



MORPHOLOGY AND BEHAVIOR OF THE SUCCESSIVE GENERATIONS OF SECONDARY ZOOSPORES OF A DAMPING-OFF PATHOGEN *APHANOMYCES COCHLIOIDES*

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SUMMARY

Laterally biflagellated first generation secondary zoospores of the parasitic peronosporomycete, *Aphanomyces cochlioides* locate host plants guided by a host-specific compound, cochliophilin A (5-hydroxy-6,7-methylenedioxyflavone), and then change morphologically to initiate infection. However, when zoospores fail to find a host during their motile life, they become cysts which generate daughter secondary zoospores up to third generation. This study aimed at comparing the morphology and chemotactic properties of the first, second and third generation of secondary zoospores of *A. cochlioides*. Scanning electron microscopic observation revealed that all three successive generations of *A. cochlioides* zoospores were structurally similar but the later generations were smaller in size than those of first generation secondary zoospores. However, all three generations of secondary zoospores responded equally to the host-specific attractants (cochliophilin A and *N-trans*-feruloyl-4-*O*-methyldopamine) and other bioactive secondary metabolites but at varying threshold concentrations. These results confirm the hypothesis of repeated zoospore emergence in parasitic *Aphanomyces* as a possible adaptation to parasitism. A detailed life cycle of *A. cochlioides* is illustrated on the basis of microscopic studies and current knowledge.

Key words: *Aphanomyces cochlioides*, zoospore emergence, life cycle, oomycetes, *N-trans*-feruloyl-4-*O*-methyldopamine, SEM.

INTRODUCTION

The genus *Aphanomyces* comprises both saprotrophic species and parasites specialized on plants and fishes (Pavizas and Ayers, 1974; Islam, 2002). These fungus-like

microorganisms belong to the class Peronosporomycetes that fall within the kingdom Straminipila, which also includes golden-brown algae, diatoms, and brown algae (Dick, 2001). The peronosporomycetes generate characteristic asexual motile zoospores propelled by two dissimilar flagella (heterokont), which are an important means of pathogen distribution and often the key infectious stage of the pathogenic species (Islam and Tahara, 2001; Islam, 2002; Judelson and Blanco, 2005). The apically biflagellated primary zoospores of *Aphanomyces* spp. become encysted just after their release from the sporangium and aggregate at the tip of the cylindrical sporangiophore (Islam, 2002). Cysts of the primary zoospores form into laterally biflagellated first generation secondary zoospores, which can be motile for several hours in sterilized tap water.

These secondary zoospores are attracted to and can locate the host plants guided by host-specific chemical signals (Yokosawa and Kuninaga, 1979; Yokosawa *et al.*, 1986; Cerenius and Söderhäll, 1984a; Diéguez-Uribeondo, 1995), such as cochliophilin A and *N-trans*-feruloyl-4-*O*-methyldopamine released from the host (Horio *et al.*, 1992, 1993). The attracted zoospores of *Aphanomyces cochlioides* Drechsler aggregate to the infection site of host plants, encyst leaving flagella, and germinate to form hyphal germ tubes to initiate infection (Islam 2002; Islam *et al.*, 2001, 2002, 2003). However, the first generation of secondary zoospores which have failed to find a host during their motile life encyst at a substrate due to the depletion of internal energy reserves or environmental stress and most of them do not promote germination. These ungerminated cysts have the ability to undergo repeated zoospore emergence up to third generation secondary zoospores (Cerenius and Söderhäll, 1984b, 1985; Diéguez-Uribeondo *et al.*, 1994, 2009). This means that a new motile zoospore (daughter) is released from the cyst and continues searching for a potential host. This phenomenon has been reported in some other genera of the pathogenic Peronosporomycetes (Deacon and Donaldson, 1993; Diéguez-Uribeondo *et al.*, 1994; Xu and Morris, 1998; Connolly *et al.*, 1999; Islam and Tahara, 2001).

Studies concerning the behavior of daughter zoospores and molecular mechanisms of repeated

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zoospore emergence are limited. Cerenius and Söderhäll (1985) proposed that repeated zoospore emergence might be an adaptation to parasitism in the genus *Aphanomyces*, which would be of great value for the survival and spread of this parasite. Evidence supporting this view has been reported in some members of the parasitic Saprolegniales (Diéguez-Uribeondo *et al.*, 2007, 2009). The repeated emergence of zoospores from the cysts in absence of the host has also been observed in some other parasitic peronosporomycetes (Deacon and Donaldson, 1993; Islam and Tahara, 2001).

A. cochlioides is a soil-borne plant pathogenic peronosporomycete, causing root rot and damping-off diseases in spinach, sugar beet and some other members of Chenopodiaceae and Amaranthaceae. Like other pathogenic *Aphanomyces*, the apically biflagellated primary zoospores become encysted immediately after release from the cylindrical zoosporangium. The laterally biflagellated first generation secondary zoospores produced from the primary cysts of *A. cochlioides* locate host root by chemotaxis, and then subsequently adhere, encyst, germinate and finally penetrate the root tissue *via* appressoria (Islam *et al.*, 2001, 2002). It has been reported that all these pre-infection events are regulated by the host-specific plant signals released from the host roots (Islam *et al.*, 2001, 2003; Islam, 2008). For example, a simple flavone, cochliophilin A (5-hydroxy-6,7-methylenedioxyflavone) released from the spinach roots can induce taxis and subsequent morphological changes of *A. cochlioides* zoospores required for the penetration at less than micromolar concentration (Horio *et al.*, 1992; Islam *et al.*, 2003). Similarly, the zoospores of *A. raphani* and *A. euteiches* show specific sensitivities toward indole-3-carbaldehyde of cabbage seedlings, and prunetin of pea seedlings, respectively (Yokosawa and Kuninaga, 1979; Yokosawa *et al.*, 1986). However, in previous studies, the attraction behavior of only first generation secondary zoospores were considered (Yokosawa and Kuninaga, 1979; Yokosawa *et al.*, 1986; Horio *et al.*, 1992; Morris and Ward, 1992). The sensitivity of second and third generations of secondary zoospores toward the host-specific compounds is completely unknown. Functional and morphological properties of the following generations of zoospores have also not been investigated.

The objectives of this study were to compare the sensitivity and morphological properties of all three generations of *A. cochlioides* secondary zoospores. This article illustrates the morphological diversity in first, second and third generations of secondary zoospores of *A. cochlioides* and discusses their responses toward the host-specific plant signals. Based on current experimental findings and previous knowledge, the life cycle of *A. cochlioides* is illustrated by light, scanning and transmission electron micrographs.

MATERIALS AND METHODS

Culture of *A. cochlioides* and production of zoospores. The *A. cochlioides* (AC-5), a gift from Prof. R. Yokosawa (Health Science University of Hokkaido, Japan), was cultured for 3-4 days on a corn meal agar (Difco) plate at 20°C and the production of first generation secondary zoospores was carried out as described previously (Horio *et al.*, 1992; Islam and Tahara, 2001). The number of zoospores in the suspension was counted as described earlier (Islam *et al.*, 2007).

Repeated zoospore emergence. Ten milliliter of the first or second generation of secondary zoospores were taken into a sterilized test tube and then induced to encystment by mechanical agitation (vortexing) for 30 sec. The suspension of mechanically induced cysts was immediately transferred into a sterilized plastic Petri dish (3 cm diameter) and incubated for 6 h at 20°C to release the following generation of secondary zoospores (Cerenius and Söderhäll, 1984b, 1985). In the above protocol, up to third generation of secondary zoospores was obtained. The number of zoospores, cysts and germinated cysts in the incubated spore suspension was counted as described previously (Islam *et al.*, 2007). At least 300 spores were counted for each treatment and data are expressed in mean values of three independent experiments \pm standard error.

Bioassay and electron microscopy. The host-specific attractants, cochliophilin A (5-hydroxy-6,7-methylenedioxyflavone) and *N-trans*-feruloyl-4-*O*-methyldopamine used in this experiment were synthesized by Horio *et al.* (1992, 1993). Chemotactic behavior of first, second and third generations of secondary zoospores was tested by using the "particle bioassay" method (Islam and Tahara, 2001). Briefly, a few particles of Chromosorb W AW were treated with varying concentrations of host-specific attractants dissolved in ethyl acetate. The particles were air-dried and carefully dropped into zoospore suspension (*ca.* 1×10^5 /ml) taken on a small Petri dish (3 cm diameter). The behavior of swimming zoospores toward the chemical-coated and control (treated with solvent alone) particles were observed under a light microscope as described earlier (Islam *et al.*, 2004).

Morphological features are considered important for cell biology and swimming behavior i.e., chemotaxis to find host plants by the zoospores of pathogenic peronosporomycetes. Therefore, detailed morphological features of three generations of secondary zoospores including flagella, cysts and germinated cysts were investigated by scanning (SEM) and transmission electron microscopy (TEM) following protocols described previously (Islam *et al.*, 2001, 2002, 2005). Briefly, the spores were fixed with 2% buffered glutaraldehyde (TAAB, UK) for 30 min on JEOL SEMPore membranes (pore

Table 1. Morphological characters and sensitivity of first and second generation secondary zoospores of *Aphanomyces cochlioides* toward host-specific attractants.

| Character | First generation secondary zoospore | Second generation secondary zoospore |
|---|--|--|
| Size of zoospore | 7.2 ± 0.3 × 5.0 ± 0.1 μm | 7.6 ± 0.1 × 4.1 ± 0.2 μm |
| Characters of anterior flagellum | | |
| Main shaft | 18.2 ± 0.6 μm long and 296 ± 1 nm diameter thick | 18.5 ± 0.3 μm long and 269.7 ± 1.8 nm diameter thick |
| Density of TTHs/ μm | 15.1 ± 1.0 | 13.9 ± 0.7 |
| TTH (shaft) | 1.57 ± 0.01 μm long and 37.3 ± 0.9 nm diameter thick | 1.50 ± 0.01 μm long and 33.2 ± 0.7 nm diameter thick |
| Length of long and short hairs | 0.52 ± 0.01 μm and 0.24 ± 0.01 μm | 0.50 ± 0.01 μm and 0.20 ± 0.01 μm |
| Characters of posterior flagellum | | |
| Main shaft | 24.0 ± 1.2 μm long and 262.1 ± 0.9 nm diameter thick | 23.1 ± 1.0 μm long and 262.3 ± 1.5 nm diameter thick |
| Density of hairs/ μm | 45 ± 2 | 44 ± 3 |
| Size of flagellum hair | 0.53 ± 0.01 μm long and 25.7 ± 0.9 nm diameter thick | 0.48 ± 0.02 μm long and 22 ± 1 nm diameter thick |
| Size of the tapered tip | 1.5 ± 0.2 μm long and 132 ± 2 nm diameter thick | 1.6 ± 0.2 μm long and 125 ± 1 nm diameter thick |
| Chemotactic behaviors to the host-specific attractants* | | |
| Cochliophilin A | Attracted at 1 × 10 ⁻⁹ M | Attracted at 2 × 10 ⁻⁹ M |
| Feruloyl-dopamine | Attracted at 1 × 10 ⁻⁸ M | Attracted at 3 × 10 ⁻⁸ M |

At least 5 spores were measured for a specific morphological character of every generation of zoospores. *Particle bioassay method was used to test chemotactic behavior of zoospores. The morphology of quaternary zoospores was almost similar to those of tertiary zoospores but nearly 5-10% deficient than the tertiary. However, the third generation secondary zoospores showed almost similar sensitivity to host-specific compounds as shown by the second generation.

size 0.6 μm), dehydrated in a graded acetone series, subjected to critical-point drying using CO₂, coated with 10-nm thick platinum-palladium using a sputter coater and observed under a JSM-6301F, JOEL SEM with accelerating voltage of 5 kV. For TEM, the glutaraldehyde fixed spores were concentrated by centrifugation at 2000 rpm for 5 min, pipetted onto copper grids with carbon-coated formvar film, rotary-shadowed at an angle of 10° with platinum and carbon on the uncooled specimen stage of a JFD-9010 freeze-fracture apparatus (JEOL, Japan) and observed under an H-800 electron microscope with accelerating voltage of 75 kV. Each experiment was repeated three times and the representative images were selected. At least 10 zoospores were selected to measure their

length and breadth, and the number and density of hairs on the flagella. The mean values ± standard errors of each parameter are presented.

RESULTS

Production of second and third generations of secondary zoospores. Release of first generation secondary zoospores from the primary cysts was 100% within 2-3 h at 20°C. Vortexing for 30 sec and subsequent incubation (6 h) of first and second generation secondary zoospore suspension yielded about 85 ± 7% of second and 55 ± 8% of third generation secondary zoospores,

respectively. The duration of normal motility of first, second and third generation secondary zoospores was recorded as 30, 18 and 15 h, respectively. After these time scales, the zoospores gradually stopped their motility and became round-shaped cysts. Light microscopic observations revealed that the second (ca. $22 \pm 3 \mu\text{m}/\text{sec}$) and third (ca. $20 \pm 2 \mu\text{m}/\text{sec}$) generation secondary zoospores moved slightly slower in sterilized tap water than those of the first generation (ca. $25 \pm 1 \mu\text{m}/\text{sec}$) secondary zoospores.

Morphological features of regenerated zoospores.

The shape and structure of the three generations of zoospores appeared similar but, although not signifi-

cantly, the size of the second ($7.6 \pm 0.1 \times 4.1 \pm 0.2 \mu\text{m}$) and third ($7.5 \pm 0.2 \times 4.0 \pm 0.1 \mu\text{m}$) generation of secondary zoospores was smaller than the first generation ($7.2 \pm 0.3 \times 5.0 \pm 0.1 \mu\text{m}$) secondary zoospores (Fig. 1 and Table 1). The detailed morphological features of first and second generation of secondary zoospores of *A. cochlioides* are presented in Table 1 and illustrated in Fig. 1. It appeared that the ventral grooves in the second (Fig. 1E) and third generations of secondary zoospores were significantly more pronounced than in the first generation of secondary zoospores (Fig. 1A). In addition, the anterior parts of the latter generation of secondary zoospore bodies were sharper and a little bent toward the ventral sides (Fig. 1E). The shape and

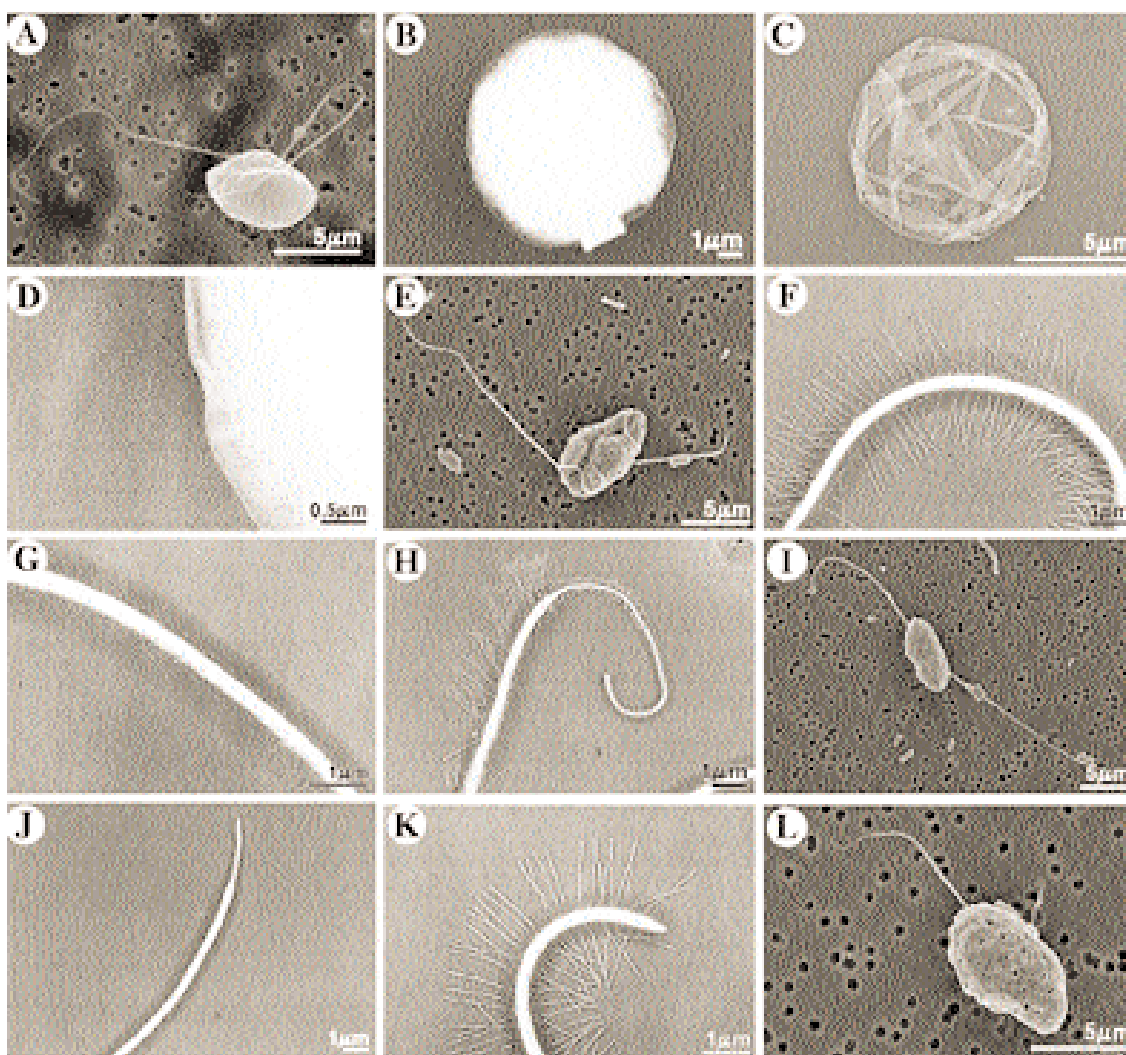


Fig. 1. Scanning (A, E, I and M) and transmission (B, C, D, F, G, H, J and K) photomicrographs of different generation of *A. cochlioides* secondary zoospores, their flagella and cysts. A, a first generation secondary zoospore; B, a first generation secondary cyst; C, a left cyst-coat (ghost) after release of a second generation secondary zoospore; D, Long hairy structures on the surface of a cysts; E, a third generation secondary zoospore; F, tripartite tubular hairs (TTHs) on the anterior flagellum of a second generation secondary zoospore; G, part of a posterior flagellum of a second generation secondary zoospore with very fine hairs; H, an unusual whiplash tip in the anterior flagellum of a second generation secondary zoospore; I, a third generation secondary zoospore; J, posterior flagellum of second generation secondary zoospore; K, TTHs on anterior flagellum of second generation secondary zoospore; L, a second generation secondary zoospore (dorsal view).

structure of the third generation secondary zoospores were almost similar but, although not significantly, a slightly smaller than the first generation secondary zoospores (Fig. 1I). Detailed morphological features of anterior and posterior flagella, such as size of main shaft, size and density of tripartite tubular hairs (TTHs), length of long and short hairs at TTHs, size of tapered tip of posterior flagellum, were measured from the micrographs obtained by TEM and presented in Table 1. TEM revealed that these flagella characters in three generations of secondary zoospores varied from a generation to another but the differences were not statistically significant. The cyst surface was ornamented with some hairy structures as observed in other peronosporomycetes (Fig. 1D) (Dick, 2001). These unbranched fine hairs varied in length (maximum 2.0 μm) and their density on the cyst surface was 5 ± 1 per μm.

Sensitivity of various generations of secondary zoospores toward host-specific compounds. The

chemotactic behavior of different generations of *A. cochlioides* secondary zoospores toward two host-specific compounds cochliophilin A and *N-trans-feruloyl-4-O-methyldopamine* were assessed by the ‘particle bioassay’ method and data are presented in Table 1. The three generations of secondary zoospore showed taxis toward the chromosorb W AW particles treated with the host-specific compounds. The first generation secondary zoospores showed the highest sensitivity in all generations so far tested, irrespective of the compound. The second and third generations secondary zoospores responded almost equally but needed approximately 2-3 times higher concentrations of the tested compounds for exhibiting bioactivity equivalent to that of first generation of secondary zoospores. All three generations of secondary zoospores showed aggregation followed by encystment at 10-20 folds higher concentration of cochliophilin A than that of threshold concentration required for clear chemotaxis. In all cases, cysts induced by cochliophilin A germinated (100%) at the bottom of

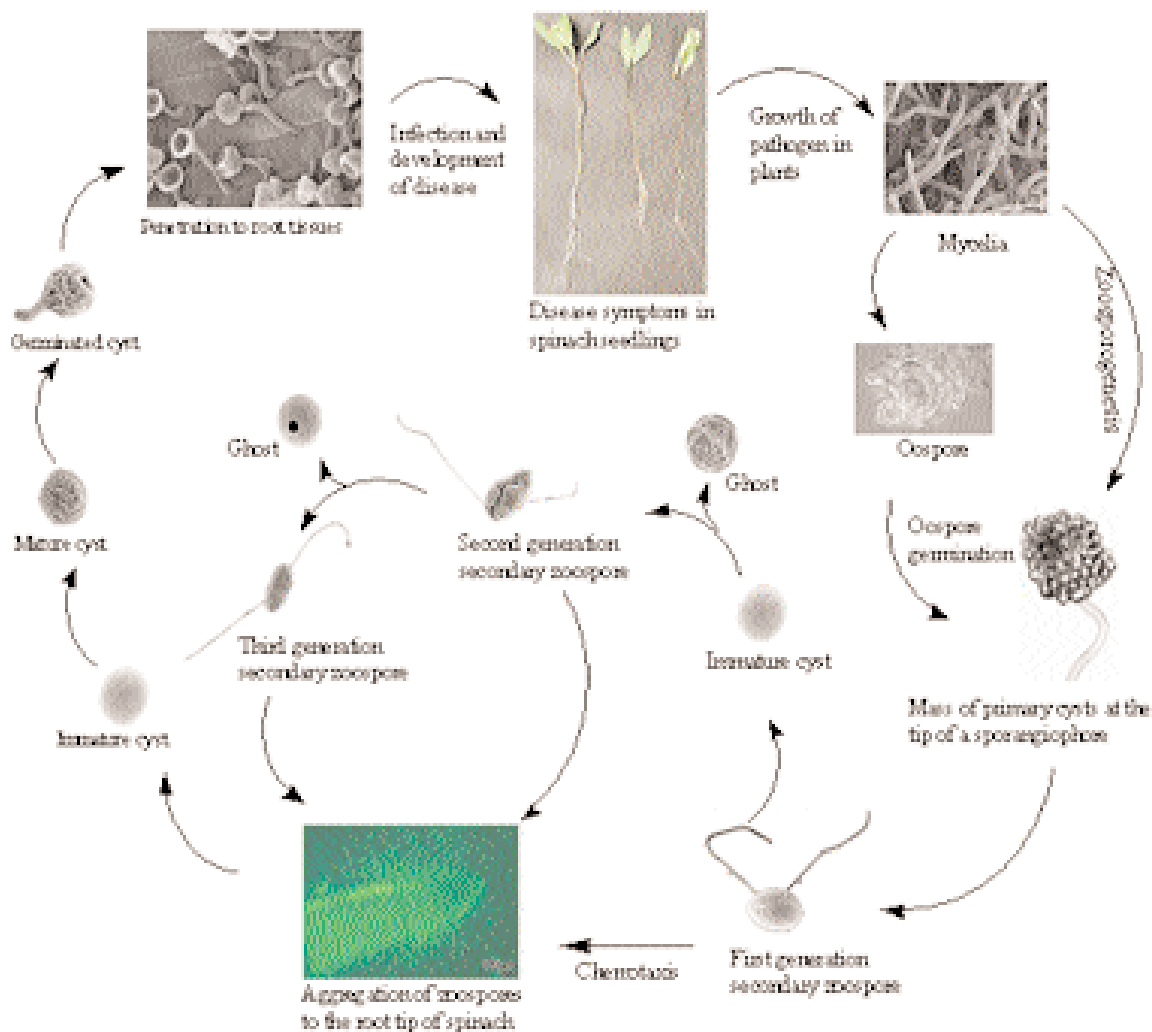


Fig. 2. Micrographical illustration of the life cycle of a damping-off pathogen *Aphanomyces cochlioides*. No scale is used for the micrographs.

Petri dish within 30-40 min. However, zoospores attracted by N-*trans*-feruloyl-4-O-dopamine did not undergo differentiation (encystment and germination) irrespective of generations of secondary zoospores.

Life cycle of *Aphanomyces cochlioides*. An elaborate illustration of different steps of the life cycle of *A. cochlioides* using light micrographs obtained from current and previous light and electron microscopic studies (Islam *et al.*, 2001, 2002, 2003, 2004) is presented in Fig. 2. This is the first detailed illustration of the life cycle of *A. cochlioides*, including possible different generations of the secondary zoospores.

DISCUSSION

For host-specific pathogens or symbionts, the ability to recognize and orient the direction of a plant signal may be critical for the survival of the organism. Chemotactic response of zoospores to host-specific signals has been described for several plant-peronosporomycete associations (Morris and Ward, 1992; Horio *et al.*, 1992). Repeated emergence of zoospores from the cysts of earlier generation under suitable conditions appears to be a common feature in the peronosporomycete phytopathogens (Cerenius and Söderhäll, 1985; Xu and Morris, 1998; Connolly *et al.*, 1999). Chemotaxis and subsequent differentiation of zoospores on host surface of the first motile generation of zoospores has been studied in many phytopathogenic peronosporomycetes including *Phytophthora*, *Pythium* and *Aphanomyces* (Morris and Ward, 1992; Horio *et al.*, 1992, 1993; Cerenius and Söderhäll, 1985; Islam *et al.*, 2003). Data presented in this paper show for the first time that the following generations of the phytopathogenic peronosporomycete *A. cochlioides* secondary zoospores are similarly attracted by the host-specific chemical signals for taxis and differentiation but at varying threshold concentrations.

The first generation of secondary zoospores swam faster and exhibited higher sensitivity (attractant activity) toward the host-specific compounds than those of second and third generations (Table 1). Furthermore, a preliminary experiment revealed that all three generations of *A. cochlioides* secondary zoospores were almost equally accumulated, encysted, and subsequently germinated to infect the roots (100%) of spinach and sugar-beet seedlings at 500 zoospores/ml/seedling in greenhouse conditions (Islam *et al.* 2002, 2005). These results clearly support the previously drawn hypothesis that repeated zoospore emergence is an adaptation to parasitism in parasitic *Aphanomyces* spp. (Cerenius and Söderhäll, 1985; Diéguez-Urbeondo *et al.*, 2009). As regeneration of zoospores is common in other peronosporomycetes, the phenomenon of equal sensitivity of zoospores toward the host signals may exist in other

plant pathogens (Xu and Morris, 1998; Connolly *et al.*, 1999; Diéguez-Urbeondo *et al.*, 2009).

SEM and TEM observations revealed that the new generation of secondary zoospores is smaller than the earlier ones (Fig. 1 and Table 1). Decrease in volume and formation of vacuoles in the later generations of zoospores has been shown also in *Saprolegnia parasitica* (Diéguez-Urbeondo *et al.*, 1994). It is known that during encystment, zoospores shed their flagella (Islam *et al.*, 2002). The potential cysts leave cyst-coats (Fig. 1C) and use some of their cellular materials for growing the flagella during regeneration processes. As the zoospores or ungerminated cysts cannot absorb nutrient from external sources, they just use and probably recycle internal resources during the process of regeneration (Lovett, 1975; Söderhäll and Cerenius, 1984; Penington *et al.*, 1989; Deacon and Donaldson, 1993). Thus, the size of new generation of secondary zoospores is reasonably smaller than that of the previous ones. Moreover, during the processes involving motility, a spore must use energy from its internal source. Perhaps, these are the main reasons why the new generation is smaller in size than those of the former ones.

The molecular mechanism of repeated zoospore emergence in peronosporomycetes has not yet been fully understood. However, Andersson and Cerenius (2002) analyzed the expression of spore-specific marker transcripts at different stages of the life cycle of *S. parasitica*. They found that one of the markers, designated *puf1*, is transiently expressed upon each of several cycles of zoospore encystment and re-emergence. The transcript was induced immediately upon zoospore encystment and is rapidly lost when a cyst is triggered to germinate. In non-germinating cysts of *S. parasitica*, *puf1* expression was maintained until a time point when the cyst could no longer be triggered to germinate and thus became determined for zoospore reemergence. The *puf1* gene encodes a putative mRNA-binding protein belonging to a conserved class of proteins including the *Drosophila melanogaster* Pumilio (Parisi and Lin, 1999), and *Caenorhabditis elegans* FBF proteins (Zhang *et al.*, 1997), which are involved in regulation of gene expression by post-transcriptional mechanisms. Since disruption of any step of the asexual development of peronosporomycetes would eliminate the possibility of plant infection, further studies are needed to elucidate in detail the molecular mechanism of zoosporogenesis and subsequent development of the phytopathogenic peronosporomycetes.

In summary, the second and third generations of secondary zoospores are structurally similar but statistically smaller than the first generation secondary zoospores of *A. cochlioides*. All three generations of secondary zoospores show attractant activity toward the host-specific plant signals or root tips of the host plants. However, the threshold concentrations required for attractant

activity of the later generations toward the host-specific compounds are 2-3 folds higher than that of first generation secondary zoospores. As mentioned above, repeated zoospore emergence may represent a possible adaptation to parasitism in *Aphanomyces* and other parasitic peronosporomycetes, as suggested by earlier investigators (Cerenius and Söderhäll, 1984b, 1985; Diéguez-Uribeondo *et al.*, 1994).

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