Factors Affecting Behavior and Developmental Synchrony of Germinating Oospores of *Pythium aphanidermatum*

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ABSTRACT

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Germination of oospores of *Pythium aphanidermatum* was greatly stimulated by desiccation of cultures prior to spore isolation. Germination of oospores from desiccated cultures was induced with good synchrony following the application of two additional activating treatments: incubation in water for 5 min at 39 C and agitation in 0.1% $KMnO_4$ for 15 min. A synthetic liquid medium containing salts and lecithin was developed for synchronous indirect germination (production of zoospores from oospore hyphal germ tubes). In the absence of lecithin and when lecithin was replaced by other organic compounds in the medium, activated oospores germinated at a very low rate. Addition of glucose and other organic compounds to the liquid germination medium resulted in oospore germination without the production of zoospores (direct germination).

Oospores are considered to be among the most resistant structures produced by the oomycetous fungi. In relation to other oomycetous spore forms (zoospores, sporangia, and chlamydospores) oospores appear to be more difficult to germinate under experimental conditions and more capable of remaining viable in the dormant state under natural conditions (2,4,12).

Intercrop survival of the plant pathogenic oomycete *Pythium aphanidermatum* (Edson) Fitzp. in arid agricultural soils is thought to be as oospores (12). The effects of nutritional, physical, and chemical factors on oospore germination in soil and in vitro have been investigated in *P. aphanidermatum* (1,2,10,12,13). Most studies have indicated that culturally produced oospores of this species are not readily germinable unless various activating treatments are applied. The following treatments have been reported to increase the percentage of germinating oospores in *P. aphanidermatum*: ingestion by water snails (13), incubation in nonsterile soil extract (2), and drying on agar disks (2). Although the percentage of germination was high following application of the above treatments, synchronous germination of oospores apparently was not attained. A further disadvantage of snail ingestion and incubation in nonsterile soil extract is that the oospores become contaminated with other microorganisms.

The nutritional requirements for oospore germination and the nature of the accompanying metabolic changes in oospores during the transition from dormant to vegetative phase in the oomycetous fungi are poorly understood. The primary obstacles to carrying out physiological studies with oospores have been the lack of suitable, defined liquid media for germination and the lack of methods for obtaining developmental synchrony during oospore germination; ie, such that individual spores in the germinating population undergo simultaneous developmental changes. The present study is concerned with the optimizing of developmental synchrony of oospores of *Pythium aphanidermatum* germinating in synthetic liquid media and with nutritional factors affecting biomodal germination.

MATERIALS AND METHODS

Isolates. Hyphal tip isolates of *P. aphanidermatum*, from infected roots of tomato (*Lycopersicon esculentum*) and pepper (*Capsicum annuum*), were used in this study. The two isolates were morphologically indistinguishable.

Oospore production and cultural conditions. Two different solid media were used for oospore production: a chemically undefined, natural medium (CMAW), which contained 1.7% Difco Corn Meal Agar and 0.1% wheat germ oil (Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 USA) in deionized water, buffered with 2.1 mM K_2HPO_4 and 0.9 mM KH_2PO_4; and a defined medium (DM), which contained 1 mM glucose, 0.1 mM asparagine, 0.1% L-α-lecithin from soybean (Sigma Chemical Company, St. Louis, MO 69178, grade II-S), 0.005 mM thiamine HCl (Sigma), 4 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 1 mM $CaCl_2$, and 1.5% Difco Bacto agar in distilled water buffered with 1.8 mM K_2HPO_4 and 1.2 mM KH_2PO_4. Sterilized buffer solutions were added to the autoclaved media at 45 C. Both media were dispensed in 20-ml aliquots in 9-cm-diameter plastic petri dishes. For inoculum, 6-mm diameter disks were taken from 48-hr-old cultures grown at 30 C on DM from which lecithin was omitted. Cultures were seeded with disks and incubated for 7 wk at 30 C in the dark.

Isolation of oospores. The contents of two petri dish cultures were homogenized in a sterile stainless steel blender (4 min at low speed and 1 min at high speed) with 100 ml of sterile deionized water (SDIW). The temperature was not permitted to rise above 25 C during blending. The blended homogenate was filtered successively through sterilized nylon cloth (Nitex, Schweizerische Seidengazefabrik, Zürich, Switzerland) of 125-μm and 74-μm pore size and centrifuged at 10,400 *g* for 10 min. A sterile pasteur pipette was used to separate the supernatant and upper layer of the pellet (containing agar and mycelial debris) from the lower pellet layer (containing free oospores). The oospore pellet was washed by centrifugation at ~ 500 *g* for 20 sec. The supernatant was discarded, the pellet was resuspended in 2 ml of SDIW and was dispersed with a Vortex-Genie mixer (Scientific Instruments, Bohemia, NY 11716). Washing by centrifugation and resuspension in SDIW was repeated four times. Oospores were then washed on sterile nylon cloth (10-μm pore size) with 250 ml of SDIW at 10 C, filtered through 48-μm (pore size) nylon cloth, and concentrated by centrifuging at 10,800 *g* for 10 min. Oospore suspensions were free of lobate sporangia, agar, and mycelial debris. Before being used in germination experiments, purified oospore suspensions were stored at 12 C for no longer than 1 wk.

Activation treatments. The desiccation treatment was carried out prior to isolation of oospores from the solid growth media CMAW and DM. Generally 10–20 cultures were dried

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simultaneously for 2 days at 30 C in a drying chamber measuring 30 × 30 × 70 cm. Five to seven cultures were arranged in a circular array on each shelf of the chamber and a dish containing about 200 g of anhydrous $CaCl_2$ was set in the center of the array. In order to promote the absorption of moisture, petri dish lids were placed askew so that the side nearest the desiccant was open. Before blending, desiccated cultures were rehydrated in cold SDIW for about 15 min. High temperature and $KMnO_4$ treatments were carried out following isolation of oospores. In the high-temperature treatment, a spore suspension in 10 ml SDIW was incubated for 5 min at 39 C or at other temperatures in a circulating water bath (Gebrüder Haake, Berlin, W. Germany), after which the suspension was cooled to 6 C and concentrated by centrifuging at 10,800 g for 10 min. Treatment with the oxidizing agent $KMnO_4$ was as follows: oospores were suspended in 10 ml of freshly prepared 0.1% $KMnO_4$ in SDIW, vigorously agitated with a Vortex mixer for 15 min at room temperature, and washed on sterile 10-μm (pore size) nylon cloth with 250 ml SDIW. Washed oospores were then filtered through sterile, 48-μm (pore size) nylon cloth and concentrated by centrifugation at 10,800 *g* for 10 min.

Germination media. Oospores isolated from the growth medium, with or without activation treatments, were germinated at 28 C, unless indicated otherwise, on the solid medium DM, or in a liquid medium (S+L) consisting of basal salts with lecithin (Sigma) as the only source of organic carbon. S+L contained 0.08 mM $(NH_4)_2SO_4$, 0.04 mM $MgSO_4$, 0.02 mM $CaCl_2$, 0.0001 mM $ZnSO_4$, and 0.01% lecithin in glass-distilled water, adjusted to pH 7.0 with KOH, and buffered with 0.024 mM KH_2PO_4 and 0.036 mM K_2HPO_4. Sterilized phosphate buffer was added to the autoclaved medium after cooling. In experiments in which the pH was other than 7.0, the medium was adjusted with HCl or KOH to the desired pH before autoclaving and the phosphate buffer was prepared with the appropriate KH_2PO_4:K_2HPO_4 ratio. Glucose, asparagine, glycine, ethanol, or sodium acetate solutions were adjusted to pH 7.0, sterilized by membrane filtration (Millipore, 0.45 μm), and added to separate quantities of the S+L medium after autoclaving. Oospores were added to the S+L medium at a density of 10,000 spores per milliliter. Spore suspensions were shaken vigorously for 10 sec with the aid of a Vortex mixer and incubated in sterile, acid-washed, glass petri dishes in liquid depths not exceeding 1 cm.

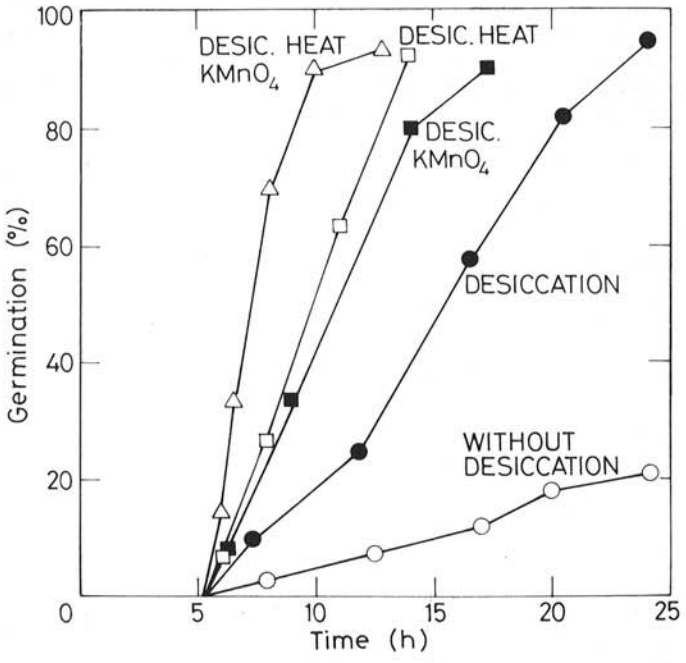

Fig. 1. Direct germination of *Pythium aphanidermatum* oospores on a defined medium (DM) at 28 C. Oospores were isolated from desiccated and nondesiccated 7-wk-old cultures and were treated at high temperature (5 min at 39 C) and with $KMnO_4$ (15 min with agitation in 0.1% solution, followed by rinsing) prior to being incubated on DM. Points represent averages of four separate experiments, each employing oospores produced and isolated under identical conditions.

RESULTS

Induction of synchronous germination. Formation of oogonia and antheridia in cultures of *P. aphanidermatum* occurred on the 4th day of incubation at 30 C on both the natural medium CMAW and the synthetic medium DM. By the 8th day of incubation, abundant oospores were present in cultures. When oospores were isolated from 4-wk-old cultures and incubated on DM, only about 5% germination occurred in 24 hr. Oospores which were isolated from moist 7-wk-old cultures showed a slightly higher rate of germination, about 20% in 24 hr. Desiccation of 7-wk-old cultures prior to oospore isolation greatly enhanced spore germination. Over 90% of the spores isolated from desiccated cultures produced on either CMAW or DM germinated within 24 hr at 28 C (Fig. 1). In spite of the greatly increased germination rate, development of desiccated spores incubated on DM proceeded with poor synchrony; ie, among individual oospores there was wide variation in the times at which hyphal emergence and elongation occurred.

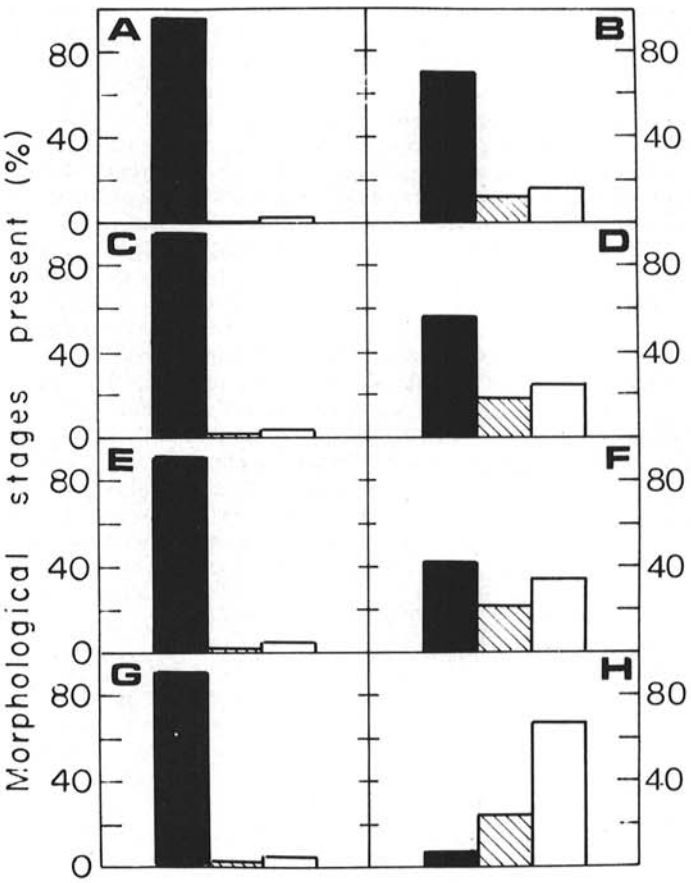

Fig. 2. The effect of various activation treatments on the development of germinating oospores. Microscopic assessment of the distribution of morphological stages among variously treated oospores was made at 10 hr of incubation at 28 C on a solid defined medium (DM). Left column (A,C,E,G): oospores isolated from moist cultures. Right column (B,D,F,H): oospores isolated from desiccated cultures. **A,B,** Without additional treatments; **C,D,** treated with $KMnO_4$; **E,F,** treated at 39 C for 5 min; **G,H,** treated sequentially at 39 C and with $KMnO_4$. Three stages of direct germination were distinguished: dormancy and preemergence (solid bars), emergence (germ tube entirely contained within oogonial wall; striped bars), and elongation (germ tube broken through oogonial wall; open bars) (200 spores were examined in each incubated sample). Data represent averages of two separate experiments, each employing oospores produced and isolated under identical conditions.

Dormant oospores from desiccated cultures, but not those isolated from moist cultures, were activated by a high-temperature treatment. Incubation of oospores in SDIW for 5 min at 39 C resulted in a further increase in the rate of germination (Fig. 1 and 2). The germination rate decreased to less than 30% in 12 hr following incubation for 5 min at 45 C. After incubation for 5 min at temperatures above 50 C, the cytoplasm of oospores lost its typical granular appearance and none of the oospores germinated.

The rate of germination also was increased by suspending oospores isolated from desiccated cultures in 0.1% $KMnO_4$ for 15 min with continuous agitation. When oospores isolated from moist 7-wk-old cultures were treated with $KMnO_4$, no increase in the germination rate occurred. Further improvement in the synchrony of germination of desiccated oospores occurred when the $KMnO_4$ treatment followed the heat treatment (Fig. 1 and 2). Lengthening the duration of the $KMnO_4$ treatment to 20 min resulted in the mortality of over 30% of the oospores.

Germination in a liquid medium. By modifying DM, a liquid medium in which activated oospores would germinate with adequate synchrony was developed. When agar, glucose, asparagine, and thiamine were omitted from DM, activated oospores germinated by the production of zoospores from the hyphal germ tube (indirect germination); in this preliminary liquid medium, however, suspensions of activated oospores showed poor developmental synchrony during germination. Improved developmental synchrony was obtained by diluting both salt and lecithin concentrations of DM. Varying the concentration of potassium phosphate buffer was found to have a marked effect on the rate of germination of activated oospores. With 0.06 mM phosphate buffer in the liquid medium S+L, the germination rate was greater and germling development more uniform than in the presence of higher phosphate buffer concentrations (0.3 mM, 3.0 mM) or in the absence of buffer (Fig. 3-A).

Poor developmental synchrony occurred at lecithin concentrations of 0.02% and above, resulting from rapid vegetative growth of early germlings and retarded development of the remainder of the spore population. Improved synchronous germination was obtained with 0.01% lecithin in S+L. With lower concentrations of lecithin in the liquid medium, the rate of germination of activated oospores was greatly reduced. At 0.005% lecithin, germination was less than 50% in 12 hr; less than 5% germination in 12 hr occurred when oospores were incubated in either S+L containing 0.001% lecithin or in the basal salt solution without lecithin (Fig. 3-B). A similar, low rate of germination occurred when lecithin was replaced by various concentrations of cholesterol, β-sitosterol, or glycerol (0.1 mM, 0.5 mM, 1.0 mM, 5.0 mM).

Germination of activated oospores was compared in S+L (0.06 mM phosphate buffer, 0.01% lecithin) adjusted to different pH values. As shown in Fig. 3-C, the rate of germination was greatly reduced at pH 5 and was the highest at pH 7.

The number of zoospores produced by oospores germinating in the liquid medium S+L was reduced by increasing the temperature of incubation to 30 C and above. Although the rate of germination in the liquid medium at 30 and 33 C was similar to that at 28 C, less than half of the germlings underwent zoospore release at the higher temperatures. Elevation of the temperature of incubation to 35 C and above resulted in a substantial decrease in the rate of oospore germination (Fig. 4). Apparently this inhibitory effect is due to the inavailability of oxygen resulting from its decreased solubility in water at elevated temperatures. When activated oospores were incubated at 35–39 C on a solid medium (S+L, to which 1.5% Bacto-Agar was added), in contrast, over 90% germination occurred between 5–7 hr of incubation.

Morphology of oospores germinating indirectly in a liquid medium. The distribution of developmental stages during germination of activated oospores in the liquid medium S+L is presented in Fig. 5. Similar results were obtained for oospores produced on CMAW and DM. Oospores were activated with sequential treatments of desiccation, high temperature, and $KMnO_4$, as described in Materials and Methods. Four developmental stages of germination were distinguished; I, dormancy and preemergence (Fig. 6A,B); II, initiation of germ tube emergence (germ tube entirely contained within surrounding oogonial wall, as in Fig. 6C); III, germ tube elongation (germ tube broken through the oogonial wall, as in Fig. 6D,E); and IV, initiation of zoospore differentiation and release (Fig. 6F).

During hours 6–8 of incubation in S+L, germ tube emergence and elongation occurred in over 90% of the oospores. During this period a vacuole surrounded the central globule at an early stage of germination (Fig. 6C,D) and underwent continuous enlargement. Cytoplasmic contents of germlings became concentrated in the elongating germ tube, completely evacuating the inner region of the oospore wall (Fig. 6E). Between 10 and 12 hr of incubation germ tube elongation ceased and a sequence of morphological events leading to zoospore release was initiated in about 80% of the germlings. The cytoplasm gradually withdrew from the base of the germ tube and became increasingly concentrated in the distal portion. The germling cytoplasm finally became localized in the enlarged vesicle formed at the germ tube apex and became differentiated into zoospores (Fig. 6F). Upon release of zoospores from the vesicle, the entire germling was devoid of cytoplasmic contents. Length of the germ tube varied considerably at stage IV,

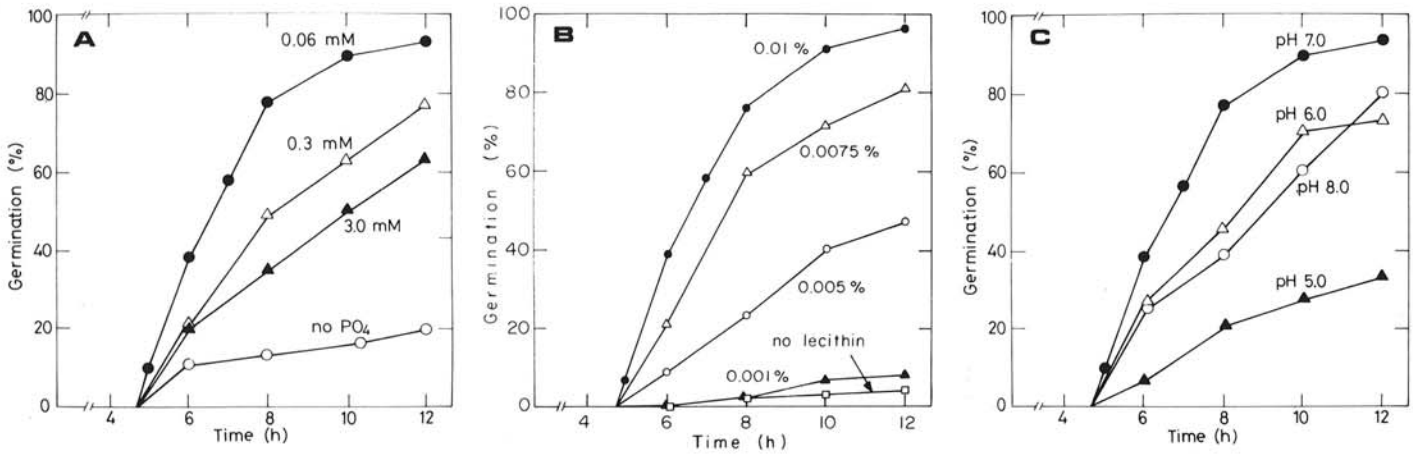

Fig. 3. Indirect germination of activated oospores of *Pythium aphanidermatum* at 28 C in a liquid basal salts medium supplemented with emulsified lecithin. Oospores from desiccated cultivars were activated by sequential incubation at 39 C for 5 min and agitation in $KMnO_4$. The effects of **A**, various concentrations of potassium phosphate buffer (pH 7, 0.01% lecithin), **B**, various concentrations of lecithin (pH 7, 0.06 mM potassium phosphate), and **C**, different pH values (0.06 mM potassium phosphate, 0.01% lecithin) are compared.

ranging from about 80–>300 μm.

Direct germination in S+L + glucose. The development of activated oospores was changed from indirect germination to direct germination (ie, without the production of zoospores) by the addition of 0.1 mM glucose to S+L. In S+L + glucose, enlargement of the vacuole surrounding the central globule occurred during stages II and III as with oospores germinated in S+L without glucose. The inner contents of the germinated oospore became largely devoid of cytoplasm during germination in both media. In S+L + glucose, however, relatively little withdrawl of the cytoplasm from the base of the emergent germ tube occurred; during the 10th to 14th hr of germination, the cytoplasm remained distributed throughout most of the germ tube. In contrast, when glucose was not present in S+L, the cytoplasm become concentrated distally at the apex of the germ tube and development proceeded as described above for stage IV. 0.1 mM was the minimal concentration of glucose which completely prevented the development of stage IV during 14 hr of incubation at 28 C. When the glucose concentration in S+L was reduced to 0.05 mM, stage IV development occurred in about 80% of the germlings after 12–14 hr of incubation. Several other organic compounds, when added to S+L, also blocked the development of stage IV; minimal concentrations of asparagine (10 mM), sodium acetate (10 mM), glycine (20 mM), and ethanol (50 mM) which blocked zoospore formation, without disturbing synchronous development during germination, were found to be greater than that of glucose (0.1 mM). To determine if any of these carbon sources is capable of stimulating oospore germination in the absence of lecithin, various concentrations (100 mM, 10 mM, and 1 mM) of glucose, asparagine, sodium acetate, glycine, and ethanol were added individually to the basal salts medium. In all of the variously amended media, the rate of germination of activated oospores was less than 5% after 12 hr of incubation at 28 C.

DISCUSSION

Activation of oospore germination in *P. aphanidermatum* was accomplished in this study by three successive treatments: desiccation, incubation in water at 39 C, and agitation in 0.1% $KMnO_4$. Desiccation was carried out prior to isolation of oospores from the growth medium and subsequent to isolation, oospores in suspension were treated with heat and $KMnO_4$. Each of the three sequential treatments employed in this work represents a modification of a previously reported technique which has been shown to increase oospore germination in *Pythium aphanidermatum* (2), *Phytophthora megasperma* (7), and *Peronospora schleideniana* (8). In this paper reproducible methods have been described which permit biochemical work to be carried out with relatively homogeneous populations of oospores germinating with a reasonable degree of synchrony in a synthetic liquid medium.

In recent years there have been several investigations of the physiological and cytological basis of spore activation in fungi. Sussman (14) suggested that chemical and heat treatments that activate spores may cause conformational changes in constituent proteins and lipids. Heat activation of *Dictyostelium discoideum* spores is considered by Cotter (3) to be based upon induced changes in regulatory proteins located in mitochondrial membranes. Evidence presented by Hohl et al (6) indicates that heat activation of *D. discoideum* spores brings about immediate structural alterations in the plasma membrane and an increase in osmolarity and permeability of heat-treated spores. In the activation of *P. aphanidermatum* oospores, the desiccation treatment appeared to be of primary importance. Only oospores that had been previously desiccated were further activated by high temperature and $KMnO_4$ treatments. Desiccation may conceivably bring about enhanced permeability of oospores by weakening the cell wall, as a consequence of contraction of wall polymers during desiccation and expansion during rehydration. The activating effect of the $KMnO_4$ treatment probably involves the chemical oxidation of wall components; in particular, oxidation of the lipid components of the oospore wall (9) would be caused by this treatment.

The germination of activated oospores in this study was found to be dependent on the presence of lecithin in a basal salts liquid medium. In the absence of lecithin, or replacement of it by other compounds (eg, glucose, asparagine, glycine, ethanol, sodium acetate, glycerol, cholesterol, and β-sitosterol), very little germination occurred. The lecithin used in this study was a natural (nonsynthetic) lipid source which presumably contained small amounts of sterols, in addition to phosphatidyl choline and esterified fatty acids. The stimulatory effect of lecithin on oospore germination may be a result of satisfying a nutritional requirement

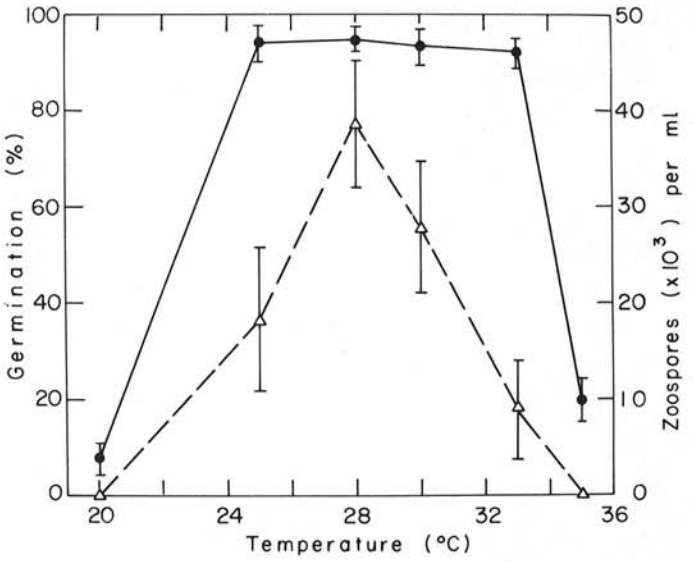

Fig. 4. Indirect germination of activated oospores of *Pythium aphanidermatum* in the liquid medium S+L (0.06 mM phosphate buffer, pH 7, 0.01% lecithin) at various temperatures of incubation. Oospores from desiccated cultures were activated by sequential incubation at 39 C for 5 min and agitation in $KMnO_4$. Percent germination and zoospore concentrations were determined after 14 hr of incubation at each temperature. Points represent mean values and vertical bars indiate the range of replicate determinations.

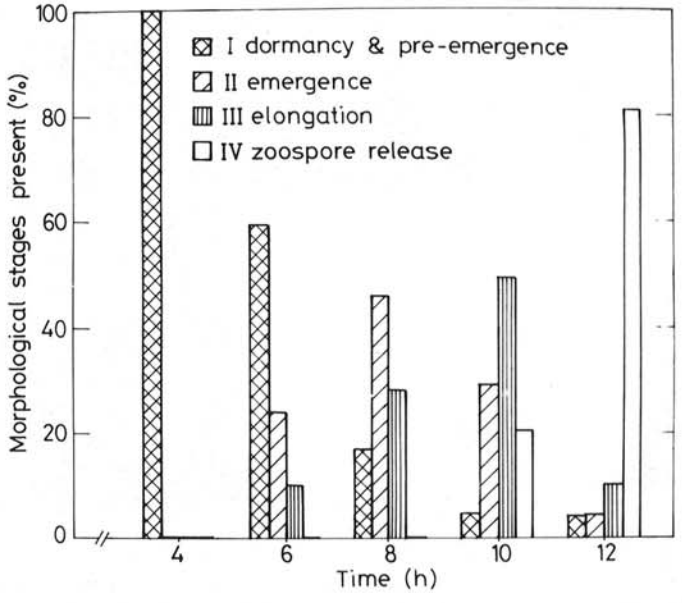

Fig. 5. Time distribution of morphological stages which develop during indirect germination of activated oospores of *Pythium aphanidermatum* at 28 C in a liquid basal salts medium, buffered with 0.06 mM potassium phosphate (pH 7) and supplemented with 0.01% emulsified lechithin.

of oospores for phosphatidyl choline or esterified fatty acids or may be related to the emulsifying properties of phosphatidyl choline which could contribute to the absorption of other lipids possibly present in small amounts (sterols). Since the outer, osmiophilic layer of the oospore wall probably is composed of lipoidal material (9), the dormant oospore would be expected to be readily permeable to lecithin. Oospores of *P. aphanidermatum* may be dependent on an external source of phospholipids in order for the necessary membrane synthesis to occur during the early stages of germ tube emergence. Stimulation of vegetative growth by lecithin and linoleic acid have been demonstrated in a number of species of *Phytophthora* (5).

Bimodal germination in oospores and sporangia of *Pythium* (4,11) and *Phytophthora* (15) is recognized to be dependent upon environmental conditions. Under natural conditions zoospore formation is favored in certain species by low temperatures, absence of nutrients, and the presence of free water (11). Although bimodal germination of oospores of *P. aphanidermatum* has been fairly well documented, the nutritional conditions determining either zoospore production (indirect germination) or germ tube elongation without zoospore production (direct germination) have not previously been rigorously defined under axenic conditions.

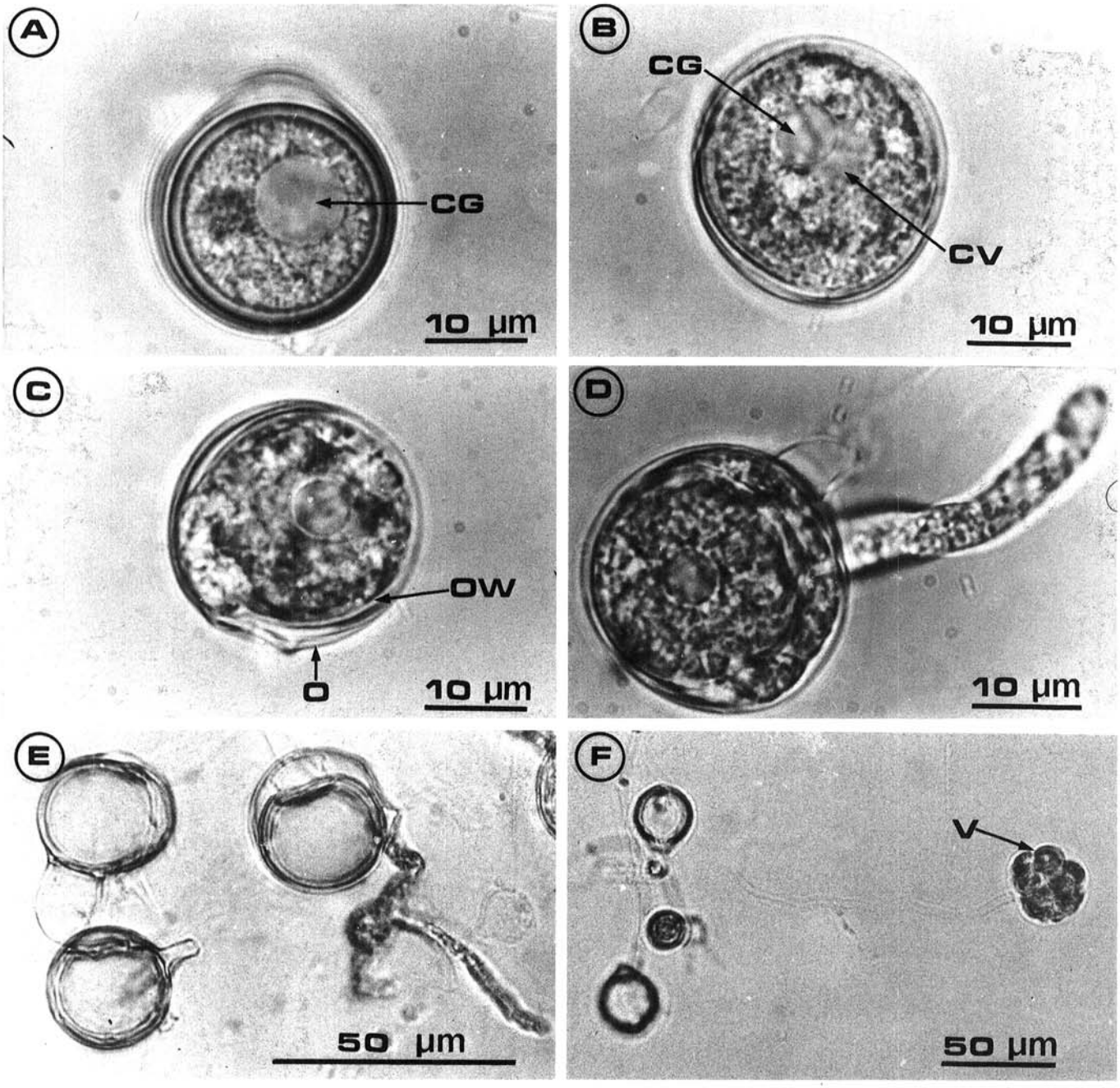

Fig. 6. Photomicrographs of developmental stages during germination of activated oospores of *Pythium aphanidermatum* in the liquid medium S+L (0.06 mM phosphate buffer, pH 7, 0.01% lecithin) at 28 C. **A,** Dormant, activated oospore, stage I at 0 hr; **B,** preemergence, stage I at 5 hr; **C,** initiation of germ tube emergence (germ tube contained within the oogonial wall), stage II at 7 hr; **D,** germ tube elongation (germ tube broken through oogonial wall), stage III at 8 hr; **E,** germ tube elongation (cytoplasm evacuated from the inner contents of oospores), stage III at 9.5 hr; **F,** formation of vesicle, in which zoospores are differentiated, stage IV at 12 hr. Abbreviations: **CG,** central globule; **CV,** central vacuole; **O,** oogonial wall; **OW,** oospore wall; **V,** vesicle. Magnifications: A–D, ×1,680; E, ×870; F, ×370.

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