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Life Cycle of the Human and Animal Oomycete Pathogen *Pythium insidiosum*

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***Pythium insidiosum*, the etiologic agent of pythiosis insidiosii, causes life-threatening infections in humans and animals. Previous studies of the epidemiology of this disease hypothesized about the possible life cycle of this oomycete. Details, however, were not provided on the steps required to cause infection. We investigated the life cycle of *P. insidiosum* by inoculating pieces of equine skin and plant leaves and then studying the ensuing events with a scanning electron microscope. Our observations revealed that zoospores had a strong tropism for skin tissue, horse and human hair, and water lily and grass leaves and a weak attraction to a variety of other leaves. Encysted zoospores were observed on the favored leaves and skin. There they produced germ tubes and later abundant hyphal filaments that penetrated leaf tissues. Young sporangia had compact, thick walls. The sporangial wall was reduced to a fragile membrane when the sporangia had produced well-differentiated biflagellate zoospores. The encysted zoospores secreted an amorphous material that permitted the zoospores to adhere to skin and plant tissues. On the basis of these findings, a model to explain the life cycle of *P. insidiosum* is proposed.**

Pythium spp. are ubiquitous in soil and aquatic environments and have a global distribution. The species of this genus are important plant pathogens, inflicting serious economic losses on a wide variety of crops (9, 22, 40). *Pythium insidiosum*, the etiologic agent of pythiosis insidiosii in humans and animals, is an ecologically and physiologically unique microorganism (10, 25, 37) that, in contrast to other species of *Pythium*, has developed the ability to cause disease in animals.

Pythiosis insidiosii is a subcutaneous, vascular disease and, less frequently, an intestinal disease in humans and animals (2, 4, 5, 28, 32, 33, 34, 39). This disease is characterized by the development of granulomatous lesions that grow rapidly and may be life threatening in some cases. Since the last century, infections caused by *P. insidiosum* have been related to rainy seasons. In India the disease is call "bursattee," a term derived from "burus," a word signifying rain. This name is based on the popular belief that the appearance of granulomas in horses was related to the advent of the monsoon season (38). In other tropical and subtropical countries similar observations led to analogous terms, the most popular being swamp cancer (2) and leeches (13). Unfortunately, because of clinical similarities to other diseases, these terms were used indiscriminately for similar clinical entities and nothing was done to distinguish them etiologically.

Austwick and Copeland (2) stimulated the production of motile zoospores in a filamentous microorganism isolated from horses afflicted with swamp cancer in New Guinea. This finding initiated the belief that zoospores may be the infecting propagules. Miller (27) showed that the zoospores of *P. insidiosum* were attracted by the hair of several animals and the leaves from an aquatic lily collected in swampy areas. He suggested a possible life cycle for this oomycete. Several questions, however, regarding the life cycle of *P. insidiosum* in nature and its relationship to infections in humans and animals remained to be answered.

Although some investigators have described the mechanisms involved in the life cycle of other species of *Pythium* (11, 19, 40), there have been no detailed studies defining the different stages necessary for *P. insidiosum* to complete its life cycle in nature or the different factors required during plant or animal tissue invasion. The aims of this study were to investigate the ultrastructural features of the asexual cycle of *P. insidiosum* and to explain in vivo events leading to the invasion of plant and animal tissues.

MATERIALS AND METHODS

Strains and plant and animal tissues. The following *P. insidiosum* strains were used during this study: ATCC 28251, isolated from a horse in New Guinea; CBS-623.85, isolated from a human in Thailand; CDC B-4301, isolated from a horse in the United States; ATCC 58643, isolated from a horse in Costa Rica; and S5, isolated from a horse in Australia. Plant tissue from different grasses (*Paspalum notatum*, *Panicum* sp. and *Setaria* sp.) and a swamp lily (*Nymphae* sp.) collected from swampy areas where equine cases of pythiosis insidiosii had been previously diagnosed (1) were used. In addition, several unidentified plants collected around the same areas were also included in this study. Plant tissue, human and horse hair, and biopsies of horse hide were used to investigate the chemotactic effect of these tissues on newly released zoospores of *P. insidiosum*. The skin tissues from horses were biopsied (4 by 4 mm, including skin and cutaneous and subcutaneous tissue, as showed by eosin- and hematoxylin-stained tissue sections) from lower extremities, collected in esteril petri dishes, and used immediately. The follicular end of human and horse

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hair was taken, kept in esteril saline solution, and used within 30 min after collection.

Asexual reproduction in plant tissue. The leaves of the different grasses and the *Nymphae* sp. were cut into small pieces (10 by 4 mm) and placed in tubes of 24-h cultures (cornmeal agar slants; Difco Laboratories, Detroit, Mich.) of *P. insidiosum* strains and incubated for 24 h at 37°C. The leaves were then transferred to a 30-ml induction medium (see below) and incubated for 1 to 2 h at 37°C. Production of sporangia and zoospores was evaluated by light microscopy. The induction medium was prepared as described previously (26). Briefly, two solutions were prepared. Solution I consisted of 87.09 g of K_2HPO_4–68.05 g of KH_2PO_4–66.04 g of $(NH_4)_2HPO_4$ in 500 ml of distilled water. Solution II was prepared by mixing 25.42 g of $MgCl_2 \cdot 6H_2O$ and 118.38 g of $CaCl_2$ in 250 ml of distilled water. Then, 0.5 ml of solution I was mixed with 0.1 ml of solution II in a 2-liter flask containing 1,000 ml of distilled water. The pH of the resulting solution was ~7.1. The solution was stored at 4°C until used. The taxis behavior of zoospores was monitored with dissecting and light microscopy by introducing the tissues under evaluation with zoospores (~450 motile zoospores per ml) obtained as described above. Control media with no introduced tissues were also included in each experiment.

Electron microscopy of the asexual cycle. The asexual cycle of *P. insidiosum* was induced as indicated above. The infected leaves were selected according to the presence of numerous sporangia in different stages of development that had been previously detected under a dissecting microscope. The material then was fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer solution at pH 7.4 for approximately 1 h and postfixed in 1% osmium tetroxide in the same buffer solution for an additional hour. The samples were dehydrated with a graded ethanol series, dried by the critical point drying method in a Hitachi HCP-2, and coated with gold at about a 20-nm thickness in a sputter coater Eiko IB-3. The zoospores were induced as described above and then centrifuged at 500 × *g* for 15 min to concentrate the zoospores. The resulting pellet was resuspended in 100 μl of phosphate buffer. Twenty-five microliters of this material was placed on poly-L-lysine (24) slipcovers and processed as described above. The Au-covered specimens were analyzed with a scanning electron microscope (Hitachi s-570). Negative staining of the zoospores was carried out after dialysis to remove residual glutaraldehyde, and the specimens were then placed on 3% parlodion-coated 300-mesh cooper grids (Pelco; Ted Pella, Inc., Tustin, Calif.) and incubated for 5 min at room temperature. The grids were washed with 2% phosphotungstic acid (pH 6.8) and stained with 2% ammonium molybdate (pH 6.5) for 1 min. They then were dried and examined with a Joel 100S electron microscope.

RESULTS

Sporangia and zoospore production evaluated by light microscopy. Microscopically, all strains colonized the grass or lily bait and developed sporangia and zoospores after 1 h of incubation at 37°C in the induction medium. The process took ~35 min between the formation of undifferentiated sporangia and the release of motile zoospores. At first the protoplasm flowed to the tip of the early-discharge tube until a small vesicle that continued to increase in size as long as the protoplasm flowed through the apical opening was formed (Fig. 1a to d). When the protoplasm stopped flowing, a plug was formed at the base of the apex and the vesicle's protoplasm began progressive cleavage and formed biflagellated secondary type zoospores (Fig. 1e to h). The motile zoospores mechanically broke the vesicle's wall and were released into the induction medium (Fig. 1i). The zoospores were globose to ovoid in form and bore two flagella. They continued to swim for ~10 to 15 min before encystment. Prior to encystment the zoospores began to swim slowly and finally came to a complete stop. At that time the two flagella were detached and the zoospores became globose. After several minutes, the encysted zoospores developed a germ tube that became elongated into a filament after 24 h of incubation at 37°C. The process of encystment and germ tube formation was more rapid when human, animal, or plant tissues were present.

Chemotaxis of zoospores. Zoospores collected within the first minute after release from mature sporangia were added to bits of grass, lily leaves, hair, and biopsied horse hide, as well as the unidentified plant leaves. The zoospores were instantly attracted toward all of the tissues and finally encysted on them. This behavior was less pronounced on leaves other than the grass and lily leaves. Some of the encysted zoospores on these tissues did not develop germ tubes. The strands of human and horse hairs also attracted the zoospores. After the zoospores reached the hairs, they accumulated at the follicular end of the hair, encysted, and produced a germ tube. An identical response was noted when equine skin tissue was tested. The zoospores began to swim toward the tissue and continued moving near it until encystment. The encysted zoospores were detected bound to the cut edges of the skin but very rarely over the normal surface. Zoospores added to the control medium tubes swam in a random manner and finally encysted.

Electron microscopy of the asexual cycle. After 15 to 20 min, the zoospores that had encysted on the different baits produced germ tubes and 24 h later a great quantity of mycelium (Fig. 2b). The hyphae also bore swollen peg-like structures that were separated from the rest of the hyphae by a septum (Fig. 2a). Only leaves bearing hyphae with these pegs were observed to produce numerous sporangia after stimulation in the induction medium. The sporangia in the process of formation were characterized by the presence of a compact vesicle wall and a turgid sporangial apex (Fig. 2c). After the protoplasm stopped flowing, a septum was observed at the base of the apex (Fig. 1d and e). The base and the vesicle at this stage remained turgid. The protoplasm inside the vesicle then began to differentiate (Fig. 2e and h). As the sporangia underwent progressive cleavage, the wall of the sporangium vesicle became thinner. The septum on the apex was clearly observed at this and later stages. Differentiation of the protoplasm into zoospores within the vesicle was evident (Fig. 2g and h). When the sporangia reached maturity, their walls had become fragile membranes that enclosed the well-differentiated biflagellated zoospores (Fig. 2h and i). The flagella were readily visualized through the vesicle's wall (Fig. 2i). The apical septum was clearly observed, and the base that connected the vesicle to the attached hyphae had lost the turgidity of its early stages and sometimes collapsed (Fig. 2h [arrow]). Zoospores then were observed being released from the vesicles (Fig. 2j). No differences in the morphological features of the sporangia that had developed on the lily or grass leaves were noted under the electron microscope.

Zoospores were noted around the ruptured sporangia and sometimes on the surfaces of the leaves under investigation

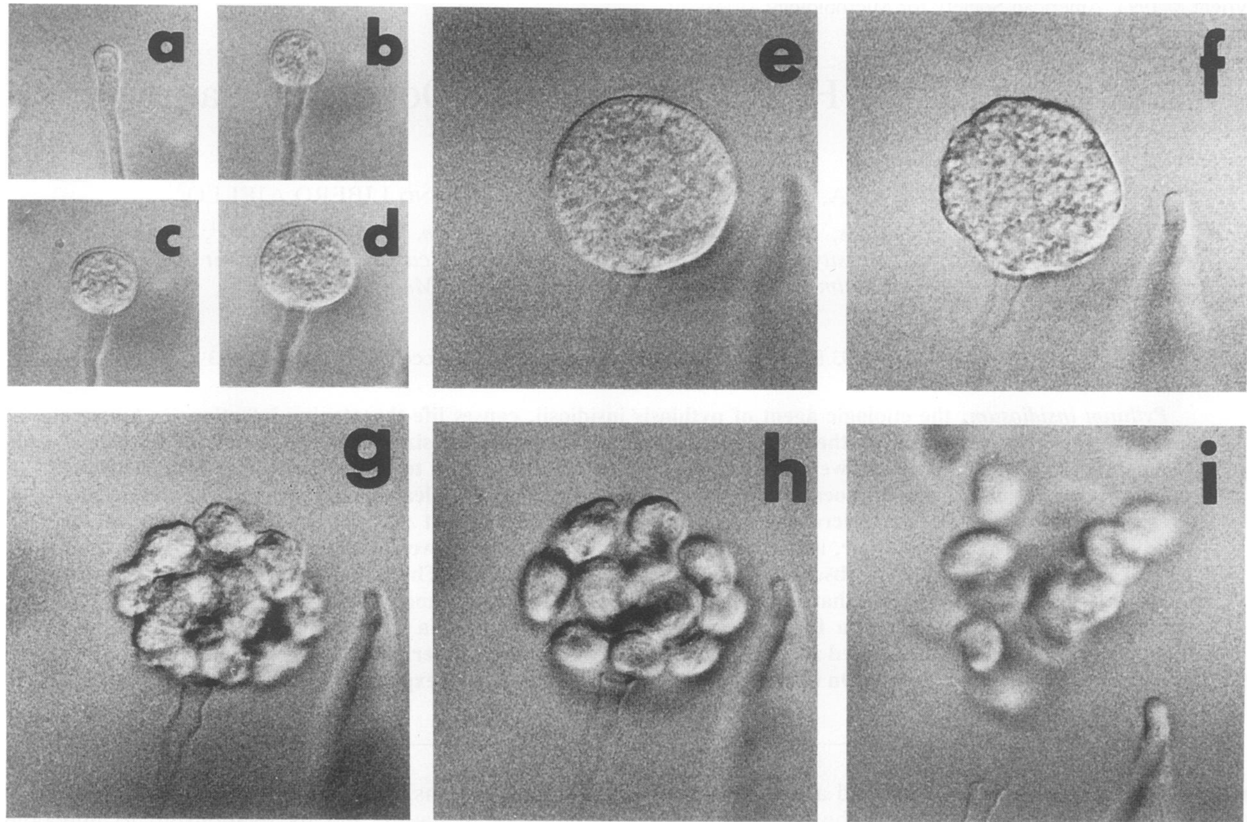

FIG. 1. Light photomicrographs show the different steps required by *P. insidiosum* to complete its asexual cycle on a grass leaf. Note the beginning of sporangial formation in an undifferentiated hyphal tip (a to d), the differentiation of a sporangium (e to h), and the release of zoospores (i). Magnification, ×35 (a to d) and ×75 (e to i).

(Fig. 3a to c). The reniform zoospores were the secondary biflagellated type. Their two flagella arose from a lateral groove (Fig. 3a and c). The anterior flagellum was the tinsel type and acquired a sinusoidal form after fixation. In contrast, the posterior flagellum was the whiplash type and appeared to be more rigid (Fig. 3c [arrow]). The central groove appeared laterally to the zoospore, and the two flagella emerged from the depth of the groove (Fig. 3a). No hairs (mastigonemes) were detected on either flagellum under the scanning electron microscope. The surface of the zoospores appeared reticulated with a pattern of ridges and mounds and sometimes randomly located small pits (Fig. 3a to c).

After encystment, the zoospores became spherical. The position occupied by both flagella was denoted by a nipple-like projection (Fig. 3d and f [arrows]) that in most cases led to the formation of a germ tube (Fig. 3e). The spherical, encysted zoospores on the surface of leaves were observed to be covered by an as-yet-unidentified amorphous material that they had secreted after encystment (Fig. 3d). Encysted zoospores, recovered from hair or equine skin tissue, also were covered with the same secreted material (Fig. 3e). Encysted zoospores in the control plates free of tissue were not covered with this material. Although they had nipple-like projections, only a few germinated and produced mycelia after 24 h of incubation at 37°C (Fig. 3f). Two strains tested (ATCC 28251 and ATCC 58643) developed oogonia identical to the ones first described for *P. insidiosum* (10) (Fig. 4k).

DISCUSSION

All *P. insidiosum* strains tested had the in vitro ability to develop sporangia on the surface or within the pieces of grass and lily pad leaves and to lesser extent within other plant leaves. Our data showed that *P. insidiosum* may use more than one species of aquatic plants to complete its life cycle in nature, indicating that not only lilies but also other plants and debris of these plants found in aquatic or humid habitats may support the propagation of this oomycete. This finding is in disagreement with those reported by Miller (27), who suggested that *P. insidiosum* may colonize only species of *Nymphaea*. Cases of pythiosis insidiosii in horses in the absence of swamp lilies (24a), however, and those reported in humans that worked in rice paddies or cornfields (34) indicate that plants other than lilies support *P. insidiosum*'s life cycle. Reports on the induction of the life cycle of *P. insidiosum* by using grass leaves strengthen this hypothesis (8, 26). Nevertheless, experiments using living plants are necessary to confirm our present data. In addition, the morphological features of the zoospores reported in this investigation are identical to those described by Shipton et al. (37) and Chaiprasert et al. (8) using strains of *P. insidio-*

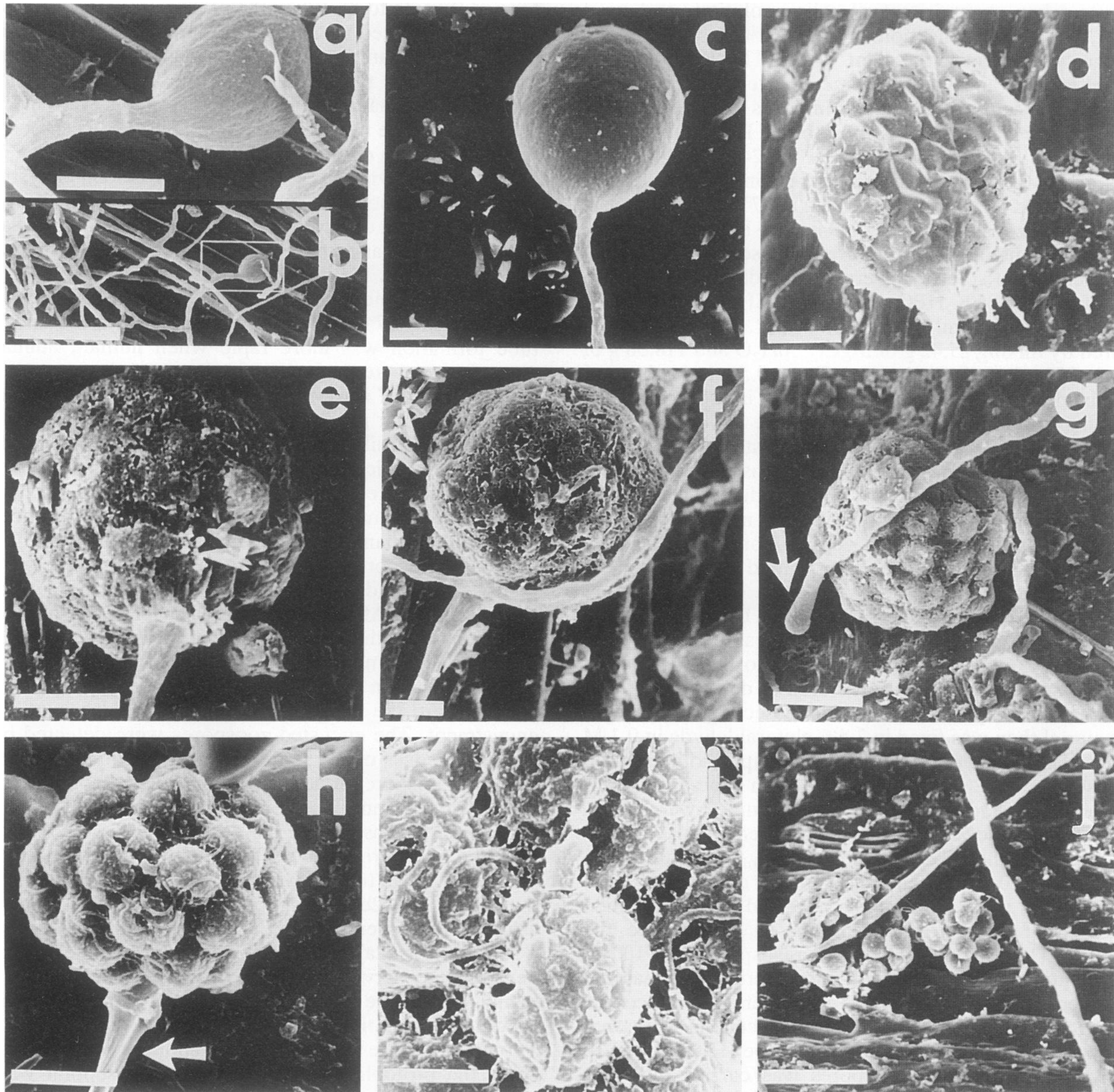

FIG. 2. Electron microphotographs of *P. insidiosum*'s asexual life cycle during hyphal parasitism in a grass leaf. A globose vesicle on a healthy leaf (a) and development of numerous hyphae (b) are the first steps in the colonization of a plant host. The electron microphotographs (c to j) show the different steps of sporangial formation leading to the release of the secondary type motile zoospores (j). Bar (in micrometers), 28 (a and c), 120 (b), 20 (d), 30 (e), 15 (f), 32 (g), 18 (h), 15 (i), and 50 (j).

sum isolated in Australia and Thailand, respectively, and they are very similar to the zoospores produced by other oomycetes (3, 9, 14, 22).

Our studies have shown that when zoospores were placed in contact with various leaf and animal tissues, they started to swim in a smoothly gliding spiral toward the target while rotating around their long axes. Near the vicinity of the attractants, the zoospores became sluggish, lost their flagella, became rounded in shape, and became motionless. A strong attraction behavior toward plant and animal tissues was noted during this investigation. Similar behavior has been reported previously for *P. insidiosum* (27, 36), other *Pythium* spp. (11, 14), and other zoosporic plant pathogens (7, 17, 22). The behavior of zoospores toward a specific target has been attributed to chemotactic substances present in plant tissues (14, 20, 22, 30). Several investigators have also shown that *P. insidiosum* has a special tropism for plant and animal tissues (27, 37). The results obtained in this study support those observations. This suggests that, in the development of the asexual life cycle of *P. insidiosum*, several pathogenic mechanisms leading to plant and animal tissue invasion have been activated.

During this study we found that all of the encysted zoospores recorded on leaf and equine tissues were surrounded by an amorphous material that they had secreted, but zoospores found in the controls lacked this material on their surfaces. This suggests that this material may act as an adhesive substance that attaches the zoospores to the host's

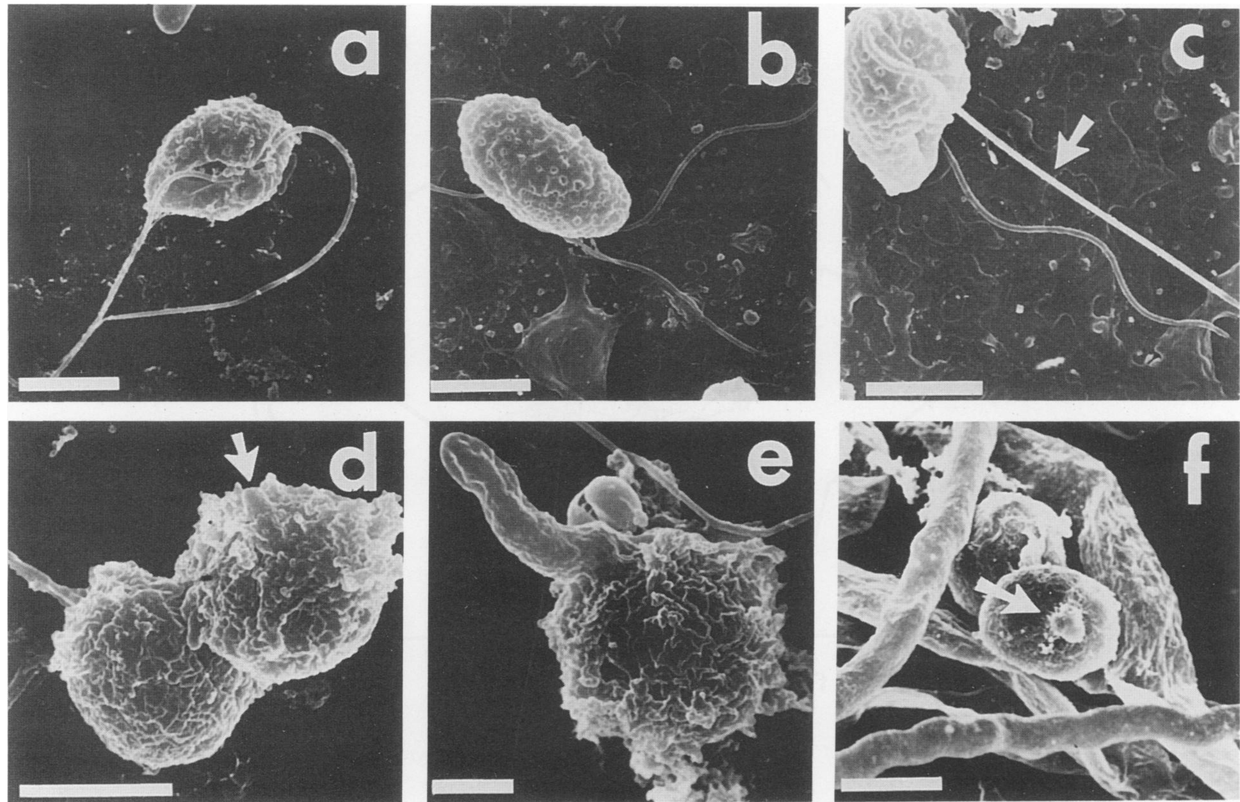

FIG. 3. Electron photomicrographs show the different morphological features of *P. insidiosum* zoospores (a to c). (a) Two flagella arising from a groove. The anterior flagellum (tinsel type) presents a sinosoidal pattern, (c) while the posterior flagellum (whiplash type) appears more rigid (c [arrow]). Under the conditions of this study, the zoospores showed ridges, papillae, and small pits (a to c). Encysted zoospores were characterized by their globose morphology. Small projections (d and f [arrows]) were detected in the place once occupied by the flagella. An amorphous substance is evident over the entire surface in those located on leaf (d) or skin (e) tissue but not in the tissue-free controls (f). Encysted zoospores develop germ tubes (e). Bars, 9 μm (a and b), 10 μm (c to f), and 5 μm (e).

surface. Several investigators also have shown a similar ultrastructural change occurring in encysted zoospores among other *Pythium* spp., *Phytophthora* spp., and chytridomycetes (6, 9, 11, 12, 16, 20–22, 40). They hypothesized that this coating functioned as an adhesive factor previous to invasion. Our findings indicate that this is the case. Such a finding is of paramount importance in explaining how the zoospores of *P. insidiosum*, attracted by a substance present in either plant or animal tissues, bind and remain attached to damaged plant and animal tissues. On the basis of the fact that most of the encysted zoospores found in the controls lacked this sticky amorphous material (Fig. 3f), we further hypothesized that perhaps the chemotactic factor may signal the cell to produce this material. This is supported by the findings for other *Pythium* species and other *Protoctista* (11, 12, 16, 17, 23, 31) showing that the discharge of a similar adhesive material from peripherally located vesicles was completed only after chemotactic stimulation by the host.

After encystment the zoospores always developed germ tubes even in cases in which the zoospores were distant from plant or animal tissues. In the case of zoospores with germ tubes bound to plant or animal tissues the germ tubes usually pointed in the direction of the tissues (Fig. 3e). This is in agreement with other reports on *Pythium* spp. and *Phytophthora* spp., which showed similar behavior upon contact with their hosts (9, 18, 29, 40). It is generally agreed that after penetration, the hyphae invaded the plant tissue intracellularly and extracellularly (14, 15, 19, 22, 29, 30, 40). In this study we noted the formation of peg-like structures (Fig. 1a) only on leaves. Thus, *P. insidiosum* may use an identical mechanism to invade plant tissue. However, we were not able to detect a similar structure in the encysted zoospores found on horse hide or hair. This is not surprising since penetration by *Pythium* hyphae through natural openings such as stomata has been also reported for plants (19, 22). Hyphal invasion of skin tissue was not detected in our histological sections because, in part, of the degenerative tissue changes that occurred after 24 h of incubation at 37°C in the induction medium. Under these conditions the skin tissue degenerated and most of its histological features were destroyed. Nevertheless, the encysted zoospores detected after 1 h of incubation were found bound to the external tissue producing germ tubes (Fig. 3e). This indicated that invasion of newly infected tissue may occur in a similar fashion during natural infections.

Several reports, on lower animals and humans that frequented swamps and became infected, have speculated about the possible role of the zoospore as an invasive agent (1, 27). Our data have shown that zoospores play an important role in the initiation of an infection in animal or plant hosts. This is due, in part, to their ability to produce a substance that allows them to maintain tight contact with the host during the initial stages of infection. Since appressoria or invasive pegs were not detected on the hair or

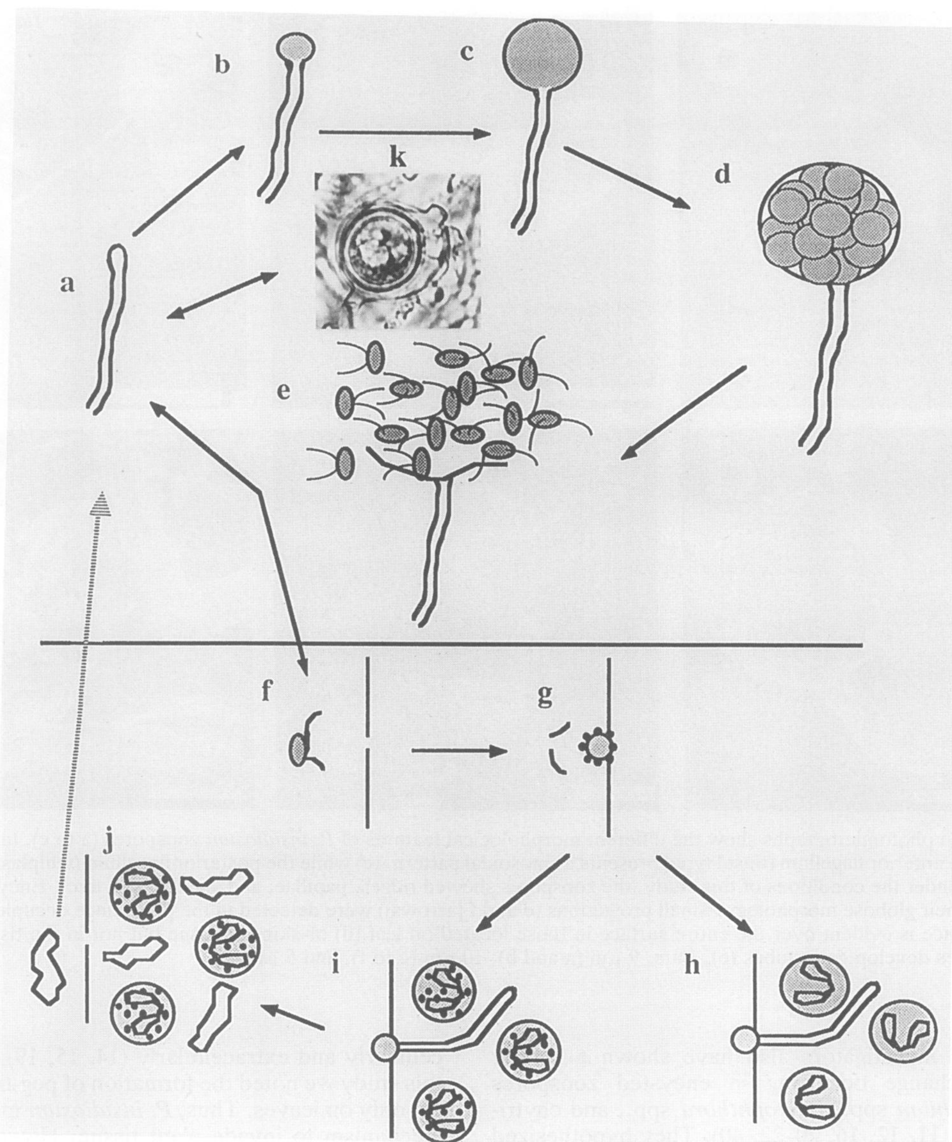

FIG. 4. The upper part of this figure shows the life cycle of *P. insidiosum* in nature. It starts when a plant is colonized (a), and then the differentiation of sporangium into mature stages (b to d) leads to zoospore release (e). The motile zoospores swim and, directed by attractants, locate another plant to start a new cycle. If an animal or human enters this ecosystem (lower part of the figure), zoospores will also be attracted by injured tissue, as showed in this study (f). There they lose their flagella attached to tissue by using a sticky substance (g [black dots]), encyst (g), germinate (h [humans] and i [animals]), invade the host, and cause pythiosis insidiosii. In horses the formation of masses, called "kunkers," occurs (j). These masses may be expelled and may produce sporangia in wet environments, but it has no impact on the epidemiology of pythiosis insidiosii. The production of oospores may occur in nature and serve as resistant spores (k [upper panel]).

skin biopsy baits during this study, and because of the fact that mycelium is not adhesive, it is more likely that zoospores act as agents of infection more efficiently than the other infectious propagules of this organism.

On the basis of the results of this ultrastructural study, we have designed a model to explain the cycle in nature of *P. insidiosum*. We also used these and previous data to illustrate the steps involved in the pathogenesis of pythiosis insidiosii (27, 35–37) (Fig. 4).

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