlesia pitysophila*. *J. South. Agric.*, 45: 980–983
- Lv, C.Q., B.L. Huang, M.J. Qiao and J.G. Wei, 2011. Entomopathogenic fungi on *Hemiberlesia pitysophila*. *Plos One*, 6: 1–6
- Maharachchikumbura, S.S.N., L.D. Guo, E. Chukeatirote, A.H. Bahkali and K.D. Hyde, 2011. *Pestalotiopsis*—morphology, phylogeny, biochemistry and diversity. *Fung. Divers.*, 50: 167–187
- Pedrini, N., 2018. Molecular interactions between entomopathogenic fungi (hypocreales) and their insect host: perspectives from stressful cuticle and hemolymph battlefields and the potential of dual RNA sequencing for future studies. *Fung. Biol.*, 122: 538–545
- Shi, L., Y. Li, N. Fei, J. Fu and X. Yan, 2017. Identification of the *Pestalotiopsis* pathogen of blueberry leaf spot. *South Chin. Fruits*, 1: 7
- Song, X.D., R.S. Zhang, G. Song, M. Liu, S.T. Wang and J.Y. Chen, 2016. Sap consumption and damage of *Aphrophora flavipes*. *For. Pest Dis.*, 35: 4–7
- Sutton, D.A., 1999. Coelomycetous fungi in human disease. A review: clinical entities, pathogenesis, identification and therapy. *Rev. Iberoamer. Mycol.*, 16: 171–179
- Tejesvi, M.V., S.A. Tamhankar and K.R. Kini, 2009. Phylogenetic analysis of endophytic *Pestalotiopsis* species from ethnopharmacologically important medicinal trees. *Fungal Divers.*, 38: 167–183
- Watanabe, K., K. Motohashi and Y. Ono, 2010. Description of *Pestalotiopsis pallidotheae*: a new species from Japan. *Mycoscience*, 51: 182–188
- Zhao, J., Z. Ma, Z. Liu, Q. Shang, X. Zhao and Y. Wei, 2016. *Pestalotiopsis clavisporea* causing leaf spot on strawberry. *Mycosystema*, 35: 114–120

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