Turk J Biol
29 (2005) 137-147
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An SEM Study of the Sporulation Process of *Pandora Neoaphidis* and *Neozygites Fresenii*

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Received: 28.04.2005

Abstract: The objective of this study was to investigate the development of fungal pathogens in aphid hosts and identify structures characteristic of each fungal species. Aphids infected by *Pandora neoaphidis* and *Neozygites fresenii* were incubated for different time periods and examined by SEM. In *P. neoaphidis*, hyphal bodies concentrated in clumps beneath the cuticle and these formed rosette patterns consisting of a central structure, the developing cystidium, surrounded by developing conidiophores. Rhizoids were the first structure emerging from the *P. neoaphidis*-killed aphid. Primary spores were formed after 6 h and secondary spores after 10 h of incubation. In *N. fresenii*, the conidiophores did not group together to form rosette patterns as in *P. neoaphidis*. Rhizoids were not formed by *N. fresenii* but infected aphids were held to the leaf by their mouthparts. The primary spores of *N. fresenii* were formed at 2 h and were generally round or ovoid in shape. These primary conidia formed long slender capillary tubes that produced secondary conidia which were almond shaped and possessed a terminal mucoid hapteron. Saprophytic fungi began to develop on *N. fresenii*-killed aphids at 24h and by 48 h the whole cadaver was covered. In *N. fresenii*, spore formation occurred earlier than in *P. neoaphidis*, but numbers of spores formed by *P. neoaphidis* were higher than by *N. fresenii*.

Key Words: Entomopathogenic fungi, *Pandora neoaphidis*, *Neozygites fresenii*, SEM

Pandora neoaphidis ve *Neozygites fresenii*'nin Taramalı Elektron Mikroskobunda (SEM) Sporulasyon Sürecinin Çalışılması

Özet: Bu çalışmanın amacı afitlerde fungus pathojenlerin gelişimi ve her fungus için karakteristik yapıların saptanması. *Pandora neoaphidis* ve *Neozygites fresenii* ile enfekte olmuş afitler belirli zaman periyotlarında SEM ile incelenmiştir. *Pandora neoaphidis*'de hyphalar integümentin altında küme oluşturur ve rozet adı verilen bu yapıların ortasında merkezi, konidi taşıyıcı (conidiophore) ile çevrelenmiş cystidium adı verilen yapılar bulunur. *Pandora neoaphidis* tarafından öldürülmüş afitlerden ilk çıkan yapılar rhizoidlerdir. Birincil sporlar 6 saat, ikincil sporlar 10 saat inkübasyondan sonra oluşur. *Neozygites fresenii*'da *P. neoaphidis*'de olduğu gibi rozet yapılar bulunmaz. *Neozygites fresenii* ile enfekte olmuş afitler rhizoidler oluşturmaz fakat bitkiye ağız yapıları ile tutunurlar. Birincil sporlar yuvarlak ve ovoid şekildedir ve inkübasyondan 2 saat sonra oluşurlar. Bu birincil sporlar uzun ince kapillari tüpler oluşturarak bunlardan, badem şeklinde, mucoid hapteron uca sahip ikincil sporları üretir. Saprofitik funguslar afitlerin ölümünden 24 ve 48 saat sonra bütün vücudu kaplar. *Neozygites fresenii*'da spor oluşturma zamanı daha kısa fakat oluşturulan spor miktarı *P. neoaphidis*'e göre daha azdır.

Anahtar Sözcükler: Entomopathojenik fungus, *Pandora neoaphidis*, *Neozygites fresenii*, SEM

Introduction

The entomophthoralean fungi, *Pandora neoaphidis* (Remaudière & Hennebert) Humber and *Neozygites fresenii* (Nowakowski) Batko, cause epizootics that greatly reduce aphid populations in the field (1-4). A detailed light microscopic study of *P. neoaphidis* and *N. fresenii* by Brobyn and Wilding (5) and a light and scanning electron microscope (SEM) study of *P. neoaphidis* by Butt et al. (6) provided some information about the infection and developmental processes of these two fungi.

Both primary and secondary conidia of *P. neoaphidis* are infective, and appressoria are formed from the spore or from a germ tube when it comes in contact with the host cuticle. Within 36-48 hours postinoculation, the fungus invades the fat body, mycetomes and muscle tissues. Pea aphids infected with *P. neoaphidis* become sluggish and change color from green to yellow. After they die (in about 84 hours), they turn dark brown in dry air or yellowish brown in a humid environment. Before the hyphal bodies differentiate into rhizoids, cystidia and conidiophores, they become localized beneath the cuticle.

Each cystidium is surrounded by a group of developing conidiophores forming a rosette pattern. The first structures to emerge from the aphid body are rhizoids that attach the host to the plant, and after this the cystidia and conidiophores develop. Soon after the tips of the conidiophores rupture the cuticle, the primary spores form (5,6).

The infective stage of *N. fresenii* is the secondary conidium or capilliconidium, which frequently attaches to the antennae, legs and tarsi (3) and forms an appressorium. After penetrating the cuticle, the fungus forms hyphal bodies that multiply in the hemocoel. The tissues are not invaded until the hemocoel is full of hyphal bodies. The conidiophores do not group together before rupturing the cuticle but they lie approximately parallel with each other. Rhizoids and cystidia are not formed by *N. fresenii* (5).

The purpose of this study was to investigate in detail the sporulation process and identify structures of *P. neoaphidis* and *N. fresenii* after various periods of incubation using a scanning electron microscope (SEM).

Materials and Methods

Pea aphids infected with *P. neoaphidis* were collected from Clemson, SC, USA and cotton aphids infected with *N. fresenii* were collected from the Edisto Research and Education Center, SC, USA.

Dried, mummified aphids infected with *P. neoaphidis* and *N. fresenii* were incubated at room temperature in moist petri dishes for various periods of time. At 2, 4, 6, 8, 10, 12, 16, 24, and 48 h, two aphids from each group were processed for scanning electron microscopy. They were placed in a sealed chamber and fixed in osmium tetroxide vapor for 2 days and then kept dehydrated for 24 h in desiccators to dry sample. Finally, they were coated with gold. Samples were examined with a Hitachi model S-570 SEM. Development of fungal structures such as conidiophores, primary conidia, secondary conidia, cystidia, and rhizoids were monitored for each fungus.

Results and Discussion

Pandora neoaphidis

Before hyphal bodies differentiated into rhizoids, cystidia, and conidiophores, and emerged through the host cuticle, they concentrated beneath the cuticle in clumps seen as raised areas of the cuticle and present on all parts of the host body except the appendages, head capsule and mid-ventral area of the thorax and abdomen (Figure 1). At 2 h incubation, the center of these clumps

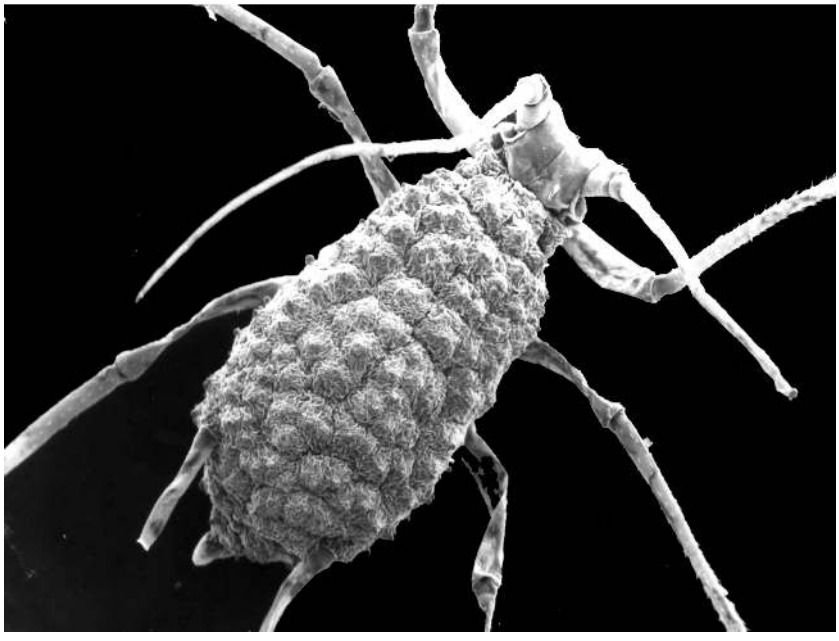

Figure 1. Overall view of a pea aphid infected by *Pandora neoaphidis*. Hyphal bodies emerged through the host cuticle and formed rosette. 45X.

began to swell (Figure 2) and at 4 h, cystidia and conidiophores began to break through the cuticle forming a rosette pattern (Figure 3). The cystidia and conidiophores surrounded most parts of the insects and at 6 h incubation these structures had completed their development (Figure 4). The cystidia were elongate, unbranched and spread among the conidiophores. Cystidia were described by Thaxter (12) as rhizoids that unsuccessful to develop completely to form holdfast since they were located on dorsal part of the infected hosts. However, Brobyn and Wilding (5) and MacLeod (13) found that the rhizoids emerged before the cystidia and the hyphal bodies which from rhizoids were larger than those forming cystidia.

As described in previous studies, the rhizoids were the first structures emerging from the aphid body. They emerged as single unbranched filaments from hyphal bodies in the midventral region of the aphids, and lengthened before branching. The ends of the rhizoids branched when they reached the substrate and combined to form the holdfast (Figure 5). The function of holdfast is to attach the host to the plant. Butt et al. (6) reported similar results and they also determined that rhizoids were formed from large, vacuolated hyphal bodies. In this study, we observed the formation of additional rhizoids during the incubation process after 6 h incubation.

By 6 h incubation, there was an extensive formation of conidiophores and cystidia, and many primary conidia had been formed and ejected and were seen on the surrounding substrate. Butt et al. (6) indicated that cystidia emerged a short time before conidiophores, but we observed that they emerged at about the same time. The function of the cystidia is not known. Butt et al. (6) suggested that cystidia may serve to breach the cuticle or may help to trap moist air over the body. The conidiophores swelled to form primary conidia soon after breaking the cuticle. Primary conidia were clavate and obovoid with a rounded basal papilla (Figure 6). Measurements of 30 spores showed that the mean length was 18.1 mm and mean width was 10.0 mm. Milner et al. (7) reported similar results with mean lengths of 19.0-25.4 and mean widths of 9.8-13.0 µm. In their study, spore size was important in distinguishing *P. neoaphidis* from *Erynia* sp. which had smaller conidia. In our study, primary spore production continued through the 8 h and 10 h incubation periods. As more conidiophores were formed, the rosette pattern on the host body surface was lost.

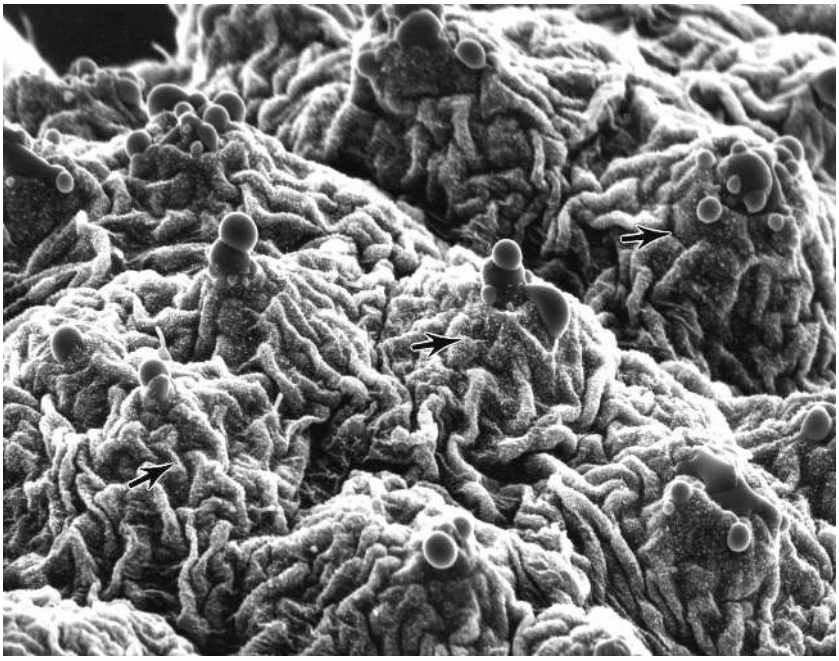

Figure 2. Rosette patterns formed by radial orientation of hyphal bodies around developing cystidia beneath the cuticle in *P. neoaphidis* infected aphids at 2 h. 350X. Solid arrows indicate the rosette patterns.

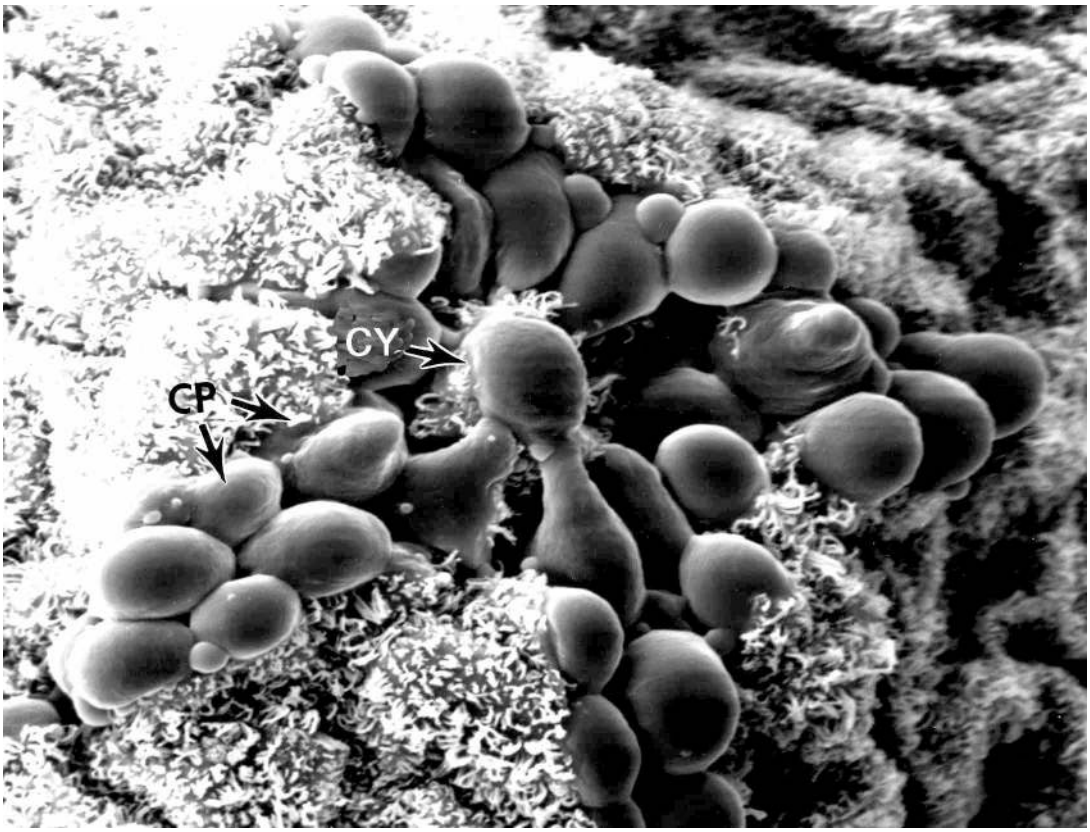

Figure 3. Early stages of cystidia (CY) and conidiophores (CP) of *Pandora neoaphidis* break through of the cuticle at 4 h. 1300X.

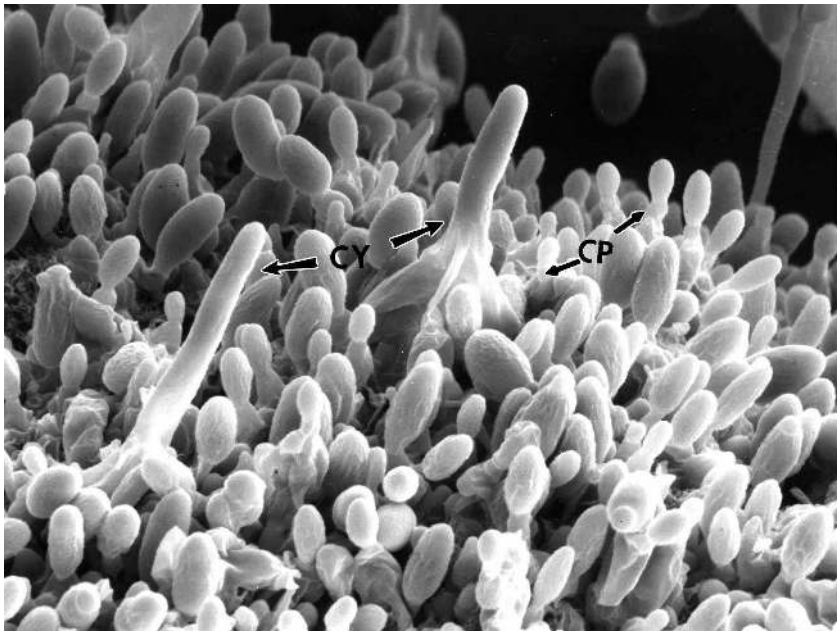

Figure 4. Conidiophores (CP) and cystidia (CY) of *Pandora neoaphidis* at 6 h. 600X.

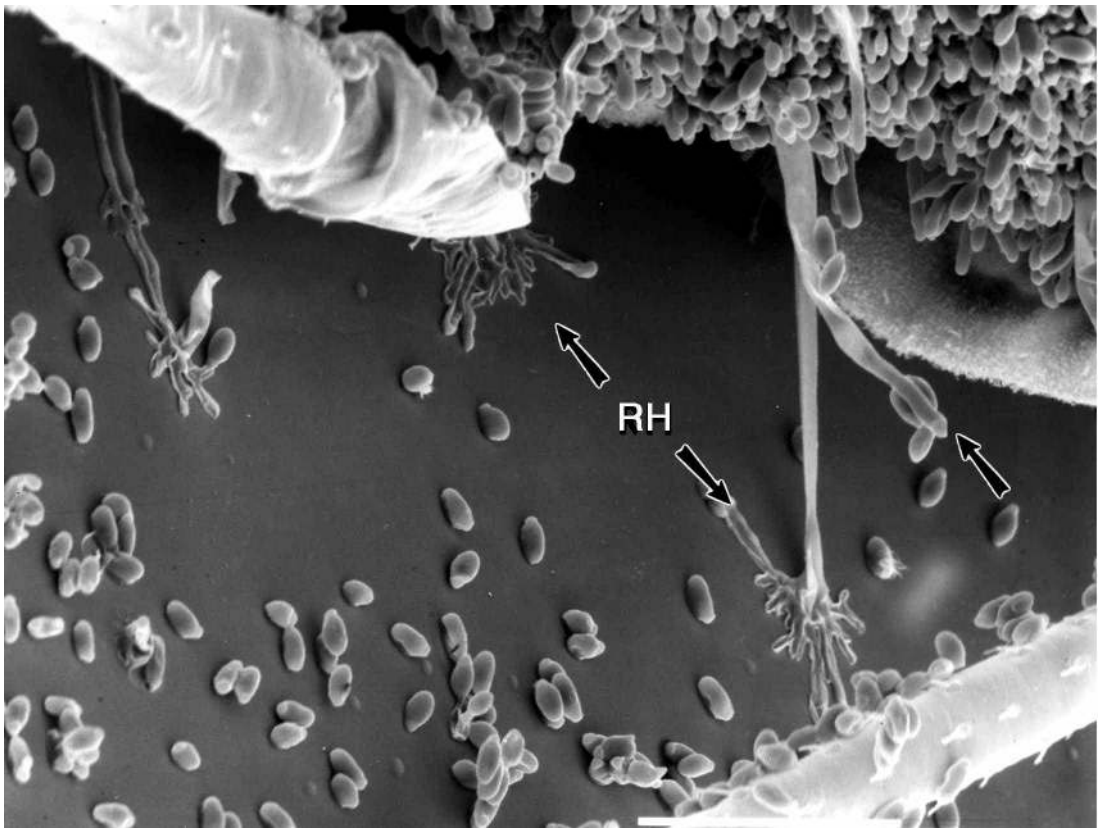

Figure 5. Rhizoids (RH) emerging from the midventral region of *Pandora neoaphidis* infected aphid at 6 h. 230X. Solid arrow indicates rhizoids without holdfast.

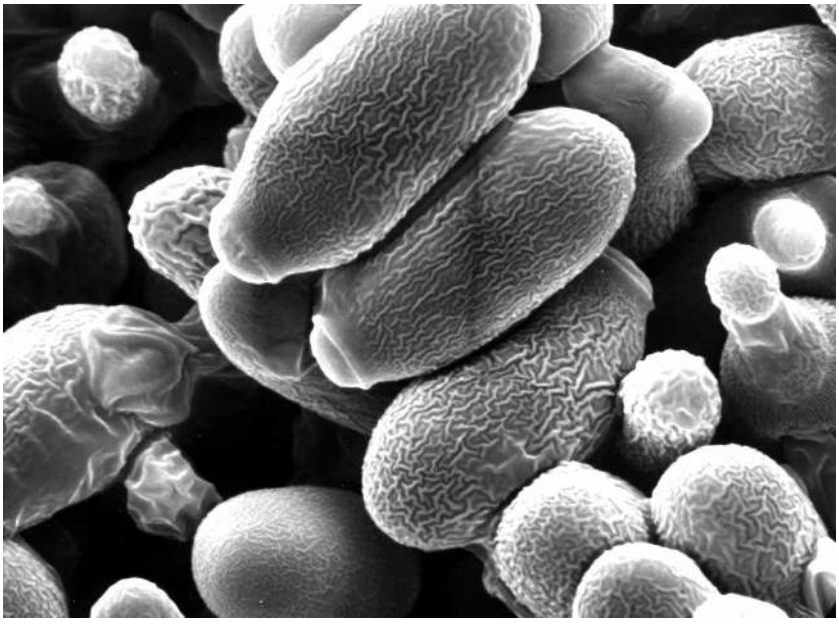

Figure 6. Primary conidia of *Pandora neoaphidis* at 8 h. 2,500X.

Secondary spore formation was first observed at 10 h, and the shape of these spores was similar to the primary spores (Figure 7). It was difficult to distinguish between primary and secondary spores. At the later incubation times, especially at 24 h, a solid mat of hyphae, conidiophores, primary spores and cystidia covered the host body surface (Figure 8). At 24 h, the body of the aphid had collapsed and by 48 h, secondary fungi had grown over the cadaver.

Neozygites fresenii

The sporulation process for *N. fresenii* occurred more quickly than for *P. neoaphidis*. At the 1 hr incubation time, the hyphal bodies below the cuticle had begun to produce conidiophores, pushing up the cuticle. Steinkraus and Slaymaker (8) determined that conidia were discharged from the air-dried aphid cadavers at 1 h after rehydration, and conidial production was completed within 5 h. In *N. fresenii* the conidiophores did not group together in clumps to produce rosettes and cystidia as in *P. neoaphidis* (Figure 9). Brobyn and Wilding (5) and Carner (9) stated that the conidiophores lay approximately parallel to each other and formed a compact palisade layer under the cuticle. In our study, at 2 h incubation, the conidiophores emerged through all parts of the aphid's body except the legs, mouthparts, and head. A primary conidium was formed at the tip of each conidiophore and was discharged from the host body (Figure 10). Primary conidia were generally round or ovoid with a basal papilla at the point of attachment to the conidiophore (Figure 11). Average measurements of 20 spores were 15.7 x 11.4 µm. Steinkraus et al. (3) reported a mean of length of 16.7 µm and a mean of width of 10.7 µm from the field-sporulated fungus.

Primary conidia formed a long slender capillar conidiophore that produced a secondary conidium (capilliconidium), and these were first observed at the 4 h incubation time. Secondary conidia were almond-shaped and possessed a terminal mucoid hapteron (Figure 12) that functions to attach the secondary conidium to the host (11). From field observations, Steinkraus et al. (3) observed that within 24 hours most primary conidia had produced capilliconidia. Average measurements of 20 capilliconidia were similar to Steinkraus et al. (3) (19.6 µm in length and 10.3 µm in width). At 6, 8, 10, 12, and 16 h incubation times, secondary conidia formation increased and almost all primary conidia completed secondary spore formation. Steinkraus et al. (3) determined that within 24 h, saprophytic fungi covered *N. fresenii*-killed aphids. In our observation, at the 24 h incubation period, saprophytic fungi had begun to develop on the *N. fresenii*-killed aphids, and by 48 h the whole cadaver was covered (Figure 13).

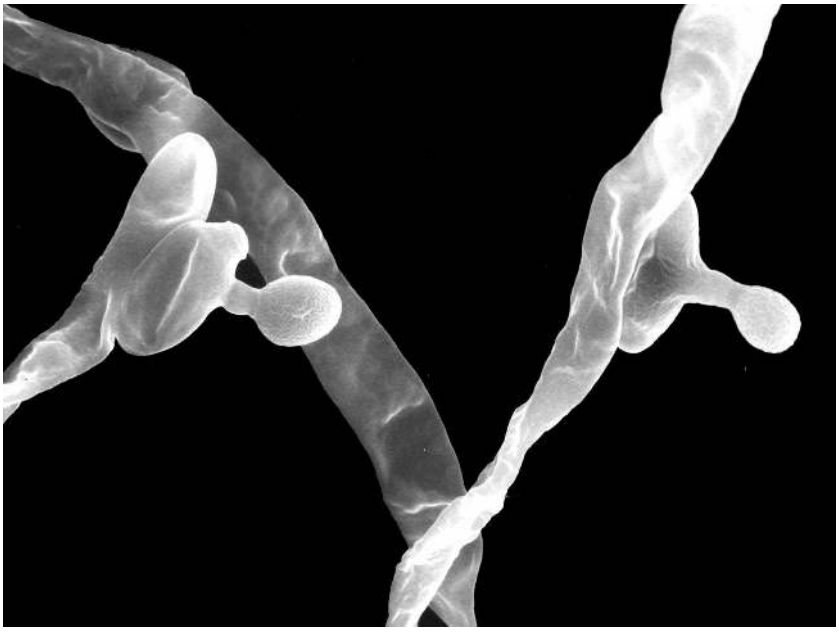

Figure 7. Formation of secondary spores of *Pandora neoaphidis* from primary spores at 10 h. 1,200X.

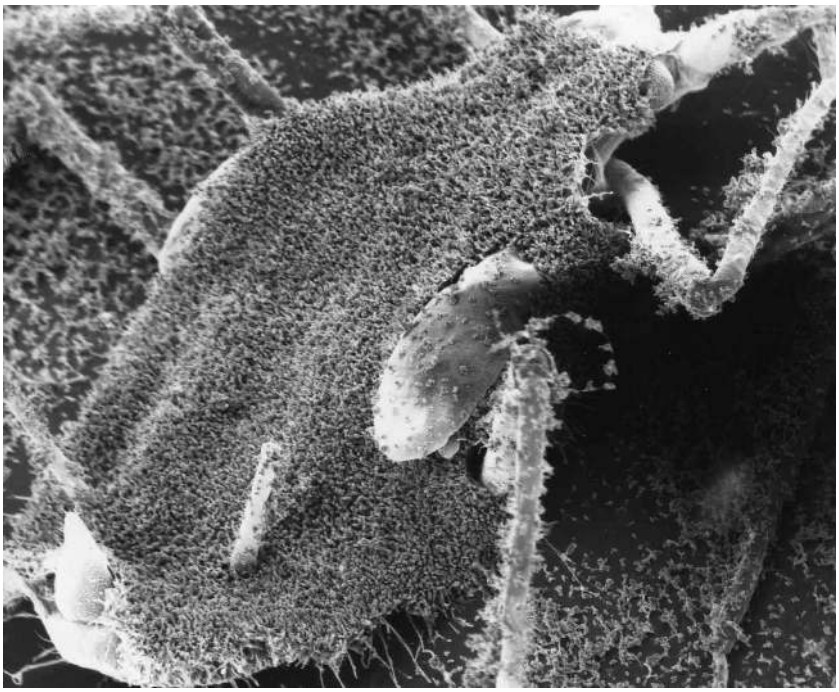

Figure 8. Hyphae, conidiophores, primary and secondary spores and cystidia of *Pandora neoaphidis* covering the host body surface at 24 hr. 45X.

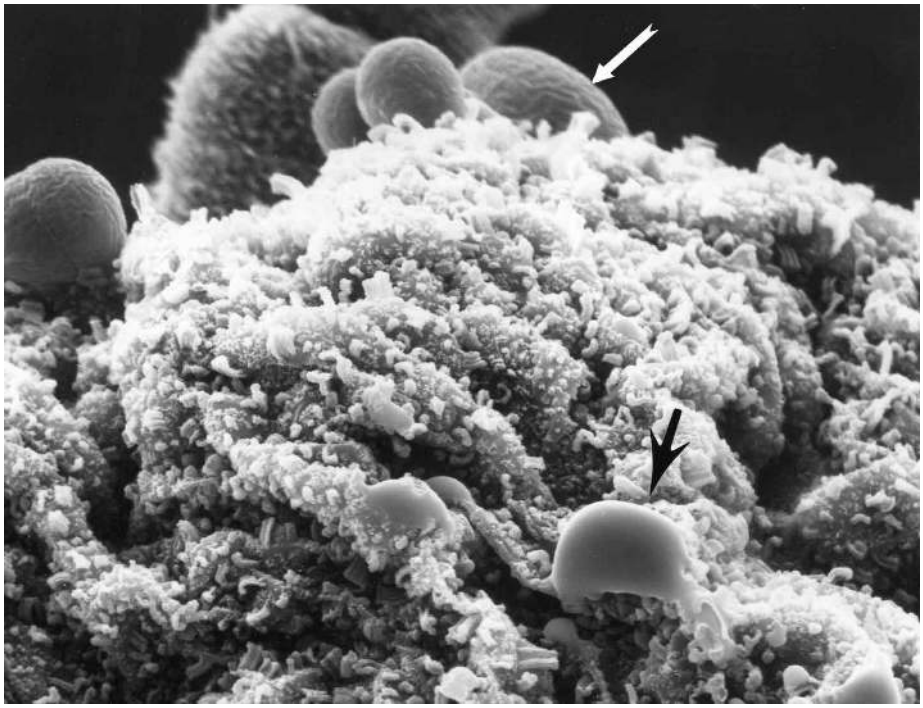

Figure 9. Solid arrows indicate emerging conidiophores from surface of *Neozygites fresenii*-killed aphid at 1 h incubation. 850X.

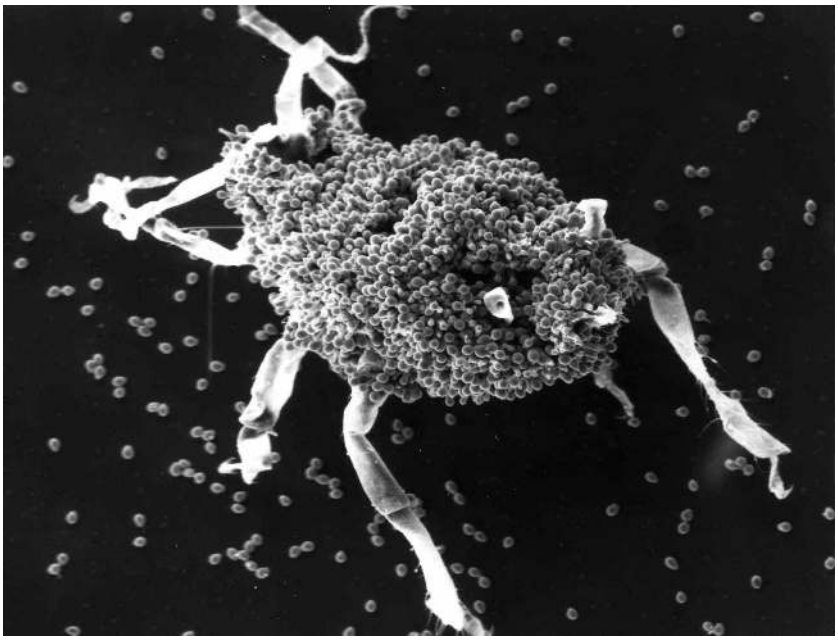

Figure 10. Conidiophores emerging through thorax and abdomen of the *Neozygites fresenii*-killed aphids at 2 h. 100X.

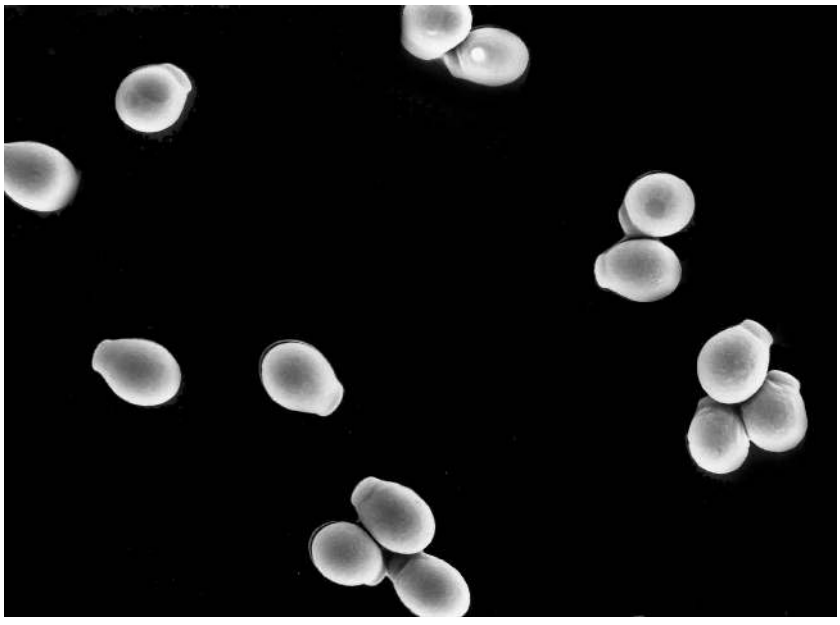

Figure 11. Primary conidia of *Neozygites fresenii* at 2 h. 600X.

During our observations, some differences in morphology and sporulation process were observed between these fungi. The conidiophores of *N. fresenii* did not group together to produce rosettes and cystidia as in *P. neoaphidis*. The conidiophores of *Entomophthora planchoniana* Cornu and *E. thaxteriana* (Petch) Hall & Bell group together before rupturing the cuticle in the same manner as *P. neoaphidis* (5). Rhizoid development was

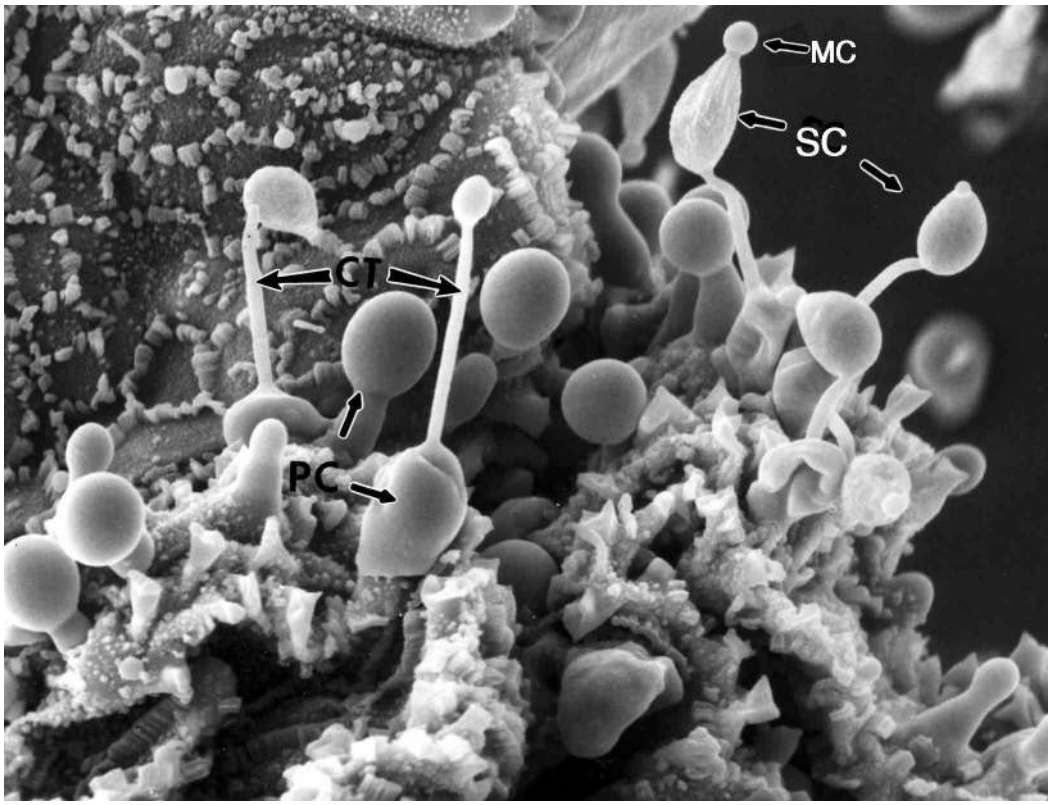

Figure 12. Primary conidia (PC), secondary conidia (SC) with terminal mucoid hapteron (MC) and capillary tube (CT) of *Neozygites fresenii* at the 4 h incubation time. 650X.

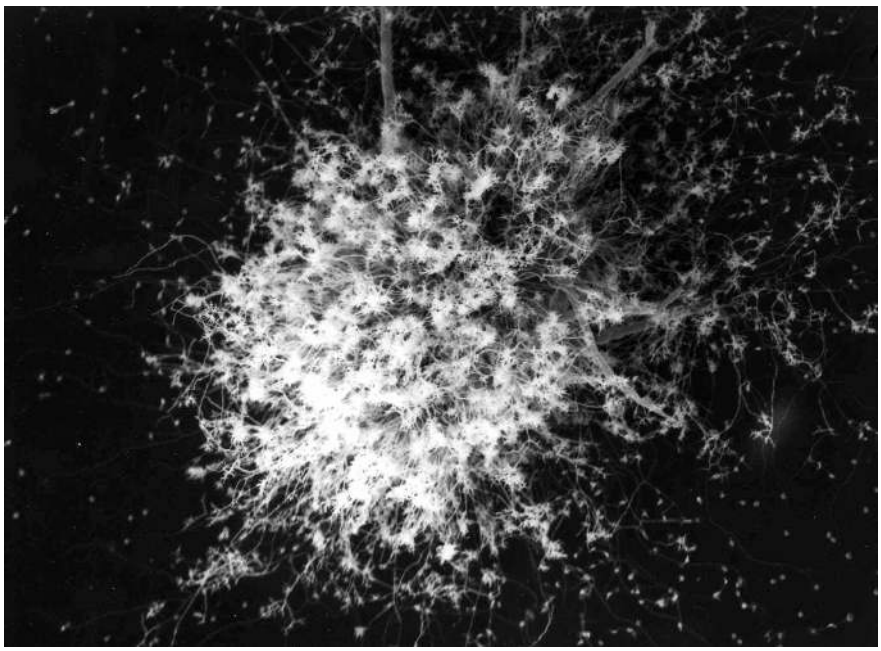

Figure 13. A shaggy, greenish-brown appearance given by saprophytic fungi that develop on *Neozygites fresenii*-killed aphids at 48 h. 50X.

not observed in *N. fresenii* as it was in *P. neoaphidis*. However, the function of rhizoids is to attach the host to the substrate, and aphids infected by *N. fresenii* were held to the leaf by their mouthparts inserted into the underside of the leaf. Other species of Entomophthorales also rely on the host to keep itself attached to the substrate by its mouthparts. Carner (10) reported that soldier beetles infected with *E. lampyridarum* (Thaxter) MacLeod & Müller-Kögler were attached to the foliage or flowers by their mandibles. This behavior is a definite advantage to the fungus because it remains on the plant where it can contact other hosts.

Primary conidia of *N. fresenii* had already formed at 2 h, whereas cystidia and conidiophores of *P. neoaphidis* had just begun to break through the cuticle at this stage. The primary and secondary conidia of *P. neoaphidis* were similar in shape while those of *N. fresenii* were quite different in shape. Formation of secondary conidia of *P. neoaphidis* was not observed until 10 h incubation so secondary spore formation by *N. fresenii* was much faster than by *P. neoaphidis*. However, numbers of spores formed by *P. neoaphidis* were much higher than by *N. fresenii*, probably because spores were formed over a longer period of time.

The entomopathogenic fungi depend on high moisture levels for survival and germination, but Steinkraus et al. (9) stated that epizootics were caused by *N. fresenii* during dry conditions. Steinkraus and Slaymaker (8) reported that primary conidia of *N. fresenii* were affected by low humidity, but that capilliconidia were much more resistant to low RH. They reported that capilliconidia remained infective for 14 days at 75 and 100% RH. In cotton aphids infected with *N. fresenii*, mortality and sporulation occur mainly during the night and early morning hours when humidity in the plant canopy is much higher than during the daylight hours. In this study, we have shown that sporulation resulting in capilliconidia can proceed quite rapidly (within 4h) probably well within the period of high humidity in the canopy. Capilliconidia would then survive and attach to moving aphids including during the lower humidity of the daylight hours. Germination and infection would then proceed during the next period of high humidity. This would partially explain the occurrence of *N. fresenii* epizootics during dry weather.

In summary, this study has revealed details of the sporulation process for *P. neoaphidis* and *N. fresenii* which had not been covered in previous studies. Information of this type is important in understanding how these fungi function in aphid populations in the field.

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