THE DEVELOPMENT AND CYTOLOGY OF THE EPIBIOTIC PHASE OF PHYSODERMA PULPOSUM [1, 2]

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ABSTRACT

LINGAPPA, YAMUNA. (U. Michigan, Ann Arbor.) The development and cytology of the epibiotic phase of Physoderma pulposum. Amer. Jour. Bot. 46(3): 145–150. Illus. 1959.—*Physoderma pulposum*, a chytrid parasite on *Chenopodium album* L. and *Atriplex patula* L., has a zoosporangial epibiotic phase. The latter consists of extramatrical sporangia and intramatrical bushy rhizoids, both enclosed in large protruding galls. The sporangia are subspherical, up to 350 μ in diameter, and may produce hundreds of planospores. If planospores settle on the host surface, they develop narrow germ tubes which penetrate the epidermal cells and develop into rhizoids. The planospore body, however, remains on the host surface and develops into a mature epibiotic sporangium in about 20–25 days at 16°C., 12–15 days at 20–25°C., or 6–8 days at 30°C. During development, its nucleus and daughter nuclei divide mitotically with intranuclear spindles until the sporangium contains several hundred nuclei. This is followed by progressive cleavage which delimits the planospore rudiments. When mature sporangia are placed in fresh water, the planospores are quickly formed within 1 hr. at 25°C. and begin to swarm within the sporangia. They escape in large numbers through an opening formed by the deliquescence of a papillum in the sporangial wall. The planospores are subspherical or elongate, 3–5 × 4–6 μ, and each has an eccentric orange-yellow refractive globule and a flagellum 18–22 μ in length. The electron micrographs of the flagella indicate that the flagella are absorbed from tip backward during encystment of the planospores. By periodic inoculation of the host plants with planospores from epibiotic sporangia, as well as from germinating resting sporangia, generation after generation of epibiotic sporangia have been obtained for 4 years. This proves the existence of a eucarpic, epibiotic, ephemeral zoosporangial phase in *P. pulposum*. Field observations on the duration and sequence of development of the fungus indicate that the endobiotic resting sporangial phase always follows the epibiotic phase. The results of infection experiments also indicate that the epi- and endobiotic phases belong to one and the same fungus, *P. pulposum*.

THE OCCURRENCE of epibiotic and endobiotic phases of *Physoderma pulposum* Wallroth (1833), their relationship, the presence of sexuality, and host reaction to infection were briefly reported by the author (1958). As emphasized in the paper just cited, Schroeter (1889) created the genus *Urophlyctis* on the basis of occurrence of epibiotic sporangia and as a result of a mistaken concept of zygomycetous sexuality in *P. pulposum*. However, the desirability of maintaining *Urophlyctis* as a separate genus from *Physoderma* was seriously questioned by several mycologists (cf. Karling, 1950). After careful study of the literature, Karling (1950) concluded that all known distinctive attributes of *Urophlyctis* were of doubtful diagnostic value as generic characters and he merged it in the older and valid genus *Physoderma*. Nevertheless, as pointed out by Karling (1950), the possibility exists that more valid distinctions may be found when all species have been fully studied. With this in mind, a comprehensive study of the life-history, development and sexuality of *P. pulposum* was undertaken, especially because it had been the type species of *Urophlyctis*. The present paper concerns only the development of the epibiotic phase, but the author proposes to present other aspects of the study in subsequent papers. However, in the interest of clarity, fig. 1 and its legend have been prepared in summary of the complete life cycle of *P. pulposum* as at present understood. The synonymy of various reproductive bodies is indicated by parentheses in the legend. Motile cells of the fungus are referred to as planospores, those that show sexual tendencies (irrespective of whether or not they fuse to form zygotes) as gametes, and asexual ones as zoospores. Inasmuch as stray and unfused gametes are not distinguishable from the zoospores when both are sedentary, they are referred to as planospores which is a noncommital term. The following account relates to the development of planospores into epibiotic sporangia.

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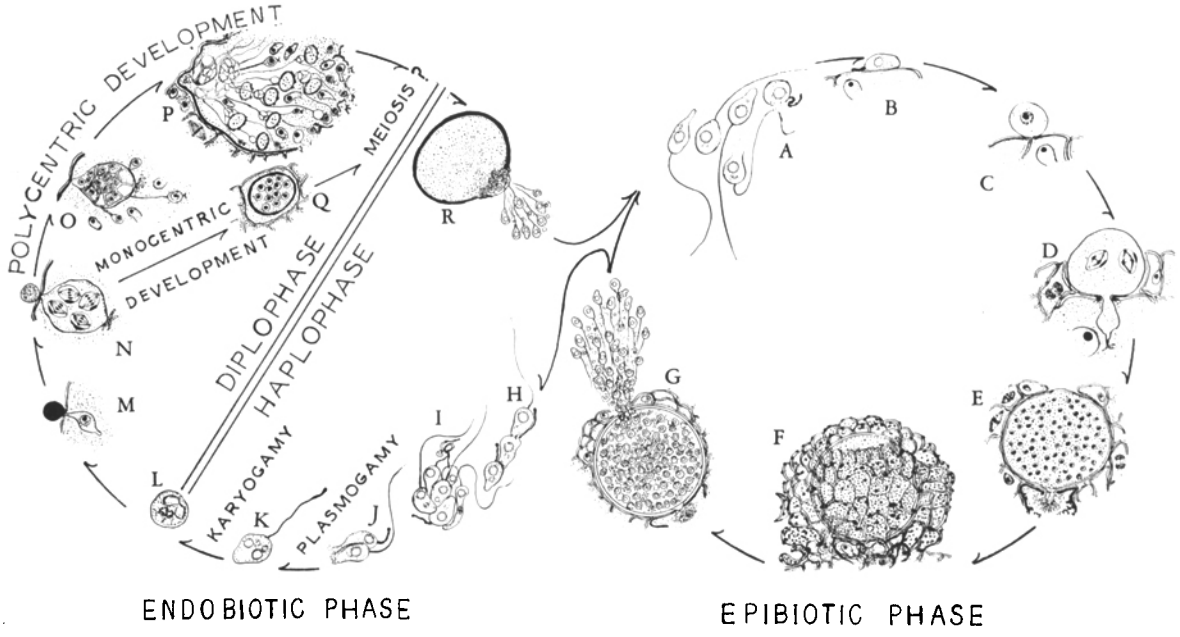


Fig. 1. Life cycle of *Physoderma pulposum* Wallroth.—A. Planospores (zoospores, gametes); B. Sedentary planospore attached to host surface; C. Infection by a planospore; D. Incipient epibiotic sporangium (sporangium, zoosporangium, gametangium) showing 2 dividing nuclei; E. Incipient multinucleate sporangium, showing proliferating neighboring host cells and bushy rhizoids; F. Gall enclosing a mature sporangium; G. Germinating sporangium; H. Chain of gametes; I. Clump of gametes; J. Pair of sedentary gametes; K. Zygote, immediately after plasmogamy; L. Zygote, after karyogamy; M. Infecting zygote; N. Incipient primary turbinate organ, showing the tuft of haustoria and dividing nuclei; O. Usual, polycentric development of primary turbinate organ. Development of incipient rhizomycelium; P. Well developed rhizomycelium, in a lysigenous cavity of the host, consisting of hyphae, and several orders of empty or growing turbinate organs which bear resting sporangia terminally; Q. Less common, monocentric development of primary turbinate organ into resting sporangia (resting spore); R. Germinating resting sporangium. As meiosis has not been observed, delimitation of haplophase and diplophase is tentative.

MATERIALS AND METHODS.—The 2 collections of *P. pulposum* on *Atriplex patula* L. and *Chenopodium album* L. reported previously (Lingappa, 1958) were maintained in the greenhouse on these hosts by periodic inoculations with planospores from epibiotic sporangia (fig. 1G). Occasionally, planospores from germinating resting sporangia (fig. 1R) were also used as inocula to start the epibiotic sporangial phase. *Chenopodium album* is hardy, quick-growing, and has broader and more crowded leaves and sturdier shoots than *A. patula* and, accordingly, is better suited for maintenance of the fungus. Therefore, it was used exclusively as a host in this study, although *A. patula* plants were inoculated also to serve as controls to determine whether or not any basic developmental differences occurred on this host. Before inoculation, the prospective host plants were wetted with a dilute solution of "Tween 80" and rinsed thoroughly with nonchlorinated water to insure a wet surface for the planospores. Small pieces of infected leaves bearing numerous mature epibiotic sporangia were then placed on the apical rosette of leaves, flooded with nonchlorinated water, and covered with a wet pad of absorbent cotton to provide the necessary moisture for dehiscence of the sporangia and dispersal of the planospores. About 2 hr. later, a drop of this water was examined to make certain that a good inoculum of active planospores was present; at the same time additional drops of water were placed on the cotton pads. The inoculated plants were grown in the greenhouse at a time when the temperature varied between 20° and 30°C; under these conditions infection occurred readily and abundantly. From November to March, the temperature of the greenhouse was thermostatically maintained at 16–30°C. Unless otherwise mentioned, the following account relates to the development of the fungus growing under the latter temperatures.

Under greenhouse conditions, epibiotic sporangia attain maturity within 2 wk. after inoculation of host plants. Infected shoots bearing abundant mature sporangia, collected and stored at 5°C. in tightly closed containers, served as a ready source of planospores. For observations on infection by planospores, small and very young leaves were removed from apical buds, washed in "Tween 80" and nonchlorinated water, and mounted in hanging drops of planospores. These preparations were kept at about 20°C. and studied microscopically at intervals for several days. Also, samples from plants,

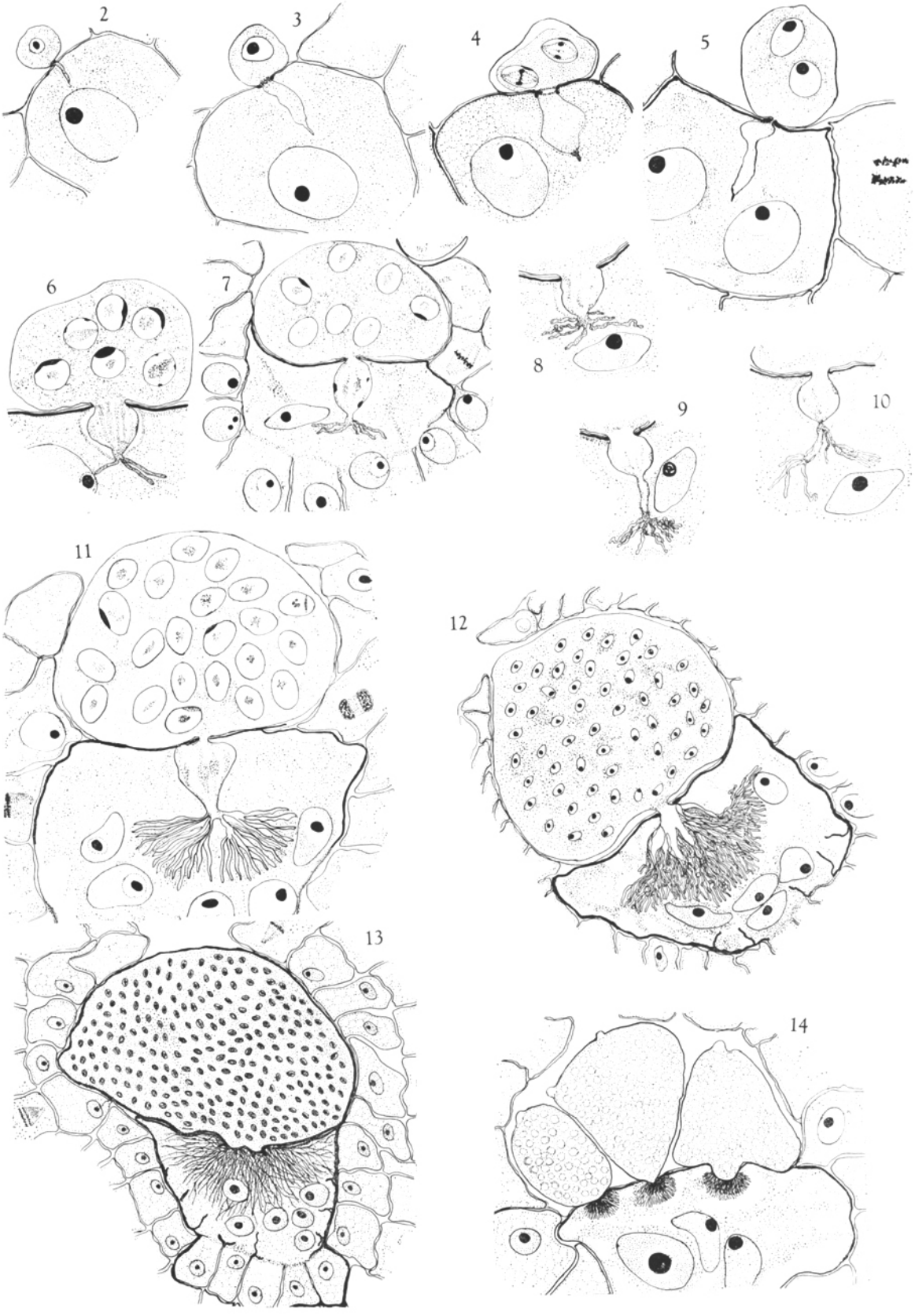
inoculated and maintained in the greenhouse, were collected and examined at regular intervals. For study of developmental stages of the fungus, both living and fixed and stained materials were used. Densely infected leaves were removed at different intervals following infection, macerated in warm N HCl or 75% acetic acid, and squashed in lactophenol-cotton blue or lactophenol-acid fuchsin. At the same time, portions of infected leaves were fixed in formalin-acetic-alcohol, Cleland's modified Bouin's fluid, Randolph's modified Navashin's fluid (CRAF) and Flemming's solutions described by Conn and Darrow (1946). This material was dehydrated through N-butanol-ethanol series, embedded in Fisher's Tissuemat, sectioned at 6–20 μ and stained with safranin-fast green, Newton's crystal violet, Flemming's triple stain, or Harris' haematoxylin. The resting sporangia were germinated on 3% agar plates as described by Lingappa (1955) for *Synchytrium*. Single or several epibiotic sporangia and resting sporangia were germinated in separate hanging drops and placed at—10 to 35°C., in order to study the behavior of the planospores, their viability, duration of activity and absorption of flagella. Such preparations were also used for inoculating the host plants.

OBSERVATIONS.—At about 25°C., the planospores discharged from epibiotic or resting sporangia swim actively for about 3 hr., but thereafter become sluggish and settle down on the host surface. They may remain motionless or whirl about and jerk with intermittent rest for about 2 hr. before becoming quiescent. Some of these are amoeboid and develop conspicuous pseudopodia, while others are spherical or oblong. As they become sedentary, the flagella are gradually absorbed (fig. 1A, B). In water suspension, the sedentary planospores become hydrated, enlarged to 15 μ or more in diameter, and eventually disintegrate without developing germ tubes. In the presence of a suitable host, however, the amoeboid, twisting and whirling movements of the planospores cease about 2 hr. after settling, and they become firmly attached to epidermal cells (fig. 1B). This attachment becomes evident when attempts are made to dislodge them by moving the cover glass or by washing with a jet of water.

About 6 hr. after attachment the spores increase in size and their heterogeneous contents become almost transparent. Their oil globules decrease in size and disappear as the germ tubes develop from the attached spores which pierce the cuticle and epidermal wall (fig. 2). The germ tube becomes the rudiment of the rhizoidal system. Several planospores may infect a single host cell so that several sporangia may develop on one such cell (fig. 14). At 20°–25°C., early stages of infection may be found within 24 hr. after inoculation. Under greenhouse conditions, infection as well as subsequent development are more rapid during summer than winter and at higher temperatures than at lower temperatures.

When 1 day old, the infecting planospore is globose to subglobose with a thin hyaline wall, finely granular cytoplasm and a centric or eccentric nucleus (fig. 2). A deeply-stained ring or collar is usually visible at the point where the germ tube has penetrated the host cell (fig. 3–5), but it disappears as the incipient epibiotic sporangium enlarges (fig. 6, 7, 11–14). Within 2 days after infection, the incipient epibiotic sporangia may average –56 × 8–10 μ in size and have swollen rhizoids (fig. 3). In living material, the developing germ tube is only faintly visible, but in fixed and stained sections it stands out clearly (fig. 2, 3). As it grows and enlarges, it may become somewhat fusiform, obpyriform or turbinate and appear like an extension of the developing epibiotic sporangium into the host cell (fig. 4). However, by about the fourth day after infection, the swollen portion of the germ tube develops into an intracellular apophysis (fig. 6–11), and radiating tubular rhizoidal branches develop from the narrow basal tip of the germ tube (fig. 6–11). The apophysis, however, is less conspicuous or not evident in older sporangia (fig. 13). Consequently, the incipient sporangia are apophysate (fig. 6–11) as in *Phlyctochytrium* and the older sporangia are non-apophysate (fig. 1, 12–14) like *Rhizophydium*. Thus, the germ tube is the rhizoidal initial, the tip of which branches into a bushy tuft of rhizoids within a week after infecting the host cell. When fully formed, the rhizoids have thick basal branches and relatively fine, richly branched and contorted tubular extremities. They form bushy tufts at 1 or more points on the base of the sporangium and may be fairly extensive filling a large portion of the limited lysigenous cavities (fig. 12–13). The rhizoidal initials (germ tubes) are intracellular, but as they branch the walls of the cells underneath them lyse, so that a limited lysigenous cavity is formed (fig. 7, 11–13). The extent of development of the rhizoids varies considerably, so much so that the size of the mature sporangia, or the lysis and division of the underlying host cells, may not always be correlated with the development of the rhizoids. The nuclei of the lysed cells usually accumulate in the vicinity of the rhizoids (fig. 11–14). The presence of 2 nuclei in the infected host cells (fig. 5), prior to lysis of the underlying host cells, suggests that the nucleus of the infected cell divides, but so far actual divisions have not been seen. The infected epidermal cells, however, do not divide. As will be described in another paper on host reaction, the mature sporangia are overgrown partly or completely by proliferation of the neighboring host cells and are thus enclosed in protruding galls on the surface of the host (fig. 1F, 12, 13).

Sporangia, Sporogenesis, and Planospores. — While rhizoids are developing, the spore body enlarges and eventually grows into the epibiotic sporangium (fig. 1). The nucleus remains in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the spore body, and, as a result, the latter becomes the center of growth and organization. In this respect, the development of the epibiotic phase is quite different from that of the endobiotic phase (to be described in a subsequent paper), where the nucleus migrates down into the germ tube which develops into rhizomycelium. The primary nucleus in the incipient sporangium is conspicuous with a deeply-stained, ovoid or spherical nucleolus, but chromatic material is not visible within the nuclear cavity. By the third day after attachment of the zoospore, the incipient sporangia are subspherical, reniform or hemispherical and usually binucleate (fig. 4, 5). Division of the primary nucleus has not been observed, but several dividing secondary nuclei have been seen (fig. 4). In preparation for division the nucleolus appears to decrease in size, becomes disc-shaped, and moves to one side of the nucleus (fig. 6, 7, 11). At metaphase, the spindle is intranuclear and extends across the nuclear cavity. Its poles are anchored on the nuclear membrane, and there appear to be only 2 chromosomes at the equator of the spindle (fig. 4), but this number has not been definitely determined. The daughter nuclei are smaller than the primary one, and, as successive divisions occur, the nuclei become progressively smaller, until they reach a definitive size of about 1.5 μ in diameter. As a result, subsequent divisions (fig. 13) are hardly recognizable as such because of the minute size of the figures.

After infection, planospores develop into mature sporangia in about 20–25 days at 16°C., 12–15 days at 20–25C., or 6–8 days at 30°C. The mature sporangia are usually subspherical and 60–350 μ in greatest diameter (fig. 1F), but when sporangia develop close together, they may be elongate, flattened or irregular in shape and smaller in size (fig. 14). In preparation for sporogenesis, highly dispersed orange-yellow refractive globules in the cytoplasm of the sporangia begin to coalesce until the definitive globules of the planospore initials are formed. Cleavage of the protoplasm then follows until uninucleate segments are delimited which mature into planospores in the presence of fresh water. Then. one or more hyaline papillae develop on the wall of each sporangium, and 1 of them deliquesces to form an exit pore. Within the unopened sporangia, the planospores begin to move slowly, and this motion gradually increases, so that within 10 min. the whole mass of planospores starts swirling. As the planospores begin to escape through the pore and as pressure within the sporangium is reduced, the movement of planospores becomes even more rapid and they stream out in great numbers. They usually remain motionless for a few seconds after emergence and then swim away. The whole process of sporogenesis takes place within 1 hr. at 25°C., but 12 or more hr. are required at 10°C. The sporangia may form and liberate the planospores at 5–30°C., but freezing, air-drying or exposure to 35°C. for 1 hr. kills the sporangia as well as the planospores. Infected shoots bearing mature epibiotic sporangia yield active and infectious planospores up to 2 months after storage at 5°C. in tightly closed containers. Also, the sporangia remain viable in the greenhouse, on host plants, for nearly 1 month after maturation if the temperature of the greenhouse is below 20°C. and water is withheld from the sporangia.

The planospores are subspherical, ovoid, oblong or elongate and measure 3–5 × 4–6 μ, each with an eccentric orange-yellow globule, a nucleus and a faintly visible nuclear cap (fig. 1A). The flagellum is 18–22 μ long with a fine lash at the distal end. Giant planospores which measure 6–8 × 8–20 μ and have as many as 20 flagella and numerous refractive globules have been observed. They occur frequently when sporogenesis takes place rapidly above 25°C. and are probably the result of unequal cleavage. The normal planospores may swim actively in water for as long as 72 hr. at 5°C., and 3–10 hr. at 23°C. At 27–30°C., some of them may become inactive within 10 min. after emerging from the sporangia.

The flagella of many planospores become crooked, beaded or looped, short and stumpy by the time the latter become sedentary. This process has been followed in hanging drops as well as on the host surface, and it is obvious that gradual shortening occurs by absorption. In hanging drop preparations, however, flagella of all planospores are not absorbed but remain intact in many. They ultimately degenerate along with the body of the spores, and as a result, component fibrils of flagella become visible in electron-microscope images. The whip lash of the flagellum has a bulbous tip as in those of other chytrids (Manton et al, 1952; Koch, 1956; Lingappa, 1958). Planospores were prepared for examination with the electron microscope by a

Fig. 2–14. Development and cytology of epibiotic phase of *Physoderma pulposum*.—Fig. 2, 3. One- and 2-day-old incipient sporangia, respectively. ×1500.—Fig. 4. Three-day-old incipient sporangium showing intranuclear spindles. × 1500.—Fig. 5. Three-day-old sporangium. Infected host cell is binucleate and nucleus of a neighboring host cell is dividing. ×1500.—Fig. 6. Four-day-old sporangium showing the development of the rhizoids. ×1500.—Fig. 7. Five-day-old sporangium showing early stages of dissolution of host cell walls and division of neighboring host cell nucleus. ×900.—Fig. 8–10. Development of rhizoids from apophysis in 4- or 5-day-old sporangia. ×1200.—Fig. 11. Six-day-old sporangium. ×1000.—Fig. 12. Seven-day-old sporangium partially enclosed by proliferated gall cells, also showing lysed host cells, rhizoids in the limited lysigenous cavity and thickening of the walls bordering the cavity. ×600.—Fig. 13. Eight-day-old sporangium showing minute dividing secondary nuclei, wall bordering the lysigenous cavity has further thickened, and gall is well developed. ×300.—Fig. 14. Three mature sporangia developing on a single host cell. They are distorted due to crowding and are much smaller than normal. Note the hyaline papillae in the sporangial walls. ×600.

method similar to that used by Ferris (1954), obtaining the planospores at timed intervals after their discharge from epibiotic sporangia. Droplets of planospore suspensions which were maintained at 28°C. were fixed ½ hr., 2 hr., 3 hr., and 6 hr. after emergence when planospores were actively swimming, sluggish, sedentary and degenerating respectively. The whip lash is shortened and terminal bulb is enlarged in sluggish planospores fixed 2 hr. after emergence, and in sedentary spores, absorption of the flagella has progressed further. Electron microscopy has confirmed the writer's observations of living as well as of fixed and stained planospores of *P. pulposum* which indicate that the planospores absorb flagella when they become sedentary.

After discharge of planospores, the sporangial walls collapse and degenerate without proliferating like those of other species of *Physoderma*. The intramatrical rhizoids and the enveloping host cell contents also degenerate, and this leads to the formation of crateriform galls. The gall cells, however, continue to proliferate and become reinfected by planospores or zygotes. This leads to the formation of crustaceous galls which may be so extensive, as in several species of *Synchytrium*, as to disfigure and distort the shoots of the host plants.

As noted previously by the author (1958), when planospores from single epibiotic sporangia or from a single or many germinating resting sporangia are inoculated on the host plants only epibiotic sporangia develop. On the other hand, both epi- and endobiotic phases develop when mixtures of planospores from many epibiotic sporangia are used as inocula. The sequence of development of the 2 phases of the fungus was carefully observed in the field for 4 consecutive years. Several ditches were selected for the purpose where severe infection of *A. patula* plants occurred year after year. Only epibiotic sporangia were found on the seedlings, showing the first infection of the season, in the second week of April, whereas both phases were present together after the end of April. However, the epibiotic phase predominates throughout summer and rapidly spreads the fungus among host plants. The epibiotic sporangia become less abundant during the latter half of October and are difficult to find a month later. In November, they may be collected on bracts and glumes of the host plants, and by the end of the month, after the first snow fall of the season (as epibiotic sporangia and planospores are killed by freezing), the spread of the fungus ceases. At this time the infected plants show only prominent, deep-seated galls which are filled with resting sporangia.

DISCUSSION.—The present observations on the epibiotic phase of *Physoderma pulposum* confirm those of Schroeter (1882), who was the first to note its occurrence in the life-cycle of *Physoderma* species. Inasmuch as the epibiotic sporangia of *P. pulposum* had not been found since that time, subsequent workers believed that Schroeter's observations related to a species of *Synchytrium*, particularly because the sporangia described by Schroeter were comparatively larger and enveloped in protruding galls. Now that the epibiotic sporangia have been obtained repeatedly by inoculating the host plants with planospores from the epibiotic sporangia as well as from the germinating resting sporangia, doubt no longer exists as to the occurrence of an epibiotic sporangial phase in *P. pulposum*. Furthermore, infection experiments as well as field observations on the sequence of development of the epi- and endobiotic phases indicate that these are interrelated and belong to one and the same fungus, namely, *P. pulposum*.

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