Scanning electron microscopic observation of the infection process of a *Metarhizium* strain Ma6 highly pathogenic to *Phyllotreta striolata*

Jianyu Li [1, 2, 3, 4], **Mengzhu Shi** [1, 2, 3, 4], **Yuechao He** [1], **Jianwei Fu** [Corresp., 1, 2, 3, 4, 5], **Lizhen Zheng** [1, 2, 3]

[1] Institute of Plant Protection, Fujian Academy of Agriculture Sciences, Fuzhou, China

[2] Fujian Key Laboratory for Monitoring and Integrated Management of Crop Pests, Institute of Plant Protection, Fujian Academy of Agriculture Sciences, Fuzhou, China

[3] Fuzhou Scientific Observing and Experimental Station of Crop Pests, Ministry of Agriculture, Fuzhou, China

[4] State Key Laboratory of Ecological Pest Control for Fujian and Taiwan Crops, Fujian Agriculture and Forestry University, Fuzhou, China

[5] Institute of Quality Standards & Testing Technology for Agro-Products, Fujian Academy of Agricultural Science, Fuzhou, China

Corresponding Author: Jianwei Fu
Email address: fjw9238@163.com

Background. *Phyllotreta striolata* is a worldwide pest that harms cruciferous vegetables. The use of pathogenic microorganisms to control pests is an important means of biological control. Using pathogenic microorganisms to prevent and control *P. striolata* has rarely been reported. **Methods.** In this study, the infection process of a *Metarhizium* strain highly pathogenic to *P. striolata* was observed by stereomicroscopyand scanning electron microscopy (SEM). **Results.**The results showed that the attachment of *Metarhizium* strain Ma6 to the body surface varied; the conidia distribution was greatest in the tibia of the posterior leg with thick bristles and in the intersegmental abdominal membrane, and the spore distribution occurred least in the smooth and hard portions of the insect's body. At the start of the infection, *Metarhizium* strain Ma6 generally grew from the body parts with gaps or connecting spaces such as mouthparts and the thoracic leg base and joints, then the spores germinated with germ tubes and penetration peg, and the penetration peg penetrated the body surface. Ten days after inoculation, the mycelia divided into conidia, and many mycelia and spores covered the entire adult insect's body. **Discussion.** Spore germination occurred on the 5th day after inoculation, and many hyphae and spores covered the entire adult insect body within 10 days after inoculation. And the invasion into tissue gaps from the weaker areas is more efficient than intruding from the body hard surface. This may be the reason for the *Metarhizium* strain Ma6's high virulence. This study preliminarily clarified the infection ability and invasion approach of a *Metarhizium* strain against *P. striolata*, providing evidence for evaluating the strain's insecticidal effect and application prospect.

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[1] Institute of Plant Protection, Fujian Academy of Agriculture Sciences, Fuzhou 350013, China

[2] Fujian Key Laboratory for Monitoring and Integrated Management of Crop Pests, Fuzhou 350003, China

[3] Fuzhou Scientific Observing and Experimental Station of Crop Pests, Ministry of Agriculture, Fuzhou 350003, China

[4] State Key Laboratory of Ecological Pest Control for Fujian and Taiwan Crops, Fujian Agriculture and Forestry University, Fuzhou 350002, China

[5] Institute of Quality Standards & Testing Technology for Agro-Products, Fujian Academy of Agricultural Science, Fuzhou, 350002, China

Corresponding author:
Jianwei Fu
#247 Wusi Road, Fuzhou, Fujian, 350013, P.R. China
Email address: fjw9238@163.com

ABSTRACT

Background. *Phyllotreta striolata* is a worldwide pest that harms cruciferous vegetables. The use of pathogenic microorganisms to control pests is an important means of biological control. Using pathogenic microorganisms to prevent and control *P. striolata* has rarely been reported.

Methods. In this study, the infection process of a *Metarhizium* strain highly pathogenic to *P. striolata* was observed by stereomicroscopy and scanning electron microscopy (SEM).

Results. The results showed that the attachment of *Metarhizium* strain Ma6 to the body surface varied; the conidia distribution was greatest in the tibia of the posterior leg with thick bristles and in the intersegmental abdominal membrane, and the spore distribution occurred least in the smooth and hard portions of the insect's body. At the start of the infection, *Metarhizium* strain Ma6 generally grew from the body parts with gaps or connecting spaces such as mouthparts and the thoracic leg base and joints, then the spores germinated with germ tubes and penetration peg, and the penetration peg penetrated the body surface. Ten days after inoculation, the mycelia divided into conidia, and many mycelia and spores covered the entire adult insect's body.

Discussion. Spore germination occurred on the 5th day after inoculation, and many hyphae and spores covered the entire adult insect body within 10 days after inoculation. And the invasion into tissue gaps from the weaker areas is more efficient than intruding from the body hard surface. This may be the reason for the *Metarhizium* strain Ma6's high virulence. This study preliminarily clarified the infection ability and invasion approach of a *Metarhizium* strain against *P. striolata*, providing evidence for evaluating the strain's insecticidal effect and application prospect.

Keywords: *Phyllotreta striolata*, *Metarhizium anisopliae*, Scanning electron microscopic, Highly pathogenic, Infection

INTRODUCTION

Phyllotreta striolata (Fabriciu), Coleoptera, Chrysomelidae), is one of the world pests of Cruciferous vegetables. Chemical control is the main method to control *P. striolata*, and the heavy use of pesticides has led to a strong resistance of *P. striolata* (Feng et al., 2000; Fu et al., etc., 2006; Liu et al., 2014; Zheng, 2015) and chemical residues in vegetables which threaten seriously to edible security of vegetables. So it is very important to study the method of prevention and control of *P. striolata* with safety, high efficient and environment friendly.

Metarhizium anisopliae is a parasitic fungus that grows naturally in soils worldwide. *M. anisopliae* is one of the most widely used entomopathogenic fungi (Shang et al., 2015; Wang and Wang, 2017) and can be used to biologically control many pests, such as *Popillia japonica* (Behle

et al., 2015), *Agriotes obscurus* (Rogge et al., 2017), *Rhynchophorus ferrugineus* (Yasin et al., 2017), and *Planococcus citri* (Ghaffari et al., 2017). Few studies on using *Metarhizium* to prevent and control *P. striolata* have been reported (Butt et al., 1995; He et al, 2017), and the invasion approach and pathogenic process of *Metarhizium* to *P. striolata* are unreported. Our laboratory screened *Metarhizium* strain Ma6 with high virulence against *P. striolata*, and its 7-day cumulative mortality against *P. striolata* reached 100% (He et al, 2017).

In this study, with *Metarhizium* strain Ma6 as the research object, the infection process of adult *P. striolata* was periodically sampled, and the conidia's attachment site, spore germination and invasive process in *P. striolata* were observed using scanning electron microscopy (SEM), to reveal the approach, process, and infection mechanism in *P. striolata* by *Metarhizium*, which is expected to provide a scientific basis for clarifying the pathogenic mechanism of *Metarhizium* on *P. striolata* to reasonably and effectively apply this strain in biological control.

MATERIALS & METHODS

Experimental materials

M. anisopliae strain Ma6 was provided by Dr. Xueyou He of Fujian Academy of Forestry Science and was cryo-preserved in our laboratory. Before the experiments, the strain was inoculated into Sabouraud's dextrose agar and yeast (SDY) liquid medium and cultured for 72 h with shaking and then inoculated into PPDA medium to culture for approximately 14 days. After ample spores were generated, the spores were collected to begin the spore germination experiment. The germination rate of the tested spores was above 95%. The spores were diluted with sterile water (containing 0.05% Tween-80), the spore density was calculated with a haemocytometer, and a spore suspension was prepared at a concentration of 1×10^8 for later use.

Experimental methods

Metarhizium* infection in *P. striolata *P. striolata* were placed in 2-mL centrifuge tubes with 20 insects per tube, and 1 mL of prepared *Metarhizium* conidia suspension was added into the centrifuge tube containing *P. striolata* to soak for 10 s. *P. striolata* were then quickly placed in bioassay bottles with 3-4 pakchoi cabbage leaves (the leaf bases were wrapped with wet cotton for moisture). All bioassay bottles were placed in a smart artificial climate chamber at 25±1 ℃ and 75% relative humidity, with the leaves replaced every 2 days. Samples were collected at 1, 2, 3, 4, 5, 6, 7, and 10 days after inoculation.

SEM Observation of *P. striolata* infected by *Metarhizium* Five *P. striolata* samples were taken at each *Metarhizium* treatment. The samples were fixed in 2%-4% glutaraldehyde fixative, washed with 0.1 mol/L H_2PO_4 buffer at pH 7.0 for 30 minutes with 3-4 buffer changes, fixed in 1% citric acid for 1-2 h, and dehydrated through 50%, 70%, 80%, 89%, 95% and 100% gradient ethanol solutions every 7 min. After the samples were dehydrated, isoamyl acetate was used to replace the dehydrating agent in the tissue. Samples were dried at the CO_2 critical point and sputter coated with gold after fixing. The prepared samples were observed by scanning electron microscopy.

RESULTS

Colony and hyphae characteristics

In the PPDA medium (25℃), the strain Ma6 colonies were initially villous or cottony and white, producing spores in grey-green with white, smooth margins (Fig. 1A). The conidiophores were solitary, aggregated or closely arranged, showing penicilli or verticils (Fig. 1B).

Macroscopic observation of adult *P. striolata* infected by *Metarhizium* Ma6

Two days after inoculating *Metarhizium* strain Ma6 into *P. striolata*, some individuals began to die. At this time, no apparent mycelium growth was observed on the surface of the newly killed adult *P. striolata* (Fig. 2A). Four days after inoculation, obvious mycelial growth began to appear in the mouthparts and the thoracic leg base and joints of the dead adult *P. striolata* (Fig. 2B). Six days after inoculation, in addition to the above-mentioned sites, obvious mycelial growth on the adult *P. striolata* began to appear in the abdomen, and the number of growing mycelia gradually increased (Fig. 2C). *Metarhizium* growth on the *P. striolata* began where the joints connected. Mycelial growth was observed on the leg, tibia, and tarsus and then spread around. Seven days after inoculation, the dorsum, chest, thoracic leg, mouthparts, and antennae of the adult *P. striolata* were covered with dense mycelia, and the growth characteristics of the mycelia on the dead *P. striolata* were similar to those on the culture medium. 10 days after inoculation, large amount of hyphae formed on the body surface, and gray-green hyphae and spores were observed (Fig. 2D).

SEM observation of *Metarhizium* infection in *P. striolata*

Adult *P. striolata* body surface structures differ greatly; therefore, *Metarhizium* attachment on the body surface also varies. The highest conidia density was observed in the thick bristled posterior

feet and intersegmental abdominal membrane (Fig. 3A, B), followed by the intersegmental membrane and thoracic leg folds, where the tissue is relatively soft and conducive to spore attachment and infection (Fig. 3C). The spore distribution was the lowest on the body's smooth and hard surfaces (Fig. 3D).

Metarhizium strain Ma6 was inoculated into the *P. striolata*, large-scale conidial growth was seen in the mouthparts, leg joints, and intersegmental membrane, while mycelial growth on the abdomen and dorsal surface was relatively delayed. In this study, scanning electron microscopy (SEM) was used to observe the *Metarhizium* growth in the infected *P. striolata*. One day after inoculation, many conidia were attached to the intersegmental membrane and the base tibia of the hind leg (Fig. 4A). Three days after inoculation, spores were attached to the intersegmental membranes and abdomen, abdominal intersegmental bristles and the hind leg joints in *P. striolata*, and some mycelia penetrated the base tibia of the hind legs (Fig. 4B). Five days after inoculation, many conidia were attached to the body surface, the intersegmental membrane, and the hind legs of the insect body. The spores were germinated, and some mycelia penetrated the body surface at the intersegmental membrane (Fig. 4C, D). Seven days after inoculation, many conidia penetrated the body wall, and the hyphae further grew on the epidermal layer of the body wall, while some hyphae penetrated out along the bristles, covering the body's surface (Fig. 4E). Ten days after inoculation, many conidia were generated from the mycelia, and many *Metarhizium* mycelia and spores were observed on the adult insects' ventral surfaces, covering the entire body (Fig. 4F).

DISCUSSION

Metarhizium differs greatly in its ability to infect different hosts or host insects' body surfaces. Butt et al. (1995) compared *Metarhizium* germination on the epidermis of aphids and flea beetles and found that the host's body wall significantly affected *Metarhizium* germination and virulence. Different structural regions on the surface of diamondback moths significantly affected appressoria production and bud length before *Metarhizium* invasion (Wang et al., 2005). With sufficient nutrients and suitable temperature and humidity, conidia germinate to form germ tubes and appressoria, and the appressoria can adhere to the host surface and form invader hyphae, which may enter the host's interior along the air hole opening or directly by piercing through the host's epidermis (Hajek and Leger, 1994; Wang and You, 1999). Special structures, such as germ tubes, appressoria, and invader hyphae, which were generated during the infection process, were observed in this study. Liu et al. (2015) observed *Beauveria bassiana* infection mechanism in

Bemisia tabaci nymphaea by scanning electron microscopy and found that the infection process was closely related to specific surface regions on the *B. tabaci* nymphaea. The spore density was high in the intersegmental membrane; spore germination was observed within 24 h after inoculation, and many conidia were found on the surface of the aleyrodids 120 h later (Liu et al., 2015). SEM was used to observe the *Metarhizium* strain CoM02 infecting hazelnut weevils. Spore attachment, mycelial germination, rapid mycelial growth, and spore germination occurred on the 3rd, 5th, 7th, and 11th days after inoculation, respectively. The entire infection cycle took approximately 11 days (Cheng et al., 2016). In this study, spore germination occurred on the 5th day after inoculation, and many hyphae and spores covered the entire adult insect body within 10 days after inoculation (Fig. 2B, D).

Spore attachment to the host insect's epidermis is a precondition for establishing the parasitic relationship between the insect and the fungi. This process is non-specific and passive, that is, after the conidia attach to the host insect's epidermis, a parasitic relationship is not necessarily established. This process is completed via the hydrophobicity of the spores and the insect epidermis (Lord and Howard, 2004; Wichadakul et al., 2015). Fungal penetration of the insect epidermis also results from a combination of mechanical pressure and enzymatic degradation. The entomopathogenic fungi secrete proteases, chitinases, lipases and other extracellular hydrolases when invading the insect body wall (Charnley et al., 1991; Hajek and Leger, 1994). Through these extracellular proteases, the conidia firmly attach to the insect body wall. This study found that 6 days after inoculation, small holes appeared on the *P. striolata* abdominal surface, with enzymatic activity near the appressoria, indicating that *Metarhizium* strain Ma6 invaded the *P. striolata*, accompanied by enzyme activity, to degrade the host's epidermis, resulting in strain Ma6 successfully invading the *P. striolata* (Fig. 4).

During the *Metarhizium* strain Ma6 infection of *P. striolata*, the *Metarhizium* mainly adhered to the intersegmental membrane, the abdominal bristles, and the tibia of the thick bristled posterior leg. Later, the spores germinated and generated germ tubes and invader hyphae to penetrate the body wall, eventually covering the entire body. This invasion into tissue gaps is more efficient than intruding from the body surface because it bypasses the hard surface to directly invade from the weaker areas, with an LT_{50} of only 4.09 d (He et al., 2017). This may be the reason for the *Metarhizium* strain Ma6's high virulence observed in this study. Many biological control agents have been developed as biocides to biologically control field pests (Güerri-Agulló et al., 2011; Wraight and Ramos, 2015); however, *Metarhizium* germination and

mycelial growth are affected by environmental conditions, such as nutrition, temperature and humidity (Athanassiou et al., 2017). Whether this strain can be developed into an effective biocide remains to be confirmed by further field trials.

CONCLUSION

Spore germination occurred on the 5th day after inoculation, and many hyphae and spores covered the entire adult insect body within 10 days after inoculation. At the start of the infection, *Metarhizium* strain Ma6 generally grew from the body parts with gaps or connecting spaces such as mouthparts and the thoracic leg base and joints, then the spores germinated and generated germ tubes and invader hyphae to penetrate the body surface, eventually covering the entire body. Ten days after inoculation, the mycelia divided into conidia, and many mycelia and spores covered the entire adult insect's body. This invasion into tissue gaps from the weaker areas is more efficient than intruding the hard surface to directly invade. This may be the reason for the *Metarhizium* strain Ma6's high virulence.

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Figure 1

Morphological characteristics of *Metarhizium* Ma6.

(A) Colonies of Ma6 on PPDA medium. (B) Spore and mycelia characteristics under electron microscopy.

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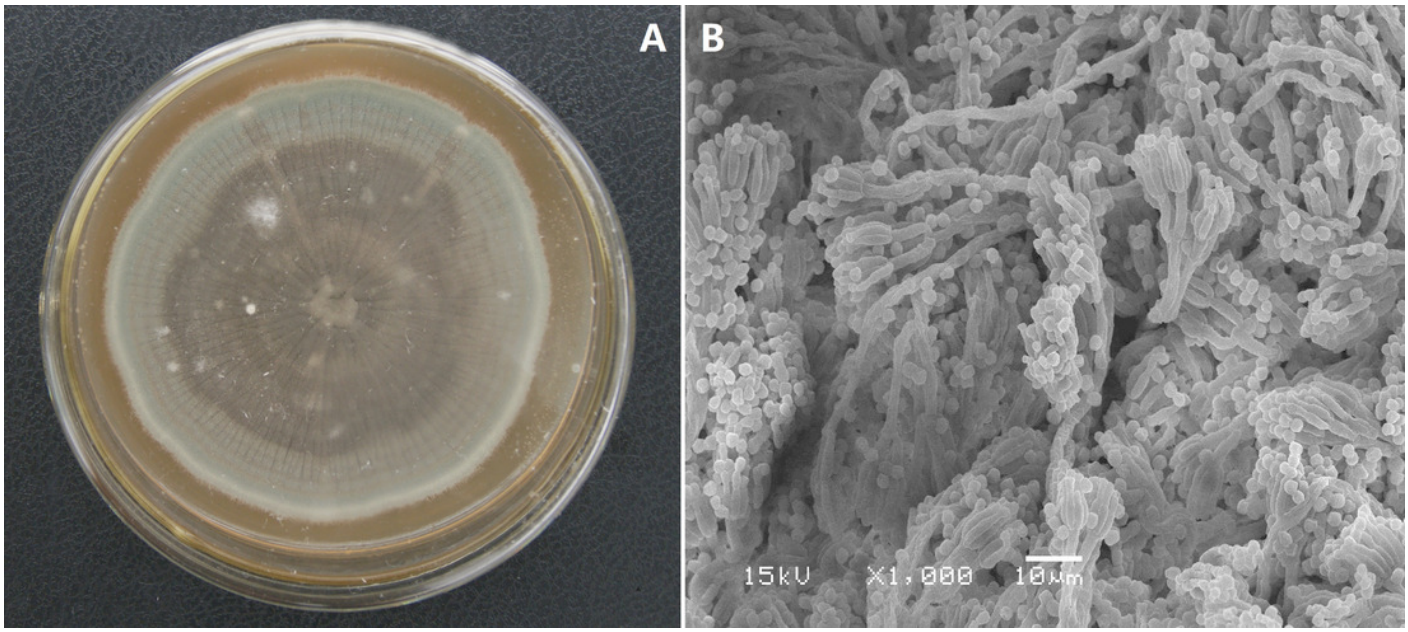

Figure 2

Process of *Metarhizium* Ma6 infecting *P. striolata* adults using stereomicroscope.

(A) Adult, 2 days after inoculation. (B) Adult, 4 days after inoculation. (C) Adult, 6 days after inoculation. (D) Adult, 10 days after inoculation, large amount of hyphae formed on the body surface.

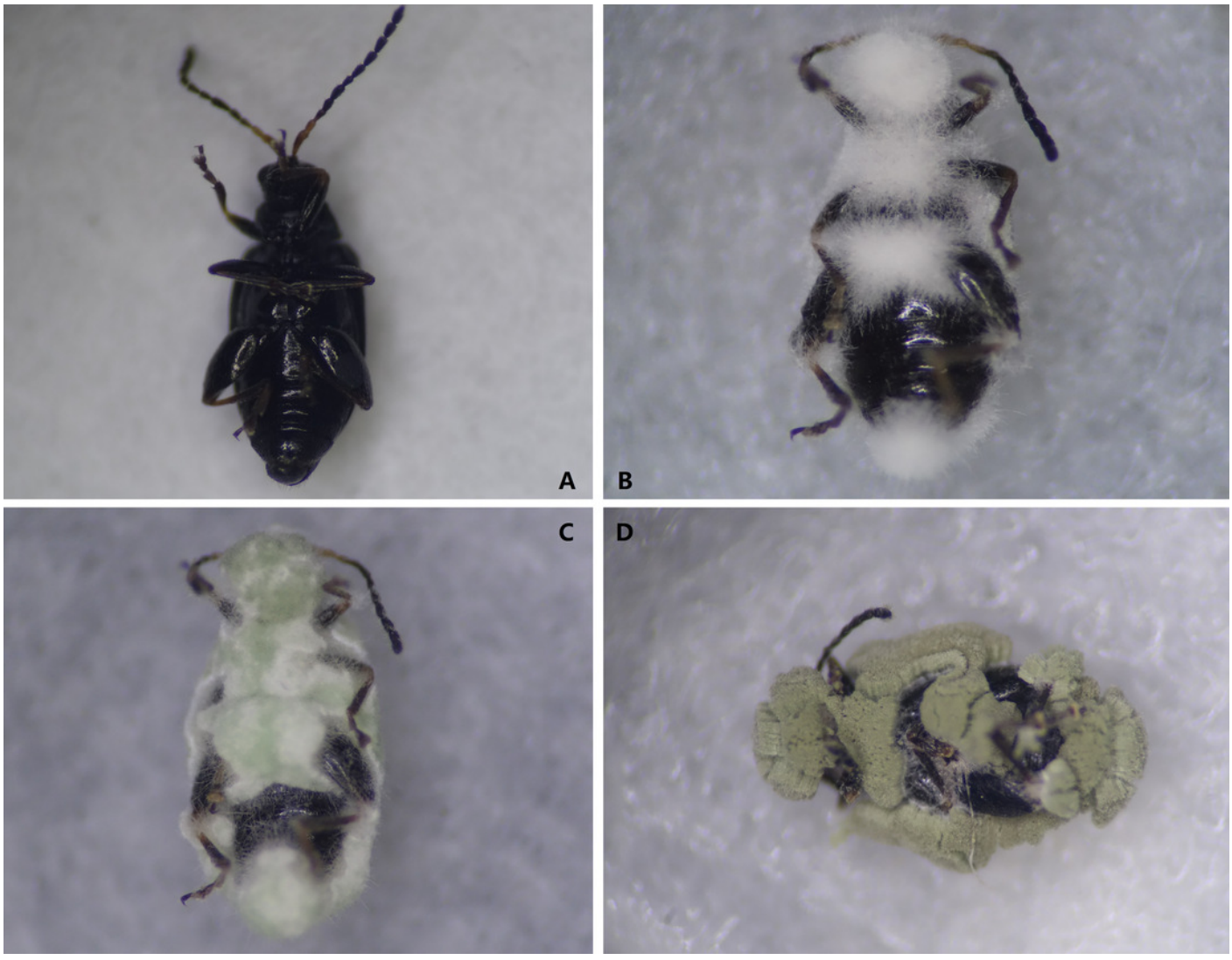

Figure 3

Main attachment of *Metarhizium* Ma6 spores on the adult *P. striolata* body surface.

(A) Conidia attached to the bract bristles. (B) Conidia attached to the intersegmental body membranes. (C) Conidia attached to the intersegmental thoracic leg membrane. (D) Conidia attached to the body surface and abdominal bristles.

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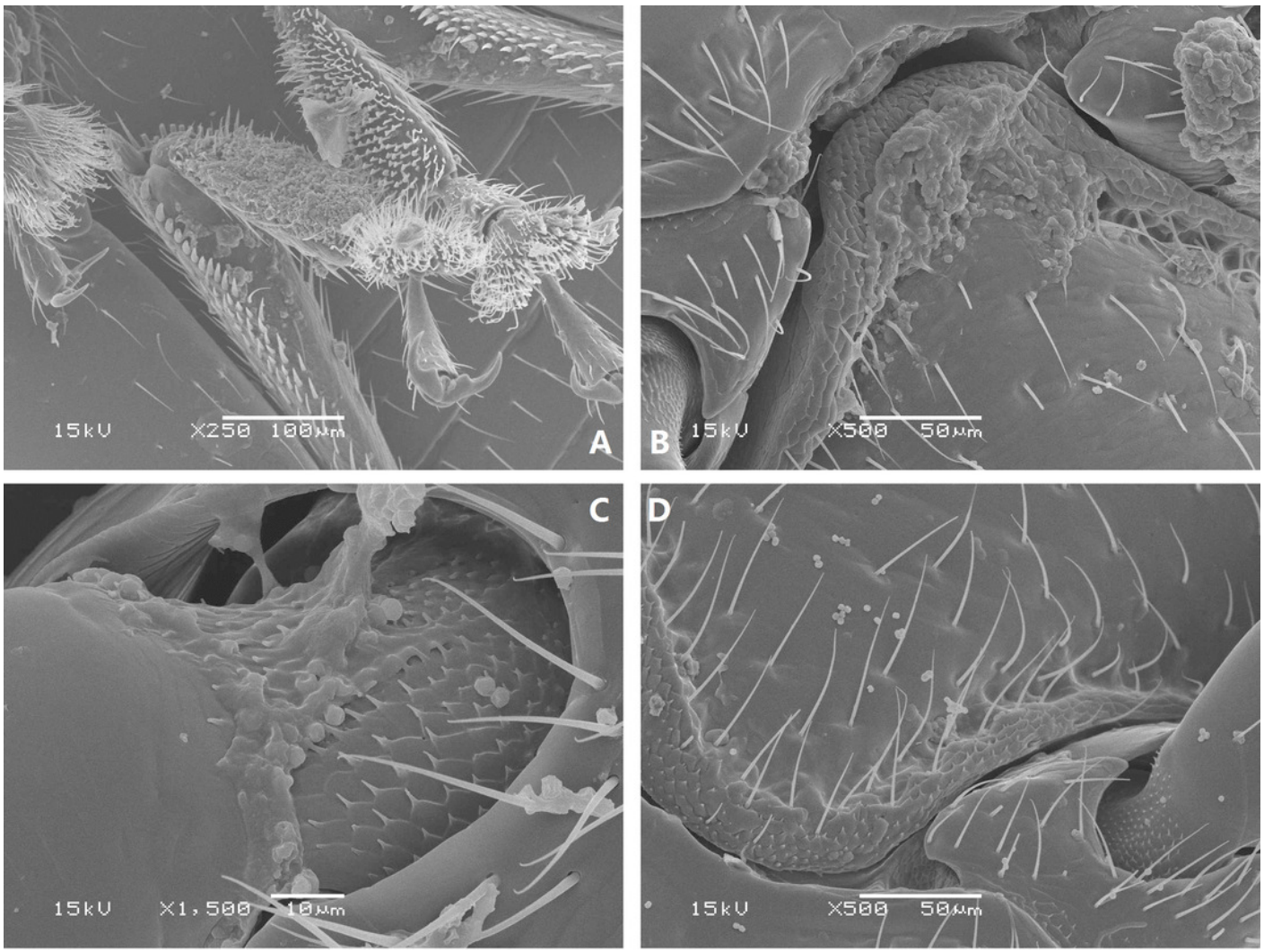

Figure 4

Metarhizium Ma6 infection process in adult *P. striolata* using scanning electron microscopic.

(A) Conidia attached to the body surface. (B) Conidia attached to the foot bristles. (C) Spore germination tube and invasion hyphae. (D) Mycelia penetrating the body surface at the tibia of the leg. (E) Many conidia growing. (F) The insect body covered with mycelia on the ventral surface.

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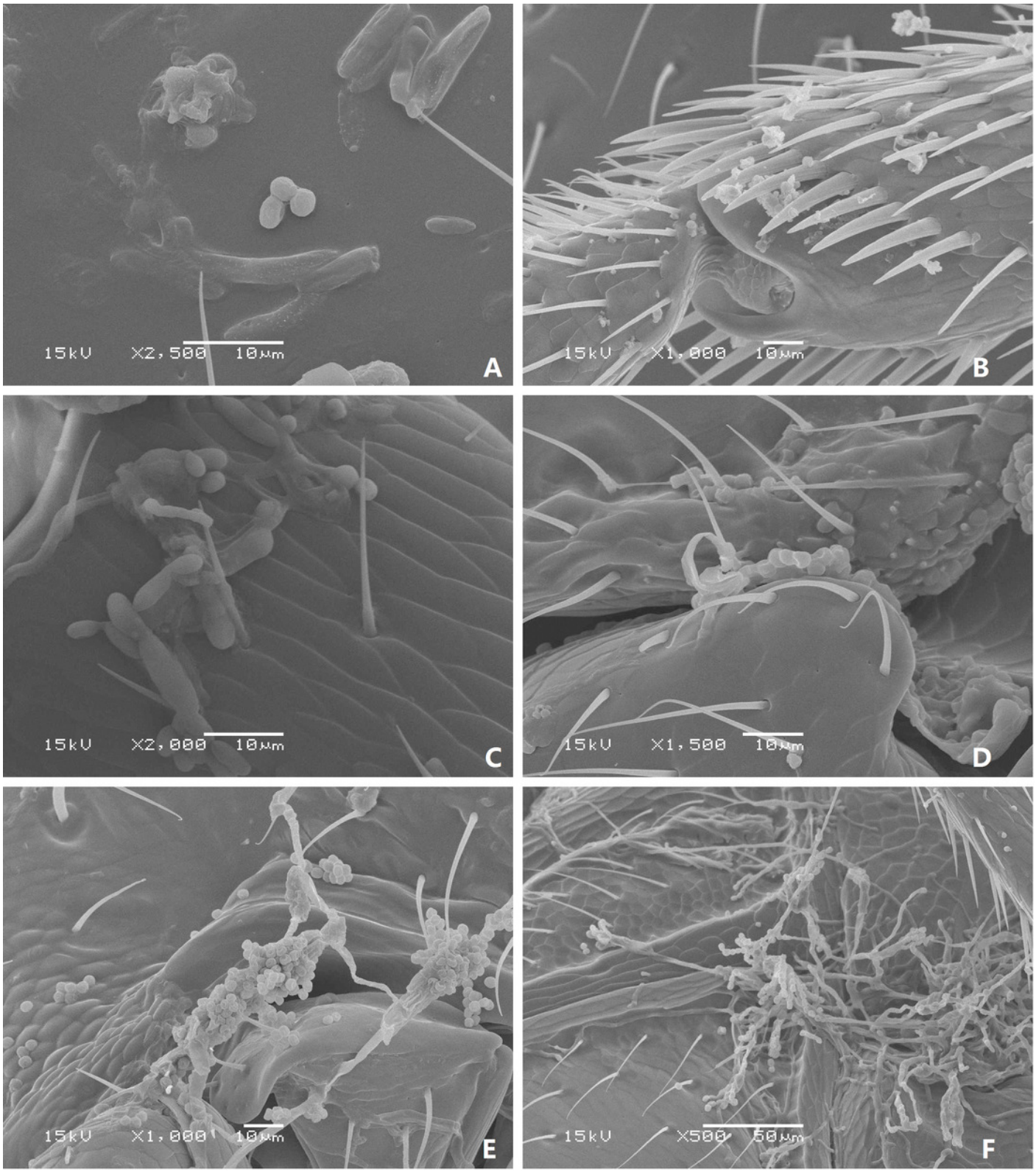

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