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# Histopathological changes of *Ceroplastes japonicus* infected by *Lecanicillium lecanii*

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ABSTRACT

The infection process and pathological changes of Japanese wax scale, *Ceroplastes japonicus* Green, by the hyphomycete *Lecanicillium lecanii* (Zimmermann) Gams & Zare were investigated by light, scanning and transmission electron microscopy. The results showed that *L. lecanii* generally infected the wax scale by penetrating the integument. The anal area, the body margin, around the base of mouthparts and legs, over the stigmatic furrow and the area around the vulva were susceptible places, while the wax test had an inhibitory effect on *L. lecanii*. Within 24 h after inoculation, conidia became attached to the cuticle, and within 48 h, hyphae adhered to the integument of the scale and their tips differentiated into specialized infection pegs. Penetration of the cuticle occurred within 72 h of inoculation; the fungus caused the insect cuticle to rupture and hyphae entered the insect body through these openings. Within 72 h after inoculation, *L. lecanii* entered the hemocoele of the scale and formed blastospores. After 96 h, blastospores were dispersed throughout the hemolymph and completely disrupted the hemocytes, resulting in damage of the cell nucleus and agglutination of chromatin. Concomitant to colonization of the hemolymph, the internal organs and tissues, e.g., tracheae, malpighian tubules and muscle fibers, were also infected. As the infection progressed, the wax test and body changed color from white and red, respectively, to yellowish. After 144 h, the internal tissue structure was totally compromised and the insects died. After this time, new conidiophores bearing conidia were produced on the surface of the cadavers.


## 1. Introduction

There are about 7000 known species of scale insects (Hemiptera: Coccoidea) recorded in the world. Many are important pests in fruit orchards, forests, horticulture and ornamental plants. Scale insects secrete wax from a variety of glands, which forms a protective coating over the body which limits the efficacy of insecticide sprays (Xie, 1998). In addition, spraying pesticides causes environmental pollution, affects natural enemies and promotes insecticide resistance. The availability of biological control agents such as entomopathogenic fungi would be highly desirable but few natural pathogens have been reported from scales; of these, *Lecanicillium lecanii* (Zimmermann) Gams & Zare (formerly known as *Verticillium lecanii* (Zimm.) Viegas) is probably the most important.

Although this fungus was originally isolated from a scale insect, *Lecanii coffeae*, by Nivter in Ceylon in 1861, it has been used as a biological control agent for aphids, whiteflies and mites in recent years (Van Driesche and Bellows, 1996; Askary et al., 1999; Gindin et al., 2000; Cuthbertson and Walters, 2005; Kim, 2007; Yuan et al., 2007). Askary et al. (1999) characterized ultrastructural and cytochemical aspects of infection of the aphid *Macrosiphum euphorbiae* by *V. lecanii*. In comparison, research on infection of scale insects by this fungus has largely focused on surveys of infected species and some simple trials to test their potential for controlling scales. To date, 40 species of scale insects have been recorded as parasitizd or controlled by *L. lecanii* on tea, citrus, coffee, megranate and other plants in twenty countries. Of these, 20 scale species have been in the Coccidae (Evans and Hywel-Jones, 1997; Cavallazzi et al., 1998; Lo and Chapman, 1998), 10 species in Diaspididae (Evans and Prior, 1990), 10 species in Pseudococcidae (Yin et al., 1996, 2000), one species in Margarodidae (Asensio et al., 2005) and one species in Phoenicococcidae (Yuan et al., 2007). Studies on the pathology of *L. lecanii* on scale insects are scarce, probably because the wax covering functions as a natural barrier to infection. It is important to understand the infection mechanism of *L. lecanii* on scale insects to identify critical processes and information that will aid development of this fungus into an effective biopesticide.

*L. lecanii* strain number 3.4505, originally isolated from a scale insect, was used in this study. Japanese wax scale, *Ceroplastes japonicus* (Hemiptera: Coccoidea: Coccidae), was chosen as the target host. *C. japonicus* is a pest throughout Asia and Europe. More than 150 host plant species have been recorded in China. The infection process and pathological changes in the scale insect were studied using light microscopy, scanning electron microscopy and transmission electron microscopy.

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## 2. Materials and methods

### 2.1. Entomopathogenic fungus and scale insects

Cultures of *L. lecanii* strain number 3.4505 was purchased from the China General Microbiological Culture Collection Center. The fungus was cultured on potato-dextrose-agar at 25 °C for 5 days, and the conidia were harvested using a sterile blade from the surface of the culture medium. Conidial suspensions were prepared and the conidial concentration was determined using a haemocytometer and adjusted to $5 \times 10^7$ conidia/ml with sterile water. Tween-80 was added as a wetting agent at a concentration of 0.1% (v/v) to the inoculum.

*C. japonicus* Green adult females were collected from densely-infested twigs of persimmon trees, *Diospyros kaki* L., in orchards in Linyi County (E110°17′30.7″ N34°58′52.9″) in Shanxi Province. For the infection trials, sample twigs were approximately 7 cm in length and 0.5 cm in diameter with 30 individual wax scales on it.

### 2.2. Inoculation of C. japonicus with L. lecanii

Infested twigs were randomly placed in seven groups and sprayed to wetness with 5 ml of conidial suspension. Control twigs were treated with sterile 0.1% Tween-80 only. Following inoculation, the scale insects were air dried for about five minutes and transferred to rearing chambers at 25 ± 0.5 °C, 85 ± 10% RH (regulated by saturated KCl solution), and a photoperiod of 16:8 (L:D). A total of seven replicate groups of seven twigs/group were treated for sampling.

### 2.3. Light microscopy (LM)

#### 2.3.1. Observation of appearance

Gross changes in the appearance of the scale insects were directly monitored undered a dissecting microscope 24 h, 48 h, 72 h, 96 h, 120 h, and 144 h after inoculation. Photographs were taken on Olympus C5050Z digital camera (OLYMPUS OPTICAL Co. Ltd).

#### 2.3.2. Slide preparation and observation of disease development in the host

*L. lecanii*-treated scale insects were collected 24 h, 48 h, 72 h, 96 h, 120 h, and 144 h after inoculation, whereas scale insects from the control treatment were collected only once, 144 h after inoculation. For each observation period, about 10 scale insects were sampled. They were first immersed in 4% (v/v) glutaraldehyde in 0.2 M phosphate buffer at pH 7.2 for 48 h at 4 °C. After rinsing three times with 0.2 M phosphate buffer, scale insects were dehydrated in a series of ethanol solutions (10 min each in 35%, 55%, 75%, 85%, 95%, and 100% (v/v)) and cleared in a xylene series (10 min each in 35%, 55%, 75%, 85%, 95%, and 100% (v/v)). The samples were embedded in paraffin wax by immersing in 1:1(v/v) xylene: paraffin mixture for 48 h at 56 °C, then in paraffin for 48 h at 56 °C. Embedded specimens were serially sectioned to a thickness of 6 μm and mounted on glass slides. They were mordanted with 2% ferrovanadium (30 min) and stained with 5% hematoxylin (1.5 h) and then destained with saturated picric acid (1.5 h). Finally, the slides were enclosed with neutral balsam and observed under an OLYMPUS BX-51 light microscope (OLYMPUS Co. Ltd. Japan).

### 2.4. Scanning electron microscopy (SEM)

Distribution, germination and development of *L. lecanii* on scale insect cuticle were observed 24 h, 48 h, and 72 h after inoculation using scanning electron microscope. Samples of infected scale insects were fixed for 48 h in 4% glutaraldehyde and rinsed three times in phosphate buffer (0.2 mol/L). They were then dehydrated in an acetone series (10–100%). Acetone was displaced by liquid carbon dioxide and samples were dried using EMS 850 critical point drying apparatus. The wax tests of the samples were removed after critical point drying. The samples were mounted on microscope slides (about 2.5 cm × 2.5 cm) and sputter-coated with gold to a thickness of about 20 μm. They were examined with a JSM-840 scanning electron microscope (JEOL Ltd. Japan) operating at 15 kV. Micrographs were taken using a Canon EOS 350D digital camera.

### 2.5. Transmission electron microscopy (TEM)

The samples were collected, fixed and rinsed as described for light microscopy. Then the samples were post-fixed in 1% (v/v) osmium tetroxide in phosphate buffer for 3 h at 4 °C, dehydrated in an ethanol series (10–100%) and embedded in Epon 812. Semithin sections (1 μm) localized for the ultrathin section were mounted on glass slides and stained with 1% (v/v) toluidine blue. These were examined under an Olympus BX-51 light microscope. Ultrathin sections (0.08 μm) cut using a Reichert Jung ultramicrotome were collected on copper grids and counterstained with uranyl acetate and lead citrate. Ultrathin sections were examined using a JEM-1200EX transmission electron microscope (JEOL Ltd. Japan) with an accelerating voltage of 80 kV. Micrographs were taken on Lucky TEM negative film (Lucky company Inc. China) using the micro-

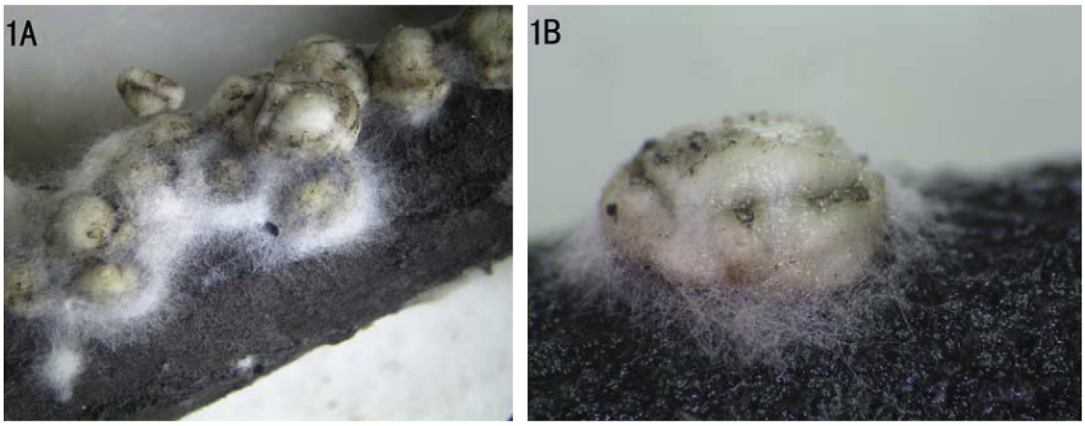


**Fig. 1.** Photographs of *Ceroplastes japonicus* infected by *Lecanicillium lecanii* observed under a dissecting microscope. (A) Mycelia developed in thick layers among scale insects on the twig, 144 h after inoculation. (B) 72 h after inoculation, the margin of the insect was surrounded by mycelia.