Germination of Oospores of *Phytophthora megasperma* f. sp. *glycinea* in the Presence of Soil

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ABSTRACT

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Oospores of *Phytophthora megasperma* f. sp. *glycinea* were produced in cultures grown 4–6 wk in darkness. Oospores were separated from mycelium by grinding for 10–20 min in a Sorvall Omni-Mixer at 5 C, collecting the spores on a 74-μm-mesh sieve, and washing them by 2–4 brief (15 sec each) centrifugations at 325 *g*. Residual mycelium was killed either with the enzyme complex beta-glucuronidase/aryl sulfatase, or by freezing at −10 C. The oospores were germinated in flooded soil smears, soybean root exudates, or other substrates. Germination in natural or autoclaved soil was high (50–60%) compared with that in deionized water (~10%). Sporangia produced in the presence of unsterilized field soil seldom germinated or released zoospores. Light stimulated germination of oospores incubated in the presence of soil. However, germination of dark-grown oospores under dark conditions was also substantial (20–25%). Light was inhibitory to sporangium production, but soybean seedlings reversed the inhibition. The optimum termperature for activation (thinning of oospore wall), germ tube production, and sporangium production was 20–24 C. Soybean seedlings and other plant tissues stimulated rapid and high percent germination of oospores incubated in soil extract in either light or darkness. Oospore germination was inhibited by glucose (0.5 mg/ml) and by a concentrated extract of sterile soil, but not by that of unsterile field soil.

Phytophthora root and stem rot, caused by *Phytophthora megasperma* (Drechs.) f. sp. *glycinea* Kuan and Erwin (syn. *P. megasperma* Drechs. var. *sojae* Hildeb.) is one of the most important diseases of soybeans *Glycine max* (L.) Merr. in Michigan as well as in other soybean-growing areas of the United States and Canada.

Oospores formed in soybean tissues are thought to be the primary survival propagules in soil. Dormancy and nonsynchronous germination of oospores and the opacity of soil have been major barriers to ecological studies of *Phytophthora* spp. The factors known to be involved in germination of oospores of *Phytophthora* spp. were reviewed by Zentmyer and Erwin (22) in 1970, and include light, sterols, temperature, maturity of the oospores, and the genetically controlled capacity of particular isolates to germinate.

Light enhances the germination of oospores of most *Phytophthora* spp., including *P. megasperma* f. sp. *glycinea,* and especially of those oospores produced and matured under continuous darkness (4,7,14,15). Soaking the oospores of *P. megasperma* f. sp. *glycinea* in water for 48 hr at 36 C increased their subsequent germination at moderate temperatures (13.).

Though the germination of oospores of *P. megasperma* f. sp. *glycinea* and other *Phytophthora* spp. has been studied in culture media (22), little research has been directed to elucidate the behavior of these spores in their natural habitat, the soil. The present investigation was undertaken to study the germination of oospores of this pathogen in the presence of soil.

MATERIALS AND METHODS

Production and isolation of oospores. *P. megasperma* f. sp. *glycinea* race 1 was used in all experiments. Races 3 and 9 were used in some experiments. Races 1 and 3 were obtained from A. F. Schmitthenner, Ohio State University, and race 9 was obtained from F. A. Laviolette, Purdue University. The fungi were subcultured on lima bean agar (Difco Laboratories, Detroit, MI 48232) bimonthly.

Oospores were produced in cultures grown in clarified V-8 juice broth, 10–15 ml per plate, containing 30 μg cholesterol per milliliter (1). Plates were inoculated with 1 ml of a zoospore suspension (10^3–10^4/ml), were sealed with Parafilm (American Can Co., Greenwich, CT 06830), and enclosed in several layers of heavy duty aluminum foil to maintain darkness.

Mycelium was killed by freezing the cultures at −10 C for 12–15 hr. Thawed mycelial mats were rinsed twice with sterile distilled water and were ground for 10–20 min in a Sorvall Omni-Mixer (Ivan Sorvall, Inc., Newtown, CT 06470), with the container surrounded by an ice water bath. The homogenate was diluted and sieved (74-μm meshes). The filtrate containing the oospores was centrifuged at 320 *g* for 15 sec. The pellet was recentrifuged two to four times to obtain mycelium-free oospores. In some experiments, instead of freezing, the enzyme complex beta-glucuronidase/aryl sulfatase, obtained from *Helix pomatia* (Sigma Chemical Company, St. Louis, MO 63178), was used to digest mycelium and antheridial remains. Mycelial mats were rinsed in sterile distilled water and ground for 10 min in 0.1 M sodium acetate buffer, pH 5.0 (9). Approximately 20 ml of the homogenate was treated with 0.25 ml of enzyme solution (2,000 units per milliliter) and incubated at 37 C for 8 hr. The digested material was passed through a sieve (74-μm mesh) to remove nonlysed mycelium. The oospores were further purified by low-speed centrifugation, as for frozen material. Oospores prepared by either method were stored aseptically in distilled water at 5 C. Spore concentrations were determined by counting in a hemacytometer or by the microsyringe method (12).

Dark-grown oospores were isolated under a "safe light" with a yellow filter (Kodak Safelight Filter, Wratten Series OA, Eastman Kodak Co., Rochester, NY 14650). Since the spectral regions responsible for oospore activation are the blue (400–480 μm) and the far-red (700–1,000 μm) (2,4,5,14–16), yellow was chosen as a likely nonstimulatory spectral area for processing the oospores.

Preparation of soil extracts. Extracts were prepared from soil collected from Michigan fields naturally infested with *P. megasperma* f. sp. *glycinea,* and were stored in polyethylene bags at 5 C. Before use, the soils were mixed for 30 min in a rotating drum, passed through a 2-mm (9-mesh) sieve and air-dried. To prepare a soil suspension, 1,000 g of soil was shaken with 1,000 ml of distilled water for 12–24 hr. Half of the suspension was autoclaved for 30

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min followed by centrifugation at 10,000 *g*, for 10 min. The supernatant was passed through Whatman #1 filter paper, and autoclaved. The other portion was passed through three layers of cheesecloth to remove coarse particles. The filtrate was centrifuged at 10,000 *g* for 10 min, and the supernatant was passed twice through Whatman #1 filter paper. The extract was sterilized either by passage through a Millipore filter (0.22-μm pore size) or by autoclaving. Soil extracts were kept at 4 C until use.

Preparation of soybean seedlings and root exudates. Soybean seeds were sterilized with propylene oxide vapors (17), followed by a 2-min soak in 5% sodium hypochlorite, then were rinsed with sterile distilled water. Sterilized seeds were germinated in 1% nutrient agar. After 2–4 days, seedlings free of contaminant bacteria or fungi were placed in petri plates (five seedlings per plate) containing 10 ml of an autoclaved extract of field soil. Plants were grown for 5–10 days under continuous light at 4,125 lux provided by four Sylvania Gro-Lux WS 40 W lamps.

The exudates were assayed for sterility by streaking a small amount from each plate on nutrient agar. Contaminated exudates were discarded and sterile exudates were pooled, vacuum filtered through Whatman #1 filter paper to remove plant material, and stored at 5–10 C.

Germination of oospores. Natural soil (0.1–0.2 g) was smeared in the bottom of 6-cm-diameter glass petri plates, using a cotton-tipped swab. The soil was moistened to saturation and the center of the smeared area was thinned to form a slight depression. The plates were air-dried on a laboratory bench before the oospores were added. Soil films were prepared from the supernatant liquid of aqueous soil suspensions (1:1, w/v) sedimented for 30 min. The supernatant was passed through two layers of cheesecloth and 1-ml aliquots were dispensed into petri dishes and air-dried. The amount of soil deposited was 10–20 mg per plate. Sand films were prepared by adding 1-ml aliquots of a 2% aqueous suspension of finely ground washed sea sand (w/v), to 6-cm-diameter petri dishes, and air-drying. Soil smears and soil films were usually used in both unsterile and autoclave-sterilized conditions; sand films were autoclaved.

About 10^4 oospores were applied per petri dish, and these were incubated under light or were enclosed in several layers of heavy duty aluminum foil for dark treatments. The light source, suspended over a laboratory bench, consisted of four ITT F40/CW fluorescent lamps with spectral peaks of 370, 410, 435, 540, and 575 μm. The distance between the light source and the cultures was adjusted so that light intensity was 4,125 lux. Temperatures inside culture plates were monitored by thermister probes inserted through small holes in the tops of the petri dishes. Dark treatment petri dishes were brought closer to the light source until they reached a temperature equal to that of plates directly exposed to light. The usual temperature was 26.5 ± 1 C.

The changes occurring during the germination process were observed with a microscope at ×100 magnification. After specified incubation intervals, usually 4–6 days, oospores were classified (2) as follows: thick-walled, dormant oospores with fine granular cytoplasm (no changes evident); activated, thin-walled oospores with coarsely granular-appearing cytoplasm; germinated oospores with germ tube; and germinated oospores with germ tube bearing sporangia. Activation was considered a stage in the germination process, and those oospores which were activated but had not progressed further were included in the totals.

Oospores (300 per replicate) were counted in each of three or four replicate petri dishes and the percentages of the different categories were determined. All experiments were done at least twice. Data were transformed to angles by the arc sine transformation for analysis of variance (21). Significant differences among treatments were estimated by Tukey's *w* procedure.

RESULTS

Germination of oospores in soil. Oospores, treated enzymatically, from 4-wk-old cultures of *P. megasperma* f. sp. *glycinea* race 1 were germinated under light in sterile or unsterile soil films. Sterilized deionized water was used as a control. Although some oospores were parasitized, unsterile field soil supported almost as many activated and germinated oospores as sterile soil (Table 1). Totals, including oospores activated but not germinated, were 70.4 and 77.4%, respectively. Those which had produced germ tubes and/or sporangia were 47.2 and 58.8%, respectively. By contrast, total oospores activated and/or germinated in water alone was only 34%; most of these were activated oospores that had not progressed further.

Similar results were obtained when enzymatically treated oospores from 6-wk-old cultures were germinated under light in unsterile or autoclaved extracts of soil. Progression through the

TABLE 1. Germination of oospores of *Phytophthora megasperma* f. sp. *glycinea* in soil

Substrate	Oospores activated and germinated (%)[a]			
	Activated	With germ tube	With sporangia	Total
Water	24.9 A	4.1 A	5.0 A	34.0 A
Sterile soil	18.6 A	27.2 B	31.6 B	77.4 B
Unsterile soil[b]	23.2 A	8.8 A	38.4 B	70.4 C

[a] Means in the same vertical column followed by the same letter are not significantly different ($P = 0.05$) by Tukey's *w* procedure. Values represent the average of three replications with 300 oospores per replication.

[b] Parasitized oospores were included among those ungerminated. Unsterile field soil.

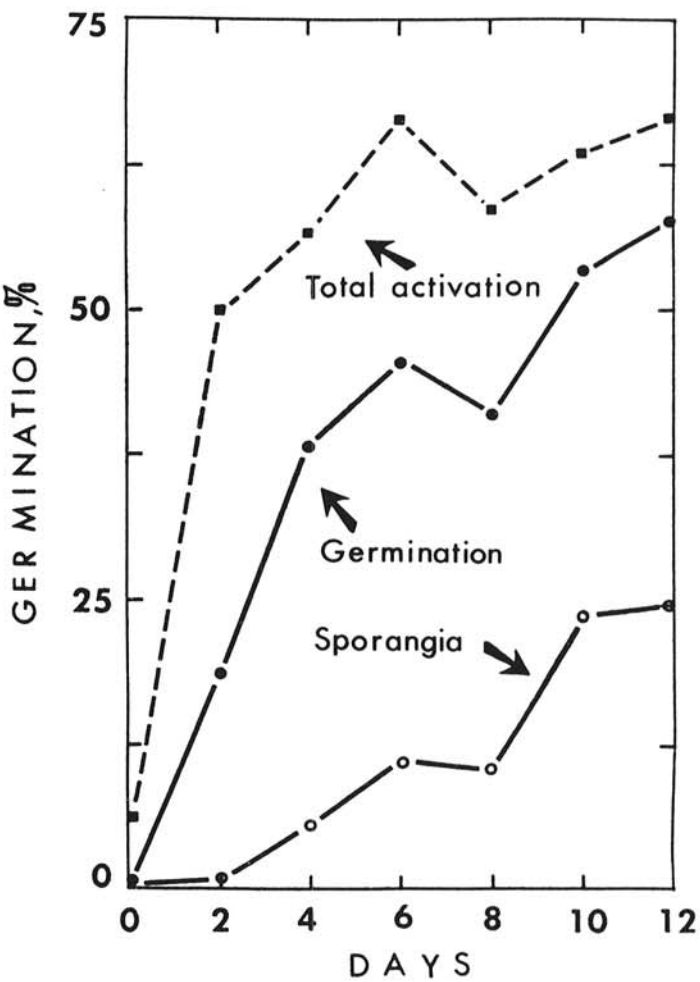

Fig. 1. Time course of germination stages of *Phytophthora megasperma* f. sp. *glycinea* incubated in sterile soil films. Total activation refers to the sum of all stages, germination refers to those that had produced a germ tube (including those that had formed sporangia), and sporangia refers to those that had formed sporangia. Activation refers to thinning of the oospore wall and granulation of the cytoplasm.

germination stages beyond activation occurred to a much greater extent in the presence of either soil extract than in the presence of water alone.

Another experiment compared germination of frozen oospores from 4-wk-old cultures of races 1, 3, and 9 in soil smears, under light. In sterile soil, oospores activated and/or germinated for races 1, 3, and 9 were 70.3, 40.2, and 59.5%, respectively. Values for natural soil (in the same order) were 73.2, 38.3, and 57.2%. There were no significant differences between soil conditions in total germination (all stages), but differences ($P = 0.05$) among races were significant.

Time course of oospore germination. To determine the time course of germination, an aqueous suspension of frozen oospores from 5-wk-old cultures was incubated under light in petri dishes containing sterile soil films. Germination stages were followed for 12 days at 2-day intervals (Fig. 1).

The numbers of oospores in the different germination stages increased as the time of incubation increased. At each interval, the number of oospores with germ tubes lagged behind the total number of activated oospores, and the number producing sporangia lagged behind those that had produced a germ tube. For example, at 6 days, total numbers of oospores activated, those with germ tubes, and those with sporangia were 70, 46, and 11%, respectively. By the 12th day, values were 70, 58, and 25%, respectively. Since germination (oospores with germ tubes) had not leveled off after 12 days, most viable oospores might have germinated with a longer incubation time.

Effect of light on oospore germination. To test the effect of light on oospore germination, 1-ml aliquots of an oospore suspension (10^4 oospores per milliliter) enzymatically prepared from 5-wk-old cultures were dispensed into petri plates containing autoclaved films of soil. Plates were sealed with Parafilm, covered with aluminum foil, and exposed to the following light regimes: 6 days under darkness; 6 days under continuous light; 4 days under darkness followed by 2 days under light; and 4 days under light followed by 2 days under darkness.

Total oospores activated and germinated were higher under treatments that included light than under continuous darkness (Table 2). This was due to greater numbers having been activated in light. However, light was inhibitory to the production of sporangia. For example, under continuous light, only 5.8% of the oospores had formed sporangia, whereas under continuous darkness 22.5% had formed sporangia, despite fewer oospores activated and germinated. Of the two treatments that included both light and darkness, larger numbers of oospores produced sporangia when light preceded darkness.

Oospores, prepared by freezing, from cultures grown for 10 days in darkness plus 5 days under light were used to test the effect of light and darkness on the germination stages of race 9 in sterile or unsterile soil films. There was little differential effect of unsterile or sterile soil on the germination process, except that oospores placed under darkness in unsterile soil had somewhat more germ tubes (12.1%) than those under darkness in sterile soil (4.7%) (Table 3). The production of sporangia was also inhibited by light in these experiments. For example, there were more oospores with sporangia in sterile soil in darkness (24.1%) than under light (2.2%), and in unsterile soil in darkness (19.4%) than under light (1.1%).

Effect of substrate concentration on oospore germination. Oospores, prepared by freezing, from 6-wk-old cultures were used. One milliliter of the following solutions containing ~10^4 axenic oospores per milliliter were added separately to sand films in petri dishes: deionized water; extract of autoclaved soil at concentrations of 1:1 and 1:10 (w/v); autoclaved extract of unsterile field soil (1:1, w/v); and glucose at 0.5 mg/ml and 0.005 mg/ml. The oospores were incubated under ambient light in the laboratory.

Concentrated substrates were inhibitory to oospore germination. Oospores activated and/or germinated were 7.8% in a concentrated extract of autoclaved soil as compared to 52.6% in a 1:10 dilution of the same extract. Glucose at 0.5 mg/ml reduced activation and germination to 30.1% as compared to 56.1% with 0.005 mg/ml. These pairs of values differed significantly ($P=0.05$). Similar values (48.2–53.5%) were obtained in water, a 1:10 dilution of extract of autoclaved soil, and an extract of natural soil; these did not differ significantly.

Effect of soybean seedlings on oospore germination. Experiments were done to test the effect of soybean seedlings on germination of oospores of *P. megasperma* f. sp. *glycinea* race 9 in light and in darkness in an autoclaved extract of natural soil (3 ml per petri dish). The oospores were obtained from 6-wk-old cultures, and after freezing the cultures, were extracted under yellow light.

The presence of seedlings greatly enhanced germination of oospores at all stages (Table 4). Whereas the results previously reported were obtained 5–7 days after exposure to a particular treatment, the data in these experiments were taken after only 36 hr of incubation. Soybean seedlings increased total oospores activated and germinated under light as well as under darkness. However, in the presence of seedlings, the change from the activated stage to the production of sporangia was greater and faster than without seedlings. The relatively large number of sporangia produced under light, 34.2%, indicated that soybean

TABLE 2. Effect of fluorescent light on the germination stages of oospores of *Phytophthora megasperma* f. sp. *glycinea*

	Oospores activated and germinated (%)[a]			
Light regime[b]	Activated	With germ tube	With sporangia	Total
6 days in dark	4.7 A	7.5 A	22.5 A	34.7 A
6 days under light	11.4 A	32.0 B	5.8 B	49.2 B
4 days in dark + 2 days of light	25.7 B	15.9 A	13.5 B	55.1 B
4 days of light + 2 days in dark	5.6 A	17.2 AB	27.3 A	50.1 B

[a] Means in the same vertical column followed by the same letter are not significantly different ($P=0.05$) by Tukey's *w* procedure. Values represent the average of three replications with 300 oospores per replication.
[b] The light source consisted of four ITT F40/CW fluorescent lamps, which provided 4,125 lux.

TABLE 3. Effect of light and darkness on germination of oospores of *Phytophthora megasperma* f. sp. *glycinea* in the presence of natural or sterile soil

	Oospores activated and germinated (%)[a]			
	Sterile soil		Unsterile soil	
Stage	Light	Dark	Light	Dark
Activated	34.0 A	16.0 B	35.6 A	19.5 B
With germ tube	18.0 A	4.7 B	19.0 A	12.1 C
With sporangia	2.2 A	24.1 B	1.1 A	19.4 B
Total	54.2 A	44.8 A	55.7 A	51.0 A

[a] Means in the same horizontal row followed by the same letter are not significantly different ($P=0.05$) by Tukey's *w* procedure. Values represent the average of three replications with 300 oospores per replication.

TABLE 4. Germination of oospores of *Phytophthora megasperma* f. sp. *glycinea* in the presence of soybean seedlings

	Oospores activated and germinated (%)[a]			
	With seedlings[b]		Without seedlings	
Stage	Light[c]	Dark	Light	Dark
Activated	15.0 A	15.0 A	30.4 B	33.3 B
With germ tube	16.7 A	12.3 A	8.5 B	1.0 C
With sporangia	34.2 A	18.0 B	1.0 C	1.0 C
Total	65.9 A	45.3 B	39.9 C	35.3 C

[a] Means in the same horizontal row followed by the same letter are not significantly different ($P=0.05$) by Tukey's *w* procedure. Values represent the average of three replications with 300 oospores per replication.
[b] Three-day-old aseptic soybean seedlings (two per replicate).
[c] 4,125 lux, provided by four cool-white fluorescent lamps.

exudates may reverse the inhibitory effect of light on sporangium production. In this experiment, the differential light stimulation of oospore activation in the absence of seedlings was not shown. This may have been due to the short incubation period used.

The effects of soybean and navy bean (*Phaseolus vulgaris*) tissues on germination of oospores from frozen 5-wk-old cultures were also determined. Soybean stem and leaf pieces, and navy bean stem tissue separately incubated with oospores in 3 ml of an extract of natural soil increased their germination, and resulted in the development of small mycelial colonies.

An experiment was done to determine the effect of soybean seedlings on zoospore release from sporangia in unsterile field soil. Oospores from frozen 4-wk-old cultures were incubated in petri dishes containing 0.2 g of a natural soil smear under light. After the oospores had germinated and produced sporangia, three cultivar Hark soybean seedlings, 3 days old, were added to each dish. Larger numbers of zoospores and more empty sporangia were observed in the presence of soybean seedlings than in their absence, after 24 hr.

Effect of temperature on oospore germination. Oospores from frozen 3-wk-old cultures were incubated under darkness in soybean root exudates in an autoclaved extract of soil at 16, 20, 24, and 28 C for 4 days. Some germination occurred at each temperature and was optimum at 20–24 C (Fig. 2). All stages of germination were similarly affected by temperature.

DISCUSSION

The study of oospore germination in soil has proved to be difficult because of the opacity of soil and the inconsistent and often low germinability of oospores. In the present study, use of thin films of soil or extracts of natural soil overcame the visual difficulties and also stimulated oospore germination. In particular, the transformation of oospores from the activated stage to the formation of sporangia was enhanced in the presence of soil. This may have been due to the presence of a favorable mineral salts environment or to low concentrations of energy yielding nutrients. Flooding natural soil is known to release occluded nutrients and to increase their distribution (8). In some experiments, there was a tendency for greater germination in the presence of natural soil than of autoclaved soil. Even where results were similar (Table 1), the germination in natural soil might have been higher had oospore parasites not destroyed some of the oospores (these were scored as ungerminated). High concentrations of nutrients such as may have been present in sterile soil are apparently inhibitory to germination of oospores of *P. megasperma* f. sp. *glycinea*. A high concentration of glucose, 0.5 mg/ml, was also inhibitory to germination, but a concentration of 0.005 mg/ml supported good germination. Inhibition of germination by excess nutrients also has been shown for oospores of *Phytophthora cactorum* (2), spores of *Glomus mosseae* (6), and was shown previously for oospores of *P. megasperma* f. sp. *glycinea* (19).

Sporangia produced in unsterile field soil seldom germinated during 10 days of observation, but remained intact and did not release zoospores. The significance of this response for the pathogen deserves further study. The sporangia could serve not only for the production of secondary inoculum, but perhaps also for dormant survival of the pathogen. The conditions for zoospore release from sporangia in natural soil are not known, but in one experiment zoospores were released in the presence of soybean seedlings.

Although light stimulated activation (but not sporangium production) of *P. megasperma* f. sp. *glycinea* oospores incubated with sterile or unsterile soil, considerable numbers of oospores also germinated and produced sporangia in darkness. Low levels of germination of oospores of several *Phytophthora* spp. incubated on water agar in darkness were reported previously (14,15). The fact that oospores produced, isolated, and incubated in unsterile soil under darkness, were able to germinate and produce sporangia casts some doubt on the importance of light for oospore germination under field conditions. Our results showing light stimulation of oospore germination of *P. megasperma* var. *sojae* are in agreement with others (4, 7,14,15), but are in contrast with those of Sneh et al (19), in which germination was greater in darkness than in light in the presence of soil or soil extracts. The reason for this discrepancy is not known, but Sneh et al (19) used larger amounts of soil than were used in our study. Sneh et al (19) found germination in water to be greater in light than in darkness, in agreement with our results using minimum amounts of soil.

The optimum temperature for all germination stages of oospores of *P. megasperma* f. sp. *glycinea* was 20–24 C, which is also near the optimum (25 C) for mycelial growth (10). This behavior differs from that for *P. cactorum*, which has an optimum of 28 C for mycelial growth and 22 C for oospore germination (2), but coincides with that for *P. drechsleri* which has an optimum at or near 24 C for oospore germination (11).

Soybean seedlings stimulated germination of oospores of *P. megasperma* f. sp. *glycinea* incubated in the presence of soil extract. All germination stages were positively affected by seedlings whether under darkness or light. The role of soybean plants and light on the germination of oospores needs to be defined more precisely. For example, germination of dark-grown oospores should be tested with seedlings in unsterile soil with the soil surface protected against light. Stimulation of oospores of *P. megasperma* f. sp. *glycinea* to germinate in response to soybean exudates was not specific to soybean or restricted to the roots. The germination response of the oospores to the presence of intact or wounded host or nonhost tissue, therefore, may be similar to that in other host-

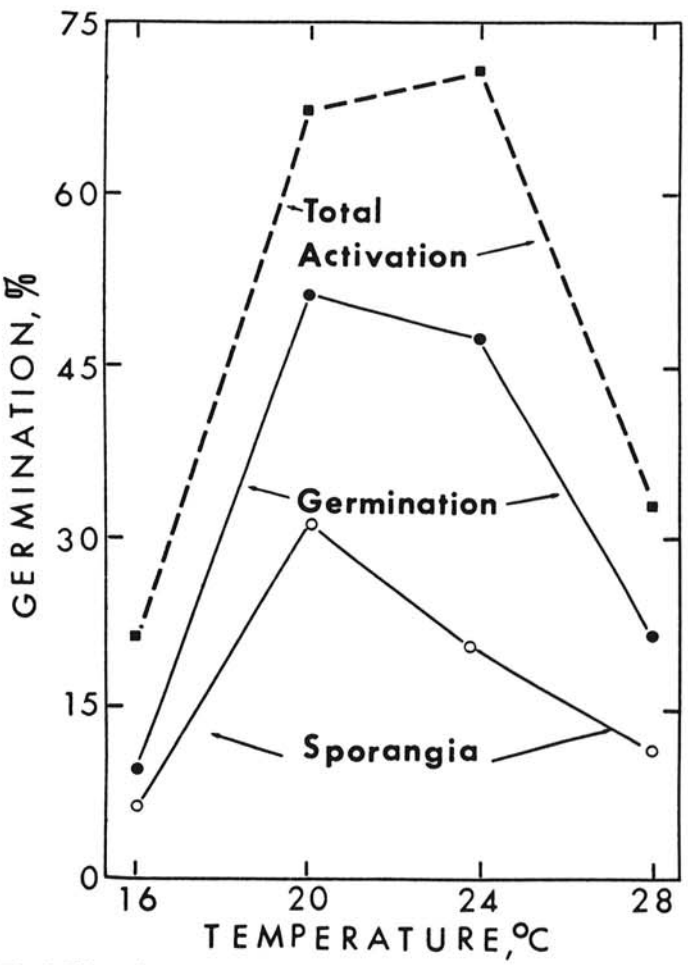

Fig. 2. Effect of temperature on germination of oospores of *Phytophthora megasperma* f. sp. *glycinea*. Least significant ranges (Tukey's *w* procedure, $P = 0.05$) for total activation (thinning of oospore wall and granulation of the cytoplasm), germination, and sporangia were 16.0, 26.4, and 20.2, respectively. Total activation refers to the sum of all germination stages, germination refers to all oospores producing a germ tube (including those that had formed sporangia), and sporangia refers to those that had produced sporangia.

pathogen systems (18).

Several of our findings are apparently new: the inhibitory effect of light on the formation of sporangia by germinating oospores of *P. megasperma* f. sp. *glycinea* and its reversibility by soybean root exudates; germination of oospores of this pathogen in natural soil in the absence of host plants and even in darkness; and the stimulatory effect of a host plant on germination of oospores of a *Phytophthora* spp. Other studies have indicated a positive host effect on the germination of *Pythium* oospores (3,20). The failure of visible and near-visible radiation to stimulate mycelial cultures of *P. megasperma* f. sp. *glycinea* to form sporangia was reported previously (15), but the effect of darkness apparently was not studied.

The oospores of *P. megasperma* f. sp. *glycinea* pose some interesting questions related to the survival of this pathogen in soil. Although the present results were obtained under laboratory conditions using only small amounts of soil, some speculations may be warranted. Under high moisture conditions, a portion of the population may germinate spontaneously, even in the absence of added nutrients. Thus, they appear to be unlike many other propagules whose germination is strongly suppressed in soil. As a consequence, compensatory characters must enable the pathogen to avoid extinction. Asynchronous germination and the possession of constitutive dormancy would confer survival value. In other respects, the fungus may behave quite conventionally. The presence of low concentrations of organic nutrients, such as occur in root exudates, appears to stimulate germination over and above the level occurring without nutrients. Moreover, sporangia appear to persist, for at least a period of some days, and zoospore release appears to be triggered by low concentrations of nutrients that could signal the presence of soybean roots, or other potentially colonizable substrate.

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