Front. Agric. China 2009, 3(2): 152–158
DOI 10.1007/s11703-009-0024-z

RESEARCH ARTICLE

Baohui WANG, Jianwei ZHENG, Dazhuang HUANG, Da WANG, Xiaoyong HAN, Xiaohong WANG

Symptoms and histopathological study of *Anoplophora glabripennis* larvae infected with *Metarhizium* (Metsch.) Sorokin MS01

Abstract Some changes in color and morphology of *Anoplophora glabripennis* larvae were observed when infected with *Metarhizium* (Metsch.) Sorokin MS01. Paraffin sections show the spores of MS01 attached to the cuticle of *A. glabripennis* larvae at 24 h post inoculation (hpi). At 36 hpi, the conidium germinated and penetrated through the cuticle. After 48 h of inoculation, the endocuticle was disintegrated and the germ tubes passed through the epithelial layers into the hemocoel and multiplied, and then invaded the tissues near the penetration point, such as the fat body, muscle, tracheal tissue, malpighian tubule, alimentary canal, etc. The infected larvae were dead and most tissues and organs were infected and disintegrated with the multiplication of the mycelium for 3 days. The hyphae emerged through the cuticle and formed a hyphal layer on the surface of the dead insect and the conidium emerged five days after inoculation.

Keywords *Anoplophora glabripennis*, larvae, *Metarhizium* (Metsch.) Sorokin, symptom, histopathology

1 Introduction

Anoplophora glabripennis (Motschulsky) (Coleoptera: Cerambycidae: Lamiini), which has caused great economic losses in ecology and society, is a wood-boring beetle native to eastern China and Korea. The insect attacks many species of hardwood trees (Xiao, 1992; Lingafelter et al., 2002; Zhang and Luo, 2003) and has become an invasive pest in other parts of the world. It has been found infesting urban shade trees in North America in the cities of New York, Chicago and Jersey (Haack, 2003), and in Europe in cities such as Braunau of Austria. Adults undergo a 1–2-week period of maturation, feeding on the foliage and tender bark of twigs of host trees before oviposition (Keena, 2002; Smith et al., 2002). Females then chew slits or holes through the bark of host trees and lay their eggs under the bark. Only a single egg is laid in each oviposition site (Lingafelter et al., 2002), and females often chew many potential oviposition sites that they do not ultimately use for oviposition (Keena, 2002). Larvae feed in the cambium during their first few stadia and then bore into the wood, where they continue to feed, eventually forming a pupal chamber. Larval boring produces structural weaknesses and disrupts the flow of water and nutrients within the host trees, leading to the death of the branches and ultimately the whole trees.

Major programs have been undertaken by the related Chinese agencies to control this insect (Zhang et al., 1999). However, there are not so many effective biological methods to control the insect pests. Therefore, it is necessary to find an effective biological way to control the pest. One way for the control that has been investigated is to apply the non-woven fiber bands containing cultures of entomopathogenic fungi. This delivery system is used for the control of adult lamiine cerambycid pests, particularly *Anoplophora chinensis* (*A. malasiaca*) and *Psacothea hilaris*, in Japanese orchards with *Beauveria brongniartii* (Higuchi et al., 1997). Fungal bands are placed around tree trunks and branches, and the pest adults will be inoculated when creeping across the bands. The key elements that make this control method effective in Japan are as follows: (1) adult lamiine pests in Japanese orchards are susceptible to *B. brongniartii*; (2) the non-woven bands serve as the environment for maintaining viable infective fungal spores for several months (Higuchi et al., 1997; Hajek et al., 2007); and (3) the normal daily activity of lamiine orchard pests in Japan is worming up and down the bark surface. The other way is to use the INA bacteria to

Received September 8, 2008; accepted October 28, 2008

Baohui WANG, Jianwei ZHENG, Dazhuang HUANG (✉), Da WANG, Xiaoyong HAN, Xiaohong WANG
College of Forestry, Agricultural University of Hebei, Baoding 071000, China
E-mail: huangdazhuang@126.com

kill the larvae of *A. glabripennis* through freezing-induction (Sun et al., 1997) and exterminate the *Nosema glabripennis* Zhang parasitizing the longhorned beetle (Zhang et al., 2003), etc.

Metarhizium (Metsch.) Sorokin, formerly known as *Entomophthora anisopliae*, is a fungus that infects insects as a biological control agent, and it has been successfully used in the control of many agricultural and forest pests. But there is no systematic study on the application of *Metarhizium* Sorokin to control *A. glabripennis*. Adults of *A. glabripennis* are susceptible to the fungal pathogen *Metarhizium anisopliae* (Dubois, 2003; Dubois et al., 2007) and they commonly walk on tree branches and trunks (Lance et al., 2000). Field trials with fungal bands made with *M. anisopliae* against *A. glabripennis* in China demonstrate a faster mortality of beetles in the treated plots compared with the control plots (Hajek et al., 2006).

Previous studies on the pathogenic mechanism shows that one of the reasons why the infected larvae died was the histopathologic dysfunction in the tissues and organs (Huang et al., 2005; Wang et al., 2005; Zhang et al., 2007), but there has been no symptomatic and histopathological study of *A. glabripennis* larvae infected with *Metarhizium* Sorokin until now. In this experiment, we observed the symptoms and studied the sections through light microscopy of the infected larvae to study the histopathologic process, the method of infection and the speed of lesion development, all of which can provide a scientific basis for the pathogenesis of *Metarhizium* Sorokin to determine the best time of use.

2 Materials and methods

2.1 Materials

Metarhizium Sorokin MS01 was selected from nine strains with high virulence against *A. glabripennis* larvae in the collecting field in 2006. *A. glabripennis* larvae were collected from the breeding adults in the laboratory, who laid eggs that were hatched in 90 days.

2.2 Methods

2.2.1 Treatment of test insects

The larvae of the same size were chosen to be immersed directly in the spore suspension of 1×10^8 conidia·mL^{-1} for 30 s so that spores could attach themselves to the surfaces of the larvae. Thereafter, the infected larvae were put into carved willow branches and sampled at 1, 2, 3, 4 and 5 days in turn.

2.2.2 Preparation of section for light microscopy

The samples were put into a fixative named Bragil Fluid with 1 g picric acid dissolved in 80% ethanol 150 mL, then 60 mL formaldehyde and 15 mL acetic acid were added. After being fixed for 24 h, the larvae were rinsed several times by 50% and 70% ethanol and stored in 70% ethanol. After a series of ethanol dehydration treatment, we used the conventional methods of section for light microscopy to cut the larvae into 8–12 μm slices. They were then observed and photographed with an Olympus optical microscope.

2.2.3 Observation of external symptom of infected larvae

For inoculation, each treatment of 20 larvae was immersed in a spore suspension of 1×10^6 conidia·mL^{-1} and 1×10^8 conidia·mL^{-1} for 30 s, and then moved to the carved willow branches. The treated insects were observed and photographed daily, with mortality and external characteristics recorded in the case of the larvae. The dead bodies were moisturized and cultured, to determine whether the larvae died of infection with MS01 when the post-mortem white mycelial and green conidia appeared.

3 Results

3.1 Observation of external symptom of *A. glabripennis* larvae infected with MS01

Early in the infection process, there was no significant change in either activities or feedings. However, 24–48 h after inoculation, the high concentration inoculation larvae changed in color gradually, and the soaked dark spots appeared in some parts (Fig. 1 (a)). A similar case occurred at 48 hpi in the low concentration inoculation larvae. This was because of the host reactions, including the accumulation of the corpuscle within the dermis, as well as the hypertrophy and melanin of the cuticle. Subsequently, the infected larvae reduced their feedings and became unresponsive, numb and died gradually at 72 hpi. The dead larvae with the high concentration inoculation became swollen and hard or rigid throughout the body because the mycelium absorbed the water in the body rapidly. Meanwhile, the dead body with the low concentration inoculation was a little softer initially, and gradually turned into a corpse. The mycelium first grew on the intersegmental membrane when moisturizing and culturing the two types of the dead bodies. Then, a white hyphal layer formed on the surface of the body (Fig. 1 (b)), and finally the whole body was covered by green conidia (Fig. 1 (c)).

3.2 Histopathologic changes of *A. glabripennis* larvae infected with strain MS01

Paraffin sections show the spores of MS01 attached to the partial cuticle of *A. glabripennis* larvae. The conidia germinated and penetrated through the cuticle, and the

Fig. 1 Symptom of *A. glabripennis* larvae infected with strain MS01
Note: (a), (b) and (c) represent the soaked spots on the cuticle of infected larvae, the white mycelium on the cuticle of infected larvae, and the green conidia on the cuticle of infected larvae, respectively.

germ tubes passed through the epithelial layers into the body cavity, multiplied and invaded the tissues nearby the penetration point, such as the fat body, muscle, tracheal tissue, malpighian tubule, alimentary canal, etc. Most tissues and organs had histopathologic changes with the multiplication of the mycelium.

The conidia attached to the cuticle of *A. glabripennis* larvae at 24 hpi, germinated and penetrated through the cuticle at 36 hpi, passing through the epithelial layers into the body cavity at 48 hpi, and the color of the penetration point deepened, resulting from the function of mechanical stress and enzyme, leading to degradation in the epicuticle of *A. glabripennis* larvae (Hajek and St Leger, 1994; Vestergaard and Butt, 1999; Wang et al., 2004; Wang et al., 2008). With the germ tubes passing through continually, the endocuticle gradually filled with hyphae, and the protein-chitin composite decomposed, leaving the exocuticle alone at last, with the cuticle separated from the epidermis (Fig. 2 (b)). Mycelium began to damage the dermis at 72 hpi, trichogen cells disintegrated, and the bristle of infected larvae fell, showing trichopore (Fig. 2 (c)). The surface had visible black spots in the process of cuticle infection, resulting from the cuticle defense of phenoloxidase.

3.2.1 Trachea

The structure of a healthy trachea was regular and tubular, and there were taenidiums on the surface (Fig. 3 (a)). The structure of spiral-wound was destroyed in the latter infection. The taenidiums were damaged and the cavity emerged partially at 48 hpi (Fig. 3 (b)), then disappeared gradually. Most trachea were disintegrated at 96 hpi (Fig. 3 (c)).

3.2.2 Fat body

Healthy fat body was irregular, structural affinity and well-defined smooth verge was also visible (Fig. 4 (a)). It became significantly deformed, loose in structure, and vacuoled, with the edge incised at 72 hpi (Fig. 4 (b)), and then it became blurred, releasing fat particles like grapes. These particles were dissolved into fat cells and mixed gradually with debris net, and the fat body was covered up with hyphae at 120 hpi (Fig. 4 (c)).

3.2.3 Muscle

The healthy muscle tissue had a tight structure, shaped like a bar or a lump (Fig. 5 (a), (c)), while the muscle tissue became loose and cracked at 72 hpi, and then separated into fragments gradually (Fig. 5 (b), (d)), and finally disintegrated.

3.2.4 Malpighian tube

Healthy malpighian tubes were composed of filamentous processes separated from each other, showing small diameters (Fig. 6 (a)), while the infected malpighian

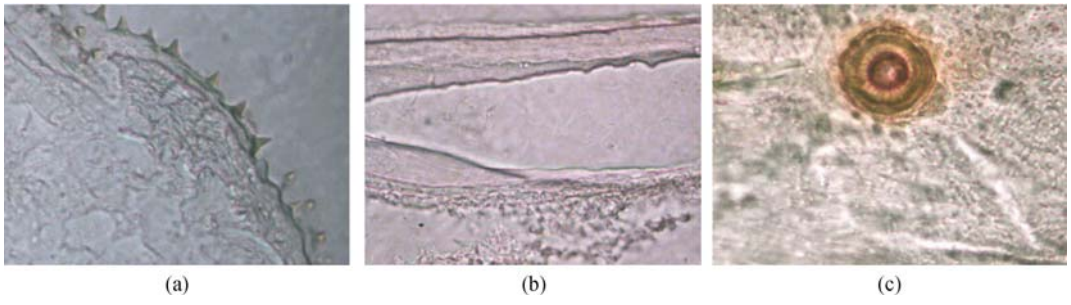

Fig. 2 The histopathological changes of integument of *A. glabripennis* larvae infected with strain MS01(×160)
Note: (a) indicates healthy cuticle; (b) indicates cuticle of infected larvae separated from epidermis at 48 hpi; (c) indicates bristle of infected larvae fell at 72 hpi.

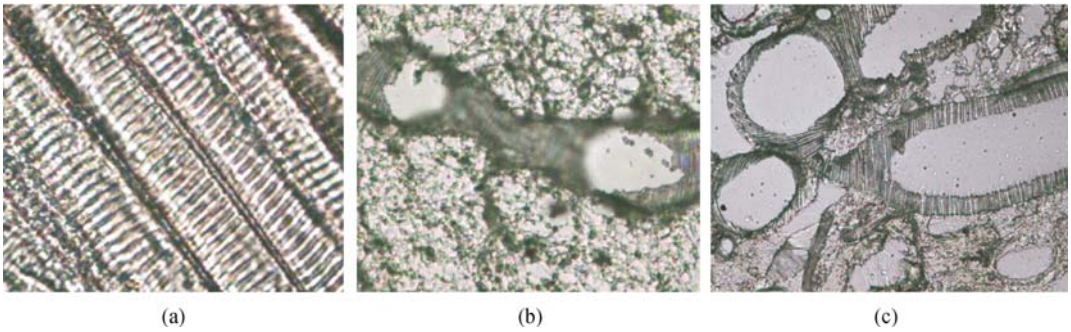

Fig. 3 The histopathological changes of trachea of *A. glabripennis* larvae infected with strain MS01 (×160)
Note: (a) indicates healthy trachea; (b) indicates that the taenidium of infected larvae was damaged and the cavity emerged at 48 hpi; (c) indicates the taenidium of infected larvae slowly disappeared at 96 hpi.

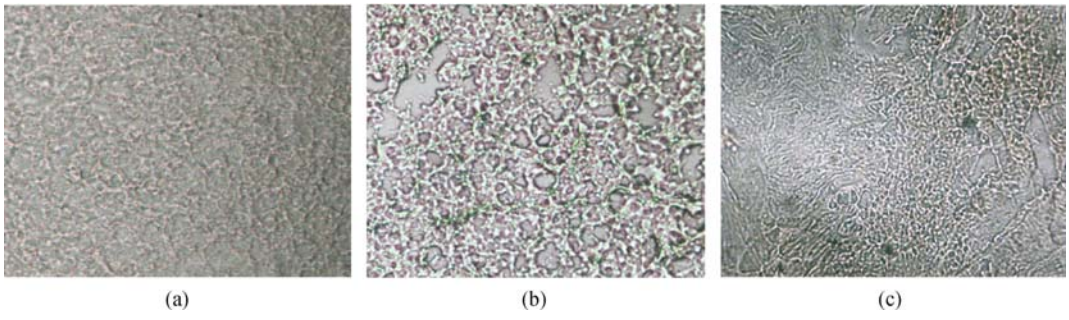

Fig. 4 The histopathologic changes of fat body of *A. glabripennis* larvae infected with strain MS01 (×160)
Note: (a), (b) and (c) represent healthy fat body, incompact fat body of infected larvae at 48 hpi, and fat body of infected larvae covered up with hyphae at 120 hpi, respectively.

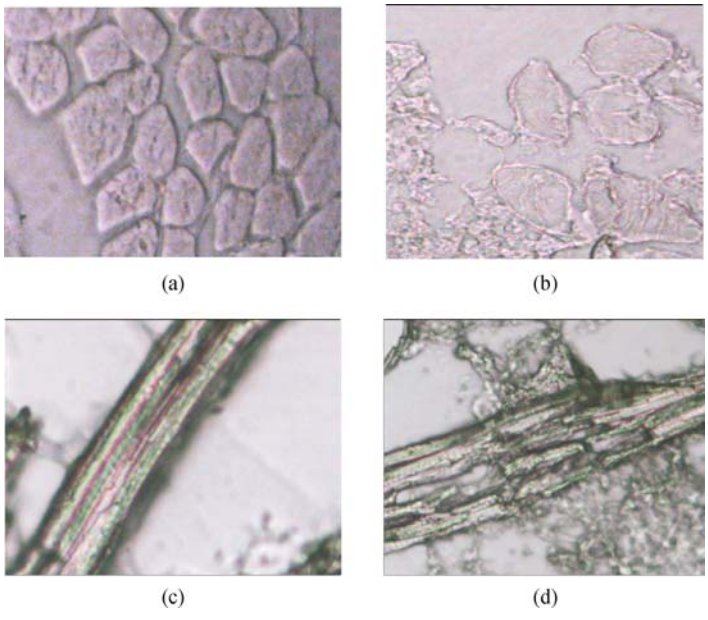

Fig. 5 Healthy muscle tissue ((a), (c)) and a crack in the loose muscle tissue of infected larvae at 72 hpi ((b), (d)) (×160)

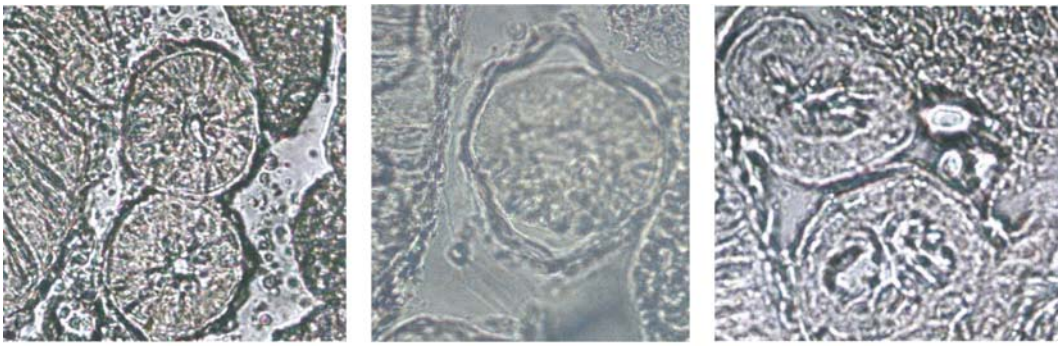

Fig. 6 Histopathologic changes of malpighian tubes of *A. glabripennis* larvae infected with strain MS01 (×800)
Note: (a) stands for normal malpighian tubes showing a small diameter; (b) stands for a malpighian tube of infected larvae showing prominent external perimeter at 72 hpi; (c) stands for malpighian tubes of infected larvae showing large diameter and covered up with hyphae at 96 hpi.

tubes shows a prominent external perimeter at 72 hpi (Fig. 6 (b)), and was covered with hyphae at 96 hpi, showing larger diameters (Fig. 6 (c)).

3.2.5 Alimentary canal

The healthy alimentary canal comprises peritrophic membrane, surrounded by regular columned cells, clinging to the muscle of wall cells (Fig. 7 (a) and Fig. 7 (d)). When the hyphae passed through the hemocoel, the alimentary canal became susceptible to being destroyed. The peritrophic membrane of the infected larvae disappeared at 72 hpi (Fig. 7 (b)). Subsequently, they invaded the mid-gut epithelium directly, and the columned cells were disorganized in the alimentary canal at 96 hpi (Fig. 7 (e)). The columned cells became slack and fell off, with parts of them having vacuoles, and were separated from the muscle of wall cells at 120 hpi (Fig. 7 (c)). All of the alimentary canal was decomposed, and cracks emerged. Part of the blood cells flew into hemocoel followed by blood, and then the alimentary canal was gradually disorganized (Fig 7 (f)). The intestinal cavity became turbid at last.

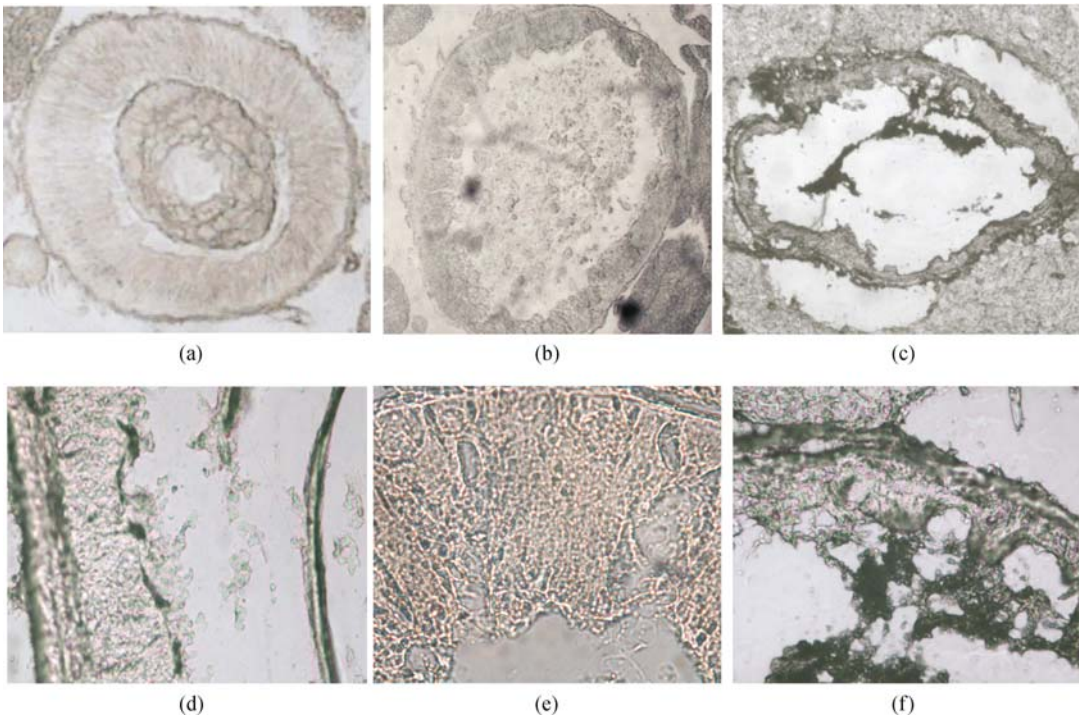

Fig. 7 Histopathological changes of the alimentary canal of *A. glabripenni* larvae infected with strain MS01
Note: (a), (b), (c), (d), (e) and (f) represent healthy alimentary canal (×160), peritrophic membrane of infected larvae disappeared at 72 hpi (×160), the disorganizing alimentary canal at 120 hpi (×160), healthy mid-gut wall cells and peritrophic membrane (×640) columned cells vacuoled in the alimentary canal at 96 hpi (×640) and remnant alimentary canal at 120 hpi (×640), respectively.

4 Discussion

In our study, we found that the intersegmental membrane to be the first invasion site when *A. glabripennis* was infected with *Metarhizium* Sorokin, as the layer of chitin was thin in the intersegmental membrane, making it possible for germ tubes to pass through. These data supported the idea proposed by Wang et al. (2004) that the head of *Plutella xylostella* susceptible to *Metarhizium* Sorokin may result from large quantities of intersegmental membrane in the head. When moisturizing and culturing the two types of the dead bodies, the white mycelium first grew on the intersegmental membrane, also further proving that the site was vulnerable to the invasion of *Metarhizium* Sorokin. Symptoms appeared earlier in the high concentration inoculation larvae than in the low one in the treatment of immersion in spore suspension, demonstrating that the quantity of conidia attached to the cuticle may be associated with the toxicity of *Metarhizium* Sorokin and the sensitivity of the host.

Based on the section for light microscopy, we knew the tissues in the whole body were damaged to different degrees, and obvious pathological changes took place. After the inoculation of insects for three days, a variety of solid organs were filled with hyphal bodies, and the external distribution had a larger density than the inner. Besides, there were two types of hyphal bodies: One was small granular or short-rod with a diameter smaller than blood cells, generally located in the hemolymph, while the other was a long filament, far larger than the length of the blood cells and was usually located with a solid-state organization around it. All these show that the reproduction of the hyphal bodies were mainly in the hemolymph of the larvae. Following the blood circulation, the small type of hyphal bodies then passed into the peripheral tissues and organs. With the growth of mycelium, the hosts died and the mycelium gradually penetrated into the inner lumen of the solid-state organs. This mode of infection was similar to the process of *Galleria mellonella* infection with *Metarhizium anisopliae* (Prasertphon and Tanada, 1968). Although the direct cause of death was not clear yet, we could infer that most tissues and organs were damaged when the hyphae passed through, so that the normal physiological activities were disrupted. The larvae hemocoel was full of hyphae with the multiplication of the mycelium, leading to the lack of nutrients and blocking of fluid circulation, which caused physical hunger and metabolic disorders.

In the near-death larvae or 48 h after inoculation, a few number of hypha were filling the bodies, while a large mount were concentrated in hemocoel in the vicinity of the invasion site. Only a very small amount passed into the adjacent body fat. This reveals that the death of the larvae was not due to direct undermining of tissues and organs of the host. We can infer that the invasion of mycelium weakened the function of all the tissues and organs, and the infected larvae died of without normal physical activities. In addition, the physiological and biochemical changes may also be the cause of death when infected with *Metarhizium* Sorokin. Ferron suggested that what really caused the larvae infected with imperfect fungi to die was a fungal toxin. The toxin could not only inhibit the immune function of the host, but also affect the central nervous system and cause some pathogenic effects, thereby speeding up the death process of the host (Sloman and Reynolds, 1993). However, Wang and You (1999) believed that the pathogenic mechanism of these toxins was not very clear, only to find that the pure toxin had effects on immune pressure, muscle paralysis and malpighian tube damage. Therefore, whether or not the infected *A. glabripennis* larvae died of toxins is a question that needs further studies.

References

Dubois T (2003). Biological control of the Asian longhorned beetle, *Anoplophora glabripennis*, with entomopathogenic fungi. Dissertation for the Doctoral Degree. New York: Cornell University, 200

Dubois T, Lund J, Bauer L S, Hajek A E (2007). Virulence of entomopathogenic hypocrealean fungi infecting *Anoplophora glabripennis*. BioControl, 53(3): 517–528

Haack R A (2003). Research on *Anoplophora glabripennis* in the United States. Nachrichtenblatt des Deutschen Pflanzenschutzdienstes, 55 (4): 68–70

Hajek A E (2007). Asian longhorned beetle: ecology and control. In: Pimentel D, ed. Encyclopedia of Pest Management, Vol II. Boca Ratonz: CRC Press, 21–24

Hajek A E, Dubois T, Lund J, Smith M, Bauer L, Li Z (2007). Developing fungal bands for control of Asian longhorned beetle, *Anoplophora glabripennis*, in the US. Journal of Anhui Agric Univ, 34: 149–156

Hajek A E, Huang B, Dubois T, Smith M T, Li Z (2006). Field studies of control of *Anoplophora glabripennis* (Coleoptera: Cerambycidae) using fiber bands containing the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria brongniartii*. Biocontr Sci Technol, 16: 329–343

Hajek A E, St Leger R J (1994). Interactions between fungal pathogens and insect hosts. Annu Rev Entomol, 39: 293–322

Higuchi T, Saika T, Senda S, Mizobata T, Kawata Y, Nagai J (1997). Development of biorational pest control formulation against longicorn beetles using a fungus, *Beauveria brongniartii* (Sacc.) Petch. J Ferment Bioengin, 84(3): 236–243

Huang D R, Lei Z R, Wang G P, Wen Z J (2005). The histopathological changes of *Locusta migratoria manilensis*after infection with *Metarhizium anisopliae*. Chinese Bulletin of Entomology, 42(2): 183–185 (in Chinese)

Keena M A (2002) *Anoplophora glabripennis* (Coleoptera: Cerambycidae) fecundity and longevity under laboratory conditions: Comparison of populations from New York and Illinois on *Acer saccharum*.

Environmental Entomology, 31: 490–498

Lance D R, Wang B, Li J, Luo Y (2000). Adult behavior of the Asian longhorned beetle. USDA, For Serv Gen Tech Rpt NE-273, 26

Lingafelter S W, Hoebeke E R (2002). Revision of *Anoplophora* (Coleoptera: Cerambycidae). Washington: Entomological Society of Washington

Prasertphon S, Tanada Y (1968). The formation and circulation, in Galleria, of hyphal bodies of entomophthoraceous fungi. J Invertebr Pathol, 11: 260–280

Sloman I S, Reynolds S E (1993). Inhibition of ecdysteroid secretion from Manduca prothoracic glands *in vitro* by destruxins-byclic depsipeptide toxins from the insect pathogenic fungus *Metarhizium anisopliae*. Insect Biochem Molec Biol, 23(1): 43–46

Smith M T, Bancroft J, Tropp J (2002). Age specific fecundity of *Anoplophora glabripennis*(Coleoptera: Cerambycidae) on three tree species infested in the United States. Environmental Entomology, 31: 76–83

Sun F Z, Xing W, Zhang Y X, Gao R T (1997). Preliminary study on effect of INA bacteria to kill larvae of *A. glabripennis* through freezing-induction. Forest Research, 10(1): 96–99 (in Chinese)

Vestergaard S, Butt T M (1999). Light and electron microscopy studies of the infection of the western flower thrips *Frankliniella Occidentalis*(Thysanoptera: Thripidae) by the entomopathogenic fungus *Metarhizium anisopliae*. J Invertebr Pathol, 73: 25–33

Wang H C, You M S (1999). Mechanism of invasion by *Metarhizium anisopliae*against insect. Microbiology, 26(1): 71–73 (in Chinese)

Wang X H, Ji H F, Huang D Z (2008). Symptom and histopathological study of *Apriona germari* larvae infected with *Beauveria bassiana* Bb00. Science of Sericulture, 34(1): 18–23 (in Chinese)

Wang Y, Lei Z R, Zhang Q W (2004). The symptom and histopathological changes of *Plutella xylostella* infected by *Metarhizium anisopliae*. Chinese Journal of Biological Control, 20(3): 156–160 (in Chinese)

Wang Y, Lei Z R, Zhang Q W, Wen Z J (2005). Microscopic observations of infection process of *Metarhizium anisopliae*on the cuticle of the diamondback moth, *Plutella xylostella*. Acta Entomologica Sinica, 48(2): 188–193 (in Chinese)

Xiao G R (1992). China Forest Entomology. Beijing: China Forest Press, 455–457 (in Chinese)

Zhang B, Bai Y, Mitsuaki S, Inoue J (1999). Microbial Control of *Anoplophora glabripennis* adults by application of non-woven fabric strips with *Beauveria bassiana*and *B. brongniartii*. Journal of Northwest Forestry University, 14(1): 68–72 (in Chinese)

Zhang B, Liu Y N, Bai Y (1999). Pathogenic fungi of *Anoplophora* spp. (Coleoptera: Cerambycidae) in Ningxia Hui Autonomous Region and their virulence. Journal of Beijing Forestry University, 21(4): 67–72 (in Chinese)

Zhang S Q, Wang J H, Huang J H (2007). Histopathologic changes of *Brontispa longissima* larvae infected by *Metarhizium anisopliae*. Chinese Journal of Tropical Crops, 28(3): 97–99 (in Chinese)

Zhang X Y, Luo Y Q (2003). Major Forest Biological Disasters in China. Beijing: China Forest Press, 32–33 (in Chinese)

Zhang Y A, Wang Y Z, Zhang L (2003). Preliminary study on a new pathogen (*Nosema glabripennis* Zhang) parasiting the longhorned beetle *Anoplophora glabripennis* (Motsch). Scientia Silvae Sinicae, 39(2): 171–173 (in Chinese)