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RESEARCH ARTICLE

Observation of the infection process of *Metarhizium anisopliae* on the cuticle of *Anoplophora glabripennis* larvae with scanning electron microscopy

Haixia YAN[1,2], Da WANG[3], Xusheng ZHAO[4], Dazhuang HUANG (✉)[1,2], Xiangchao MA[1]

[1] *Forestry College, Agricultural University of Hebei, Baoding 071000, China*
[2] *Key Laboratory of Forest Germplasm and Protection of Hebei Province, Baoding 071000, China*
[3] *Shanghai Academy of Agricultural Sciences, Shanghai 201106, China*
[4] *International Cooperation Office, Agriculture University of Hebei, Baoding 071000, China*

Abstract **The invasion behavior and infection process of *Metarhizium anisopliae* on different cuticle areas of *Anoplophora glabripennis* larvae were observed by scanning electron microscopy. The results indicated that *M. anisopliae* infected *A. glabripennis* larvae mainly through the intersegmental membrane where more attaching conidia, faster germination, and higher germination and penetration rates were observed. The secondary invasion area was around the valve. The two areas were vulnerable to infection from *M. anisopliae*. Twelve hours after vaccination, conidia germinated and bud-shaped protrusions formed on the cuticle of *A. glabripennis* larvae, then germ tubes and various attaching structures were produced. Conidia penetrated the integument into the hemocoele of *A. glabripennis* larvae 36–48 h later.**

Keywords *Metarhizium anisopliae*, *Anoplophora glabripennis*, different areas of cuticle, invasion behavior, infection process, scanning electron microscopy

Introduction

Anoplophora glabripennis, as one kind of dangerous boring-beetle pests, has done great damage in 27 provinces, municipalities or autonomous regions in China (Wang, 2004) and has seriously reduced the value of timber and resulted in huge losses to forests. Nowadays, more attention is being paid to personal health and environmental protection. A series of environmental problems, such as pest resistance to pesticides, the death of natural enemies of pests and the destruction of the ecological environment, have become more and more prominent, which makes people seek better ways to carry out biological prevention or bio-based comprehensive treatment. *Metarhizium anisopliae*, with its many advantages and valuable potentials, is one kind of pathogenic fungi with very broad prospects for application and high practical values.

Some researches (Fan et al., 1987; Tong et al., 1991; Xu, 2002; Wang et al., 2004; Xia et al., 2005; He et al., 2005, 2007) have applied *M. anisopliae* to the cinontrolg of longhorned beetles, but so far, no studies on the infection process of *Metarhizium anisopliae* to *Anoplophora glabripennis* larvae have been reported. As a result, the invasion behavior and infection process of *M. anisopliae* to different cuticle areas of *A. glabripennis* larvae were observed and studied in this research.

Materials and methods

Experimental materials

A. glabripennis adults were collected in Baoding in Hebei Province, China. By a 3∶1 female–male sex ratio, *A. glabripennis* adults were raised in 5-year-old weeping willows to let them lay eggs. All weeping willows were

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Correspondence: Dazhuang HUANG
E-mail: huangdazhuang@126.com

caged to stop the *A. glabripennis* adults from escaping. Fifty days later, we collected the larvae from the branches as our research material.

Fungi test and preparation of spore suspensions

M. anisopliae MS01 is a highly virulent fungus to the *Anoplophora glabripennis* larvae. The MS01 fungi was grown on PPDA medium at 26°C for two weeks. Conidia were harvested from the PPDA surface by scraping. Spore suspensions were made by placing the harvested spores in 20 mL sterile distilled water in a small beaker containing 0.1% Tween 80. Beakers were agitated on a vibrating shaker for 10 min to produce a homogenous conidial suspension. The spore concentrations were then quantified with the Neubauer hemocytometer and adjusted to 1×10^8 conidia/mL.

Method of selecting sample for test

After feeding for 50 d, the *Anoplophora glabripennis* larvae were put in the spore suspensions for 30 s, with the concentration of *Metarhizium anisopliae* MS01 strain at 1×10^8 conidia/mL, and then raised to the man-made nest in the branches of the weeping willow. Water cultivation was done in an artificial climate box, with the branches of the weeping widow in the water, the top of the branches covered by wet gauze, and their bottom in the water. The temperature and the humidity in the artificial climate box were kept at 26°C and 90%, respectively. The infected larvae were kept in the artificial climate box for 12 h, 16 h, 20 h, 24 h, 36 h, 48 h, 60 h, about 72 h (the larvae just died), and 72 h, when hyphae grew out of the body of the larvae by penetration, as the samples for scanning electron microscopy.

Sample preparation and observation

The samples were treated with 2.5% glutaraldehyde for 10 h, followed by osmium tetroxide treatment for 2 h, PBS treatment for 1 h, ethanol dehydration, then replacing the ethanol with isoamyl acetate. When the treated samples were dried at the critical point and gold-painted after pasting, they were photographed by scanning electron microscopy to observe the invasion behavior and infection process of *Metarhizium anisopliae* to different cuticle areas of *Anoplophora glabripennis* larvae.

Results

The different invasion behaviors of *M. anisopliae* among different cuticle areas of *A. glabripennis* larvae

It was shown that, from the results of scanning electron microscopy, *M. anisopliae* had different invasion behaviors among different cuticle areas of the *A. glabripennis* larvae. There were more attached conidia of *M. anisopliae* gathering in the intersegmental membrane of *A. glabripennis* larvae than in other regions of the cuticle areas. The secondary actively intruded area was around the valve. The conidia germinated by generating germ tubes, most ends of which expanded swiftly, forming the attaching spore with large morphological changes and a sticky material on the cuticle areas (Fig. 1 C). Some of the attaching spores were oval (Fig. 1 E), while some looked like mulberries because of the accumulation of mucus layers attaching to the flat region of the cuticle areas tightly (Fig. 1 F), then penetrating into the body walls of the host. A few conidia extended from the original diameter by a long distance and oriented their growth toward the body wall after several bends, finally getting into the body of the host through the body walls.

The morphological changes of *M. anisopliae* larvae while germinating and invading

The infection process of *M. anisopliae* to different cuticle areas of *A. glabripennis* larvae included attachment, germination, penetration, extension and proliferation of mycelium. Twelve hours after vaccination, many conidia of the MS01 strain had attached to different regions of the body walls successfully, showing signs of germination and a bud-like body growing out of one end of the conidia (Fig. 1 A). Sixteen hour later, the conidia germinated, forming the germ tubes, some of which expanded into the attaching spores to penetrate the body walls (Figs. 1 B–D). From 20–24 h, the germinating spores and germ tubes secreted a viscous material to adhere to the body walls of the host, and a large number of germ tubes and all kinds of attaching structures penetrating the body walls was visible (Figs. 1 E–F), many of which were longer in length than the longitudinal diameter of the conidia, forming the shape of a depression slightly at the point of penetration (Figs. 1 G–H). From 36–44 h, some germ tubes grew to attach to the body walls through several bends in order to penetrate easily at some point (Figs. 1 I–J). In addition, the conidia living in the valves generated long mycelium to grow into the body (Fig. 1 K). During the process of the experiment, it was observed that surface attachment fell without spores and mycelia (Fig. 1 L). After penetration of the mycelia into the hosts, the spores competed with the hosts for nutritional materials, destroying the tissues and organs, which led to the death of the hosts, and finally the spore and mycelium grew out of the inner body of the dead hosts (Figs. 1 M–R).

Discussion

In this research, it was discovered that *M. anisopliae* infected the *A. glabripennis* larvae mainly through their intersegmental membrane where there were more attaching conidia with faster germination, higher germination rate and invasion

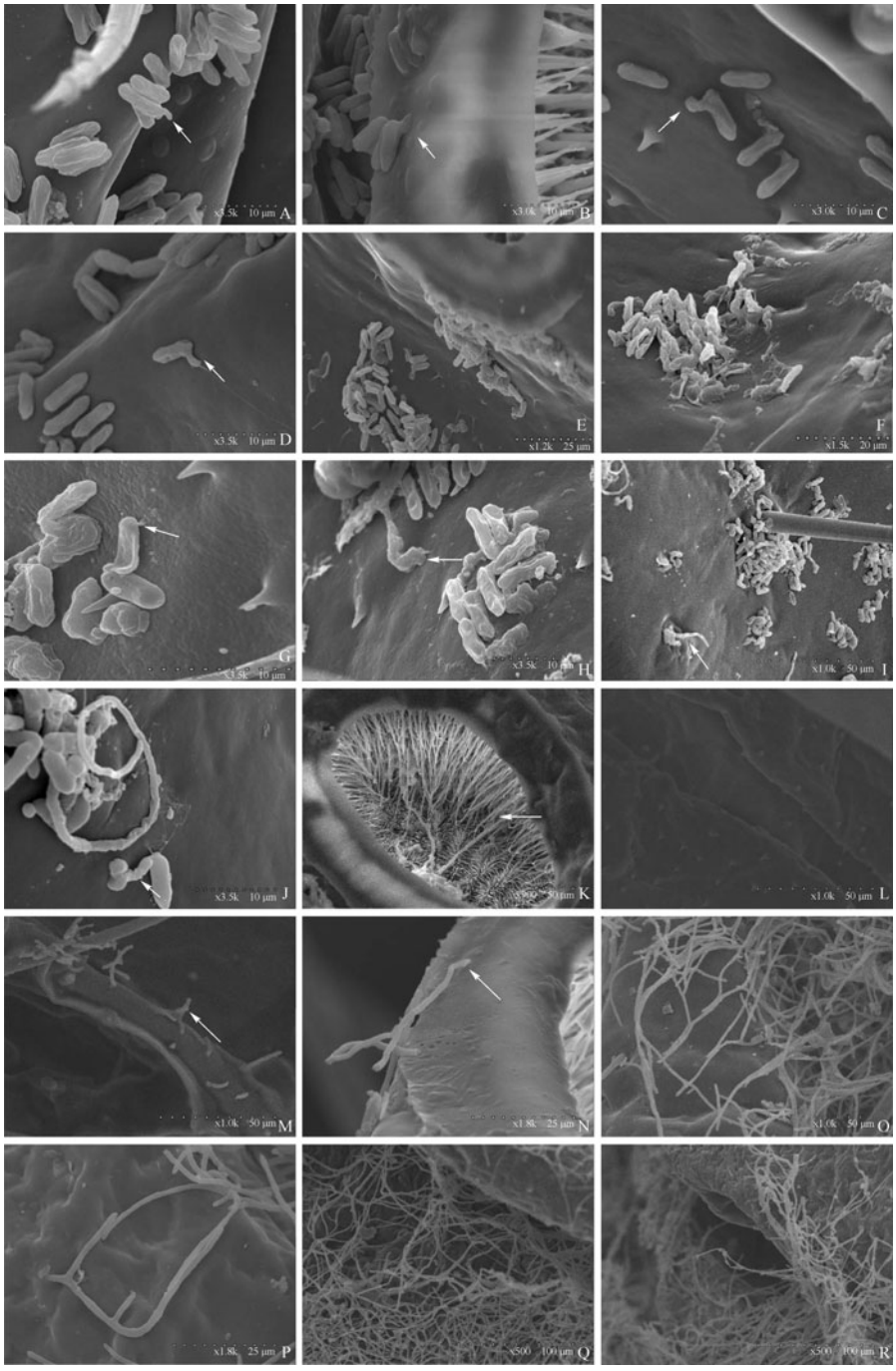

Figure 1 Infection progress of *Metarhizium anisopliae* on *Anoplophora glabripennis* larvae. A shows gemmiform protuberance (arrow) appearing on the top of the conidia upon integument 12 h post-inoculation; B–C indicate aspersoria penetrating the integument, resulting from conidia germination at the edge of the valve and intersegmental membrane 12 h post-inoculation; D indicates aspersoria resulting from conidia penetrating the integument,16 h post-inoculation; E–F indicate a large amount of germ tubes and various attaching structures covered with mucilage secretion penetrating the integument, 20 h post-inoculation. E shows the area around the valve; F is the body wall; G–H indicate conidia penetrating the integument, the depression (arrow) at the point of penetration, 24 h post-inoculation; I–J indicate oriented growth of some germ tubes toward the cuticle and penetration into the cuticle. I is 36 h post-inoculation; J is 48 h post-inoculation; K is 48 h post-inoculation, shows long hyphae from conidia germination in the valve and penetration; L shows conidia and hyphae had disappeared and the attachment exfoliated at the intersegmental membrane of the just dead *A.glabripennis* larvae; M–N show a few hyphae grew out of the surface and the valve fringe of the dead *A.glabripennis* larvae, 72 h post-inoculation; O–P show extensive hyphae growth out of the integument; Q shows intensive hyphae growth out of intersegmental membrane; and R means intensive hyphae growth out of the open valve of the just dead *A.glabripennis* larvae.

rate. The intersegmental membrane is the favored part of the invading *Metarhizium anisopliae*, probably because of the microclimate around the intersegmental membrane and the chemical composition of the surface, which is similar to the discovery of Fan and Li (1994) of the infection process of *M. anisopliae* to *P. xylostella* and *G. mellonella*, in that there were more attaching spores generating in the intersegmental membrane than in other parts of the larvae, but the generating process of attaching spores was decided by many factors.

The valves of terrestrial insects usually have a brush-shaped filter structure called sieve with dense villus, and this structure can prevent dust, pathogens and rainwater from invading. A research has discovered that the dense villus outside the valves of *Anoplophora glabripennis* larvae and the chrysanthemum-shaped villus in the valves of *Anoplophora glabripennis* can filter bacteria (Zhang et al., 1997). In this experiment, it was discovered that most valves of *Anoplophora glabripennis* larvae were all closed at the beginning of the infection process by the pathogen and without any conidia attaching to the valves, which had something to do with the dense microvilli preventing the spores from invading. The regions around the valves, however, had more conidia attached, whose vitality was second only to the conidia in the intersegmental membrane, because the microenvironment around the valves was helpful to their attachment and germination. With the time of the pathogen infection progressing, the defense mechanism wore out gradually, and the conidia in the valves germinated, generating long hyphae and penetrating the body walls.

The conidia of *M. anisopliae*, just as with many other pathogens, attached to the surface of the host first, then generated the mycelium at a suitable condition, invading into the body of the host to reproduce rapidly, finally leading to the death of the host (Wang et al., 1999a). In this experiment, it was also discovered that 12 h after inoculation, *M. anisopliae* began to germinate, generating the bud-shaped protrusion and then forming germ tubes and all kinds of attaching structures. From 36–48 h, the conidia could penetrate into the hemocoele and in the process of self-reproduction, the conidia competed with the host for nutritional materials, destroying the tissues and organs, which led to the death of the host. Finally, the new mycelia and the conidia grew out of the dead body of the host. It was proved that *M. anisopliae* can make a quicker infection and kill the *A. glabripennis* larvae swiftly.

Generally, penetration into an insect's body of the entomogenous fungi is supposed to be the result of the joint action of mechanical pressure and enzymatic degradation. The major components of the insect cuticle include proteins, chitin and lipids. Secreting extracellular enzyme is one of the strategies for pathogenic fungi to degrade the cuticle of the host (Wang et al., 1999b). The experiment indicated that the body-wall degrading enzymes (protease, chitinase, esterase etc.), secreted during the invasion process of *Metarhizium anisopliae* into *Anoplophora glabripennis* larvae, resulted in the secretions of the germinating conidia and germ tubes, and the formation of a depression at the point of penetration.

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