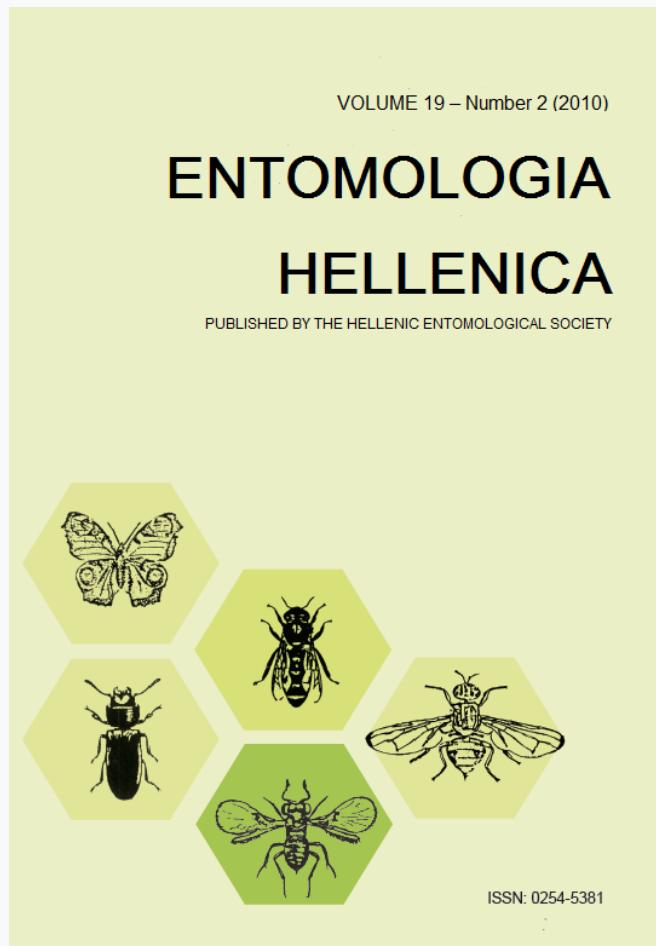


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## Integument of soft scale insects and the invasion of the pathogenic fungus *Lecanicillium lecanii*

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### ABSTRACT

In order to understand how the entomopathogenic fungi infect soft scale insects (Hemiptera: Coccoidea: Coccidae), the integument of four species, namely *Ceroplastes japonicus* Green, *Didesmococcus koreanus* Borchsenius, *Rhodococcus sariuoni* Borchsenius and *Coccus hesperidum* L. were infected with the pathogenic fungus, *Lecanicillium lecanii*, strain NO. 3.4504. The invasion process was studied using the electron microscopy and chemical analyses methods. The results showed that the fungal conidia easily become attached on the surface of the cuticle, especially the sites with furrows and ridges. The hyphae penetrate the integument using mechanical force and also by the cuticle degradation by the extracellular enzymes. The fungal penetration results in the structural anamorphosis and disruption of chitin in the procuticle, causing damage and separation of cuticle from the epidermis with wax gland cells. The rise in fungal protease activity occurs prior to the production of chitinase and the activity values correlate with the quantities of the protein and chitin contained in the cuticle of the target scale insects.

KEYWORDS: scale insect, *Lecanicillium lecanii*, cuticle, ultrastructure, protease, chitinase.

### Introduction

Entomopathogenic fungi invade their host insect primarily through penetrating the integument. Thus, successful infection is thought to depend primarily on the fungal ability to adhere to and penetrate the host integument (Charnley 1991, Schreiter et al. 1994, Askary et al. 1999). Penetration of the insect integument involves both mechanical pressure from the hyphae and degradation action by extracellular enzymes secreted by the fungi (Goettel et al. 1989). Entomopathogenic fungi in culture produce enzymes capable of digesting the major components of insect integument (St. Leger et al. 1986). The insect integument is composed of up to

70% protein and so particular attention has been focused on the role of proteases in the penetration process (Natasha and Charnley 2008). St. Leger (1991) considered that protease activity determines the virulence of entomopathogenic fungi to some degree. Feng (1998), who studied the reliability of extracellular protease and lipase activities of *Beauveria bassiana* isolates used as their virulence indices, suggested that protease activity could be used as a virulence index of the fungus infecting host insects. Chitin is also an important component of the insect integument (Gullan and Cranston 2005). However, investigations into the degradation of chitin by chitinase secreted by the fungus have been relatively scarce.

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Scale insects are important pests in forests, fruit trees and ornamental plants (Xie 2006). *Lecanicillium lecanii* (formerly named *Verticillium lecanii*) is an effective pathogenic fungus against the scale insects (Evans and Hywel-Jones 1997, Zare and Gams 2001). However, there have been few studies on the mechanism of invasion of the scale insect integument by *L. lecanii*.

In this study, four species of soft scale insects: *Didesmococcus koreanus* Borchsenius, *Rhodococcus sariuoni* Borchsenius, *Ceroplastes japonicus* Green and *Coccus hesperidum* L., were chosen as the target hosts. Their integument structure before and after *L. lecanii* infection was observed using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The fungal proteases and chitinases were also investigated in order to understand their role in integument infection of the scale insects.

## Materials and Methods

### Entomopathogenic fungus and scale insects

The entomopathogenic fungus, *L. lecanii*, strain NO. 3.4504 (ex scale insect, species unknown), was purchased from the China General Microbiological Culture Collection Center. Conidia were obtained from potato-dextrose-agar culture medium at 25°C for 5 days. The conidia were harvested from the surface of the culture medium using a sterile blade. 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. This preparation was used to inoculate the scale insect and cuticular substrates.

Scale insects: The four species of soft scale insects (Hemiptera: Coccoidea: Pseudococcidae) were *C. hesperidum* (collected off leaves of *Citrus reticulata* Banco), *C. ja-*

*ponicus* (collected off the twigs of *Diospyros kaki* Linn.f.), *D. koreanus* (collected off the twigs of *Prunus persica* (L.) Batsch) and, *R. sariuoni* (collected off the twigs of *Malus pumila* Mill.), all in Shanxi Province of China.

### Scale insect inoculation

The scale insects on the host twigs and leaves were first surface sterilized in 1% sodium hypochlorite for 1 min and rinsed in sterile distilled water. Then the scale insects were sprayed to wetness either with the conidial suspension of *L. lecanii* or treated similarly with sterile 0.1% Tween-80 only. A total of 10 replicates of 30 scale insects of both control and conidia-treated samples were used. Following the inoculation, the scale insects were air dried for about five minutes and then transferred to a rearing chamber to culture at  $25 \pm 0.5^\circ\text{C}$ ,  $85 \pm 10\%$  RH (regulated by saturated KCl solutions), and under a photoperiod of 16:8 (L:D) h.

### Infected scale insect sampling and observation

Gross changes in the appearance of the scale insects were directly monitored under a dissecting microscope 24, 48, 72, 96, 120 and 144 h after inoculation. For each observation period, about 30 scale insects were sampled. They were first immersed in 4% (v/v) glutaraldehyde [prepared in 0.2M, pH 7.2 phosphate buffer (PB)] for 48 h at  $4^\circ\text{C}$ . The fixed samples were used for scanning and transmission electron microscopy.

### Scanning electron microscopy (SEM)

The distribution, germination and development of *L. lecanii* on the scale insect cuticle was observed 24, 48 and 72 h after inoculation using a scanning electron microscope. Samples of infected scale insects were fixed for 48 h in 4% glutaraldehyde and rinsed three times in 0.2M PB. They were then dehydrated through 70, 80, 90,

100% ethanol (5 min in each stage) and three changes in 100%. The ethanol was then displaced by liquid carbon dioxide and samples were dried using an EMS 850 critical point drying apparatus. The samples were mounted on microscope slides (about 2.5×2.5cm) and sputter-coated with gold to a thickness of about 20 $\mu$ m. 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.

#### Transmission electron microscopy (TEM)

After being pre-fixed in 4% (v/v) glutaraldehyde and rinsed in PB, the samples were post-fixed in 1% (v/v) osmium tetroxide for 3 h at 4°C. They were then dehydrated in an ethanol series in 10% steps and embedded in Epon 812. Semithin sections (1 $\mu$ m), 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 $\mu$ m) cut using a Reichert Jung ultramicrotome were mounted on Formvar-coated nickel grids and either counterstained with uranyl acetate and lead citrate or processed further for cytochemical labeling. Ultrathin sections were examined using a JEM-1200EX transmission electron microscope (JEOL Ltd. Japan) with an accelerating voltage of 80 kV.

#### Chitin labelling

In order to label the chitin in the scale insect's cuticle, an indirect cytochemical labeling technique, wheat germ agglutinin/ ovomucoid-gold cytochemistry (WGA/Ovo-G) was used. Wheat germ agglutinin (WGA; Sigma Chemical Co.), a lectin with N-acetylglucosamine-binding specificity, was used as the first-step reagent. Ovomucoid (Ovo; Sigma Chemical Co.), a high molecular-weight glycoprotein from chicken egg white, with a high affinity for WGA, was chosen as

a second-step reagent, and combined with colloidal gold (G, particle diameter of 10nm; Sigma Chemical Co.) into a complex at pH 5.4 before cytochemical labeling. A blocking liquid (BL), composed of 0.01M, pH 7.2, phosphate-buffer saline (PBS), 0.02% polyethylene glycol (PEG) 20,000 (Sigma Chemical Co.) and 0.02% NaN<sub>3</sub> (Sigma Chemical Co.), was prepared for stabilizing the colloidal gold.

For gold-cytochemical labeling, ultra-thin tissue sections of the scale insect were first incubated on a drop of BL for 30 min and then transferred to a drop of WGA (10 $\mu$ g/ml) in PBS (pH 7.2) for 30 min at 25°C in a moist chamber. Following washing twice with distilled water to remove superfluous WGA, the samples were treated with a drop of BL for 30 min and then incubated on the dilution Ovo-G complex (1:80 in BL) for 30 min. After rinsing with distilled water, the sections were stained with uranyl acetate and lead citrate before examination with the JEM-1200EX transmission electron microscope.

#### Extracellular enzymatic activity assay

Three species of scale insects, *C. japonicus*, *D. koreanus* and *R. sariuoni* were chosen for extracellular enzymatic activity assay. The scale insects were first obtained from their host plants using a tweezer, then washed with distilled water several times in order to get rid of any impurity. The integument was obtained by removing internal tissue and organs from scale insects under a dissecting microscope. The integument was then immersed in chloroform and vibrated repeatedly to dissolve the waxes on the insect surface. Following washing with ethanol to remove chloroform, the cuticle was rinsed with distilled water to eliminate the ethanol. The cuticles were then either prepared for cuticle chemical component measurements or for cuticle substrates.

For chemical component assay, the integument was dried to a constant weight ( $W_0$ ) in a sterile-air-flow cabinet at 60°C. Petroleum ether (weight 10:1 to the cuticle) was added to the cuticle and extracted in ultrasonic for 5 min, then petroleum ether replaced the solvent. After the extraction process had been repeated for five times, petroleum ether (20-30 times of the cuticle weight) was added again and the sample shaken for 8 h to degrease the cuticle. The degreased cuticle was dried to constant weight ( $W_1$ ) at 80°C, and then saturated potassium hydroxide was added and incubated in a glycerol bath to alkaline hydrolysis protein in the cuticle with chitin residual at 160°C for 30 min. Following rinsing with running water and dehydrating with 95% or 100% ethanol, the residual was weighed as the content of chitin ( $W_2$ ). The content of lipid is  $W_1$  subtract form  $W_0$ , and protein content ( $W_{21}$ ) is  $W_2$  subtract form  $W_1$ .

For cuticle substrate preparation, the cuticle was dried in a sterile-air-flow cabinet at 100°C, milled to a fine powder using a mortar and sieved through a 40 $\mu$ m sieve. The cuticle substrate was performed in glass Petri dish viz. Each dish contained 0.2g of cuticle powder and distilled water, which was added as a wetting agent. The cuticle substrates were sterilized in autoclave.

After the cuticle substrates were prepared, a conidial suspension ( $5 \times 10^7$  conidia/ml, 1ml in each substrate) was inoculated onto the substrate and cultured at 25°C and 70% RH for eight days. *L. lecanii* was sampled everyday during its culture. The sample was dissolved in 5ml phosphate buffer (pH 7.0) and centrifuged at 8,500g for 20 min, the supernatant was collected as an enzyme solution for enzymatic assay.

Protease activity was assayed in a reaction mixture containing 1.0ml substrate [1% (W/V) casein solution in Tris-HCl buffer (50mM, pH 8.5)] and 1.0ml enzyme solution. After 30 min at 37°C, the reaction was terminated by the addition of 3ml trichlo-

roacetic acid (0.4M), followed by filtration. Then 1ml filtrate was collected and mixed with 5ml Na<sub>2</sub>CO<sub>3</sub> (0.4M), followed by the addition of Folin reagent (1ml) and mixed immediately. After chromogenic reaction for 30 min, the absorbency at 680nm ( $A_{680\text{nm}}$ ) was measured by spectrophotometer. One protease unit is defined as the amount of enzyme which produced 1 $\mu$ g L-tyrosine by decomposing protein in each minute. Assays were performed in duplicate. All results are representative of at least two similar experiments using different enzyme and cuticle preparations.

Chitinase activity was assayed in a reaction mixture consisting of 0.5ml colloidal chitin and 0.5ml enzyme solution. After 4h at 37°C, the reaction was terminated by centrifuging at 8,500g for 5 min. 0.2ml supernatant was added to 0.08ml potassium tetraborate. After shaking and boiling for 5 min, the mixture was cooled to room temperature using tap water. Then 1.2mL, 10% DMAB reagent was added and reacted at 37°C for 20 min. The reaction system was cooled to room temperature using tap water and the absorbency ( $A_{585\text{nm}}$ ) was measured at 585nm under a spectrophotometer. One chitinase unit is defined as the amount of enzyme which produced 1 $\mu$ g N-acetylglucosamine by decomposing chitin in each minute.

## Results

### Ultrastructural characteristics of the scale insect integument

In the TEM photographs, it was observed that the integument of the scale insect was composed of cuticle, formation zone, epidermis and basement membrane (Fig. 1A). The cuticle, about 1.5-5.0 $\mu$ m in thickness, consisted of two principal layers, epicuticle and procuticle. The epicuticle was outermost and thin (0.15 $\mu$ m) while

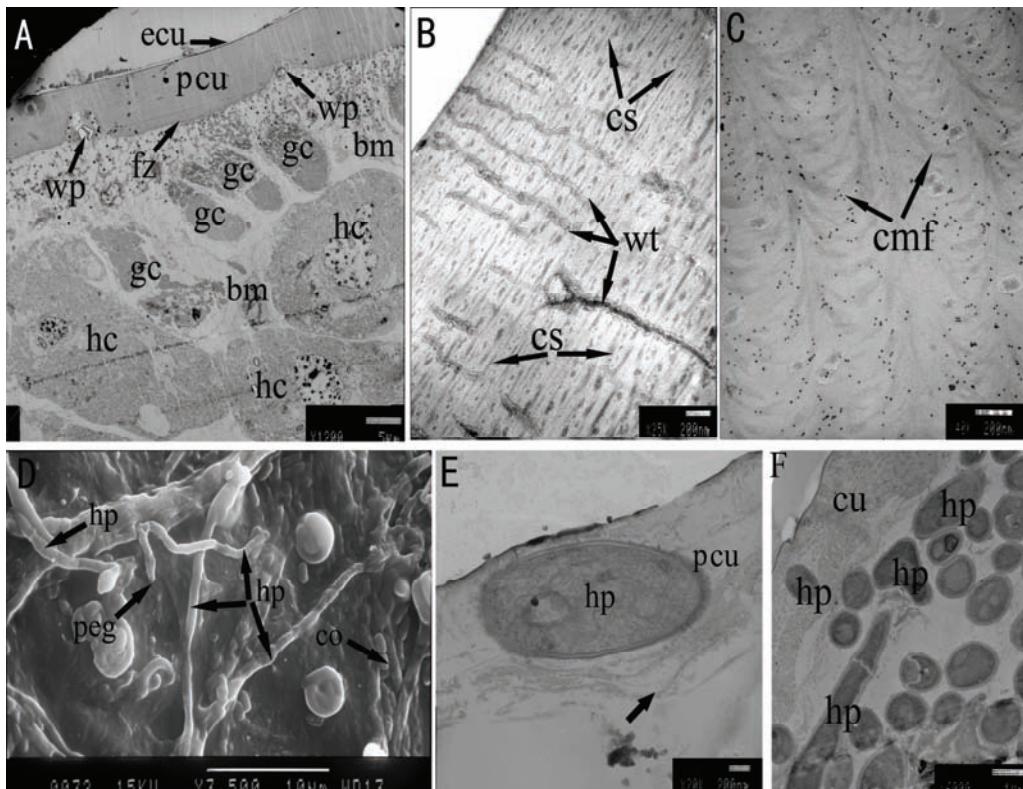


FIG. 1. Ultrastructural photographs of soft scale insect integument and fungal penetration. (A-C) Integument ultrastructure of *C. japonicus*. (A) An entire view of the integument, showing epicuticle (ecu), procuticle (pcu), formation zone (fz), epidermis and basement membrane (bm), wax gland cells (gc), wax pores (wp), and hemocyte (hc). Magnification 1200 $\times$ , bar = 5 $\mu$ m. (B) showing transverse parallel sheet (cs) of the chitin and longitudinal wax-secretion tubes (wt) in the procuticle. Magnification 25,000 $\times$ , bar = 200nm. (C) chitin labelled with WGA-Ovo/G complex, showing chitin microfibrils (cmf) in a helical structure. Magnification 40,000 $\times$ , bar = 200nm. (D-F) infection of the cuticle of *Coccus hesperidum* by *Lecanicillium lecanii*. (D) SEM photograph, showing the conidial attachment and hyphal invasion on the surface of the integument. co: conidium; hp: hyphae; peg: penetration. (E) Hyphae (hp) penetrating into the procuticle (pcu) causing disruption (arrow) of the parallel sheet of chitin. Magnification 20,000 $\times$ , bar = 200nm. (F) Masses of hyphae (hp) colonizing the integument, resulting in damage to the cuticular structure. Magnification 6000 $\times$ , bar = 1 $\mu$ m.

the underlying procuticle was thick (1.3-4.8 $\mu$ m). The epidermis possessed wax glands that were closely packed large complex cells. One wax secretion duct from each wax gland passed through the cuticle to the surface. The basement membrane lies beneath the epidermis. In

the procuticle, under high magnification, the chitin labeled with the WGA/Ovo-G complex was observed to be arranged in a parallel sheet (Fig. 1B). When magnified to 40,000 $\times$ , the chitin microfibrils, combined with gold particles, was in an evenly helical arrangement (Fig. 1C).

### Invasion by *L. lecanii* of the scale insect integument

It was observed under SEM that the fungal conidia became attached on the surface of the integument after inoculation, particularly where there were furrows and ridges. The conidia germinated and developed into hyphae. The hyphae adhered to the integument surface and developed penetration pegs at their tips to invade the integument (Fig. 1D).

The penetrating hyphae entered the procuticle 48 h after inoculation. The hyphae penetrated vertically through the cuticular layer or travelled transversely in the procuticle. The fungal invasion damaged the integument causing transmutation and disruption of the parallel sheet structure of the chitin (Fig. 1E). With the growth of the hyphae, the integument became almost entirely occupied by the fungus, causing the cuticle and epidermis to separate (Fig. 1F).

### Protease and chitinase activity assay

Protein and chitin are the primary components of the scale insect integument. From Table 1 it can be seen that the protein contents in *D. koreanus*, *R. sariuoni* and *C. japonicus* cuticle were 2.0g, 2.1g and 1.8g, while their

chitin contents were 0.2g, 0.1g and 0.1g respectively.

The protease and chitinase activity of *L. lecanii* cultured on the cuticular substrate of the three scale insect species were assayed. The results showed that the protease activity of *L. lecanii* cultured on *D. koreanus* and *R. sariuoni* cuticle was similar, their peak value emerging at the sixth day with  $34.58\pm 1.01$  U/g and  $33.94\pm 1.61$  U/g respectively, and with a trend to gradual increase during the first six days, followed by a rapid reduction. On the other hand, the protease activity on *C. japonicus* cuticle was obviously lower during the first six days and peaked at  $34.30\pm 0.48$  U/g on the seventh day (Fig. 2).

Chitinase activity of *L. lecanii* exhibited a slow increase during the first four days followed by a rapid increase during the second four days. The chitinase activity of the culture on the cuticle substrate of *D. koreanus* was much greater than that on *R. sariuoni* and *C. japonicus*. The value for *D. koreanus* was  $13.82\pm 0.35$  U/g on the eighth day, while that for *R. sariuoni* and *C. japonicus* cuticle substrates were  $7.28\pm 1.36$  U/g and  $8.33\pm 0.20$  U/g respectively (Fig. 3).

TABLE 1. Cuticular components of the three scale insects.

Scale insects	<i>D. koreanus</i>		<i>R. sariuoni</i>		<i>C. japonicus</i>	
Cuticular components	Content (g/2.5g)	Percent (%)	Content (g/2.5g)	Percent (%)	Content (g/2.5g)	Percent (%)
Protein	$2.0\pm 0.01$ b	80.0%	$2.1\pm 0.02$ b	84.0%	$1.8\pm 0.03$ a	72.0%
Chitin	$0.2\pm 0.03$ b	8.0%	$0.1\pm 0.01$ a	4.0%	$0.1\pm 0.01$ a	4.0%
Lipid	$0.3\pm 0.02$ a	12.0%	$0.3\pm 0.01$ a	12.0%	$0.6\pm 0.02$ b	24.0%
Total	2.5	100%	2.5	100%	2.5	100%

Note: The data for cuticle components in the table are the means from the three independent experiments, each with two replicates; and the letters "a" and "b" behind numerals indicate their significant difference ( $P = T_{0.05}$ )

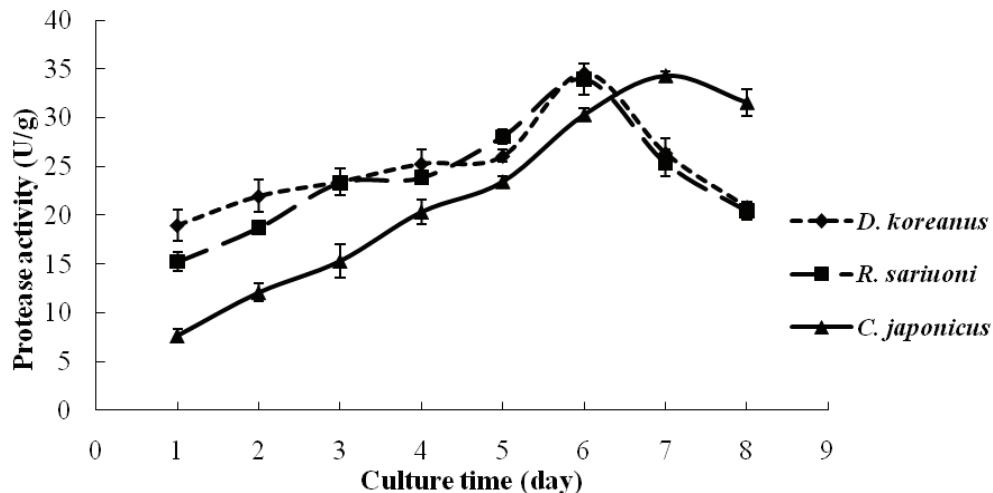


FIG. 2 Protease activity curve of *L. lecanii* No.V3.4504 cultured on cuticle substrate of *D. koreanus*, *R. sariuoni* and *C. japonicus*.

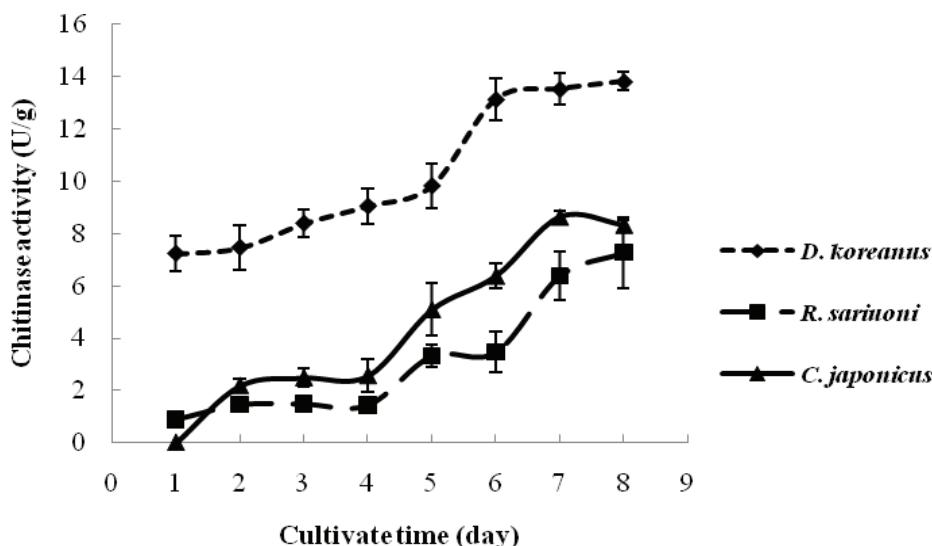


FIG. 3. Chitinase activity curve of *L. lecanii* No.V3.4504 cultured on cuticle substrate of *D. koreanus*, *R. sariuoni* and *C. japonicus*.

By comparing the protease and chitinase activity, an interesting phenomena was found. The protease activity of the fungus was

obviously higher than chitinase activity, corresponding with the much higher protein content than the chitin content in the in-

tegument. The protease activity occurred earlier than the chitinase activity, indicating that protein degradation occurred prior to chitin degradation. For the three experimental scale insects, the higher protease activity on the cuticles of *D. koreanus* and *R. sariuoni* was correlated with the higher protein content in their integument. Similarly, the higher chitinase activity on *D. koreanus* cuticle corresponded with the greater chitin content in its integument.

## Discussion

The insect integument is the exoskeleton and forms a protective layer that prevents the insect body from attack. The general structure of the insect integument has been well studied (Gullan and Cranston 2005). However, the structure of scale insect integument and its penetration by a pathogenic fungus has not been previously reported. In this study, the integument ultrastructure of four species of soft scale was observed using an electron microscopy. The results showed that the integument of scale insects was also composed of epicuticle, procuticle, epidermis and basement membrane, and that the epidermis possessed various closely packed wax-secreting glands. The most significant find in this study is that the chitin and its arrangement in the procuticle could be directly seen using the gold-cytochemical labelling technique, when the chitin was combined with gold particles. On the TEM photographs, the arrangement of chitin microfibrils appeared as helically arranged and parallel sheets. This is the first time that this structure has been observed in the scale insect studies.

It has been reported that entomopathogenic fungi, such as *Metarrhizium anisopliae*, *B. bassiana* and *L. lecanii*, infected their host insects primarily by penetrating through the integument (Goettel et al. 1989, Schreiter et al. 1994, St. Leger 1996, Feng 1998, Askary

et al. 1999). We found that *L. lecanii* infecting the scale insect integument involved conidial adherence to the integument surface, germination, hyphal formation and integument penetration. In the infection process, the fungal conidia mainly became attached to the areas with furrows and ridges, and the hyphae penetrated the integument using their penetration pegs. It was important that the damage to the chitin by the fungus was directly observed by using WGA/Ovo-G labeling technique. In the procuticle, the parallel sheet structure of the chitin was disrupted around the hyphae and the density of the gold particles became obviously decreased, indicating that the chitin was pierced by the mechanical pressure of the fungus and degraded by the fungal chitinase. This is the first direct visible evidence of chitinase activity in the scale insect integument penetration process by a pathogenic fungus. Similar results were found in the potato aphid by Askary et al. (1999).

Charnley (2003) pointed out that the insect integument was composed of up to 70% protein and so it is not surprising that extracellular fungal protease appears to be particularly important in the penetration process. In the present study, the protease activity of the fungus was obviously greater than the chitinase activity, corresponding to the greater protein content in the integument of the four species of soft scale insects (72-84% compared with the chitin content of 4-8%). Also, the protease activity occurred earlier in the process than the chitinase activity, indicating that protein degradation occurred prior to chitin degradation. This confirmed that the chitin was embedded in and linked to the protein in the procuticle. Thus, it was only once the protein had been degraded by the protease that the chitin was exposed and could be degraded by the fungal chitinase. By comparing protease and chitinase activity of the fungus cultured on the three scale insects cuticle, it is concluded that the greater the protein content in the

integument, the higher the protease activity. The same relationship was found between the fungal chitinase activity and the chitin content in the procuticle.

### Acknowledgements

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## Εξωσκελετός ειδών της οικογένειας Coccidae και η μόλυνση από εντομοπαθογόνο μύκητα *Lecanicillium lecanii*

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### ΠΕΡΙΛΗΨΗ

Προκειμένου να κατανοήσουμε τον τρόπο που οι εντομοπαθογόνοι μύκητες μολύνουν είδη κοκκοειδών εντόμων (Hemiptera: Coccoidea: Coccidae), ο εξωσκελετός τεσσάρων ειδών *Ceroplastes japonicus* Green, *Didesmococcus koreanus* Borchsenius, *Rhodococcus sariuonii* Borchsenius και *Coccus hesperidum* L. μολύνθηκε με τον μύκητα *Lecanicillium lecanii*, στέλεχος NO. 3.4504. Για τη μελέτη χρησιμοποιήθηκαν τεχνικές ηλεκτρονικής μικροσκοπίας και χημικής ανάλυσης. Παρατηρήθηκε ότι τα κονίδια του μύκητα προσκολούνται εύκολα στην επιφάνεια του εξωδερματίου, ειδικά σε σημεία με εσοχές και αυλακώσεις. Οι υφές του μύκητα διαπερνούν τον εξωσκελετό με μηχανικές μεθόδους αλλά και μέσω της λύσης του δερματίου από εξωκυτταρικά ένζυμα. Η είσοδος του μύκητα προκαλεί δομική αναμόρφωση και διακοπή της συνέχειας του δερματίου. Αυξημένη δραστηριότητα των πρωτεασών του μύκητα συμβαίνει πριν την παραγωγή χιτινάσης και το μέγεθος της δραστηριότητάς της σχετίζεται με την ποσότητα πρωτεΐνης και χιτίνης στο δερμάτιο των εντόμων.