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Phytophthora megasperma Drechs. var. sojae.: I. A laboratory method for assessing "field tolerance" in soybeans. II. Germination of the oospores in soil.

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# PHYTOPHTHORA MEGASPERMA DRECHS. VAR. SOJAE HILDEB.: I. A LABORATORY METHOD FOR ASSESSING "FIELD TOLERANCE" IN SOYBEANS. II. GERMINATION OF THE OOSPORES IN SOIL.

By

Benjamin Jimenez-A

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# ABSTRACT

## PHYTOPHTHORA MEGASPERMA DRECHS. VAR. SOJAE HILDEB.: I. A LABORATORY METHOD FOR ASSESSING "FIELD TOLERANCE" IN SOYBEANS. II. GERMINATION OF THE OOSPORES IN SOIL.

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This research was undertaken to develop a laboratory method for studying "field tolerance" in soybean (Glycine max (L.) Merr.) seedlings to Phytophthora megasperma var. sojae, and to study the germination of the oospores in soil.

Part I. Seedlings 2-4 days old of several varieties of soybean with known levels of "field tolerance" were planted in slitted styrofoam cups filled with vermiculite. The cups were placed 0.5-1.0 cm deep in a soil suspension in plastic trays 17 X 25 X 6 cm containing 100 g steamed greenhouse mix and 200-250 ml water. Inoculum consisted of 20-40 zoospores/g soil obtained by flooding lima bean agar cultures of races 1, 2, 3, 4, 5, 7, 8, and 9 of the pathogen. The plants were allowed to grow for 15-20 days in a growth chamber at 28°C and a 12 hr photoperiod (17,000 lux). Disease was assessed by measuring dry weights and lengths of individual plants.

With race 3, the reductions in growth (length) of the varieties Hark, Williams, and Agripro 26, as

compared with uninoculated control plants, were 64%, 57%, and 45% respectively. With race 7, corresponding reductions in growth were 41%, 34%, and 26%. Dry weight measurements gave similar results. Comparable results were obtained with a natural soil infested with race 4. These results agree with relative "field tolerance" reported previously, i.e., low or none for Hark, medium for Williams, and medium to high in Agripro 26.

The response of the "field tolerant" variety Agripro 26 was maintained against all eight races. However, the variety Williams demonstrated variability in its response. The varieties Woodworth, Agripro 25, SRF 307 P, and Wayne, with known levels of "field tolerance," also showed less reduction in growth than did varieties without "field tolerance."

Part II. Oospores for germination experiments were obtained from cultures 4-6 weeks old grown under darkness. To obtain oospores free of mycelium, cultures were ground for 10 to 20 minutes in a Sorvall Omnimixer at 5°C. The homogenate was diluted and sieved (74 μm meshes). Mycelial contamination was removed with 2-4 brief (15 seconds each) centrifugations at 320 X g. In some experiments the enzyme complex beta-glucuronidase/ aryl sulfatase was used to lyse mycelium. In other experiments freezing at -10°C was used to kill mycelium.

The oospores were germinated in flooded soil smears, soybean root exudates, and other substrates under various conditions of light and temperature. The data were expressed as percentage of germination stages: i) activation, ii) germ tube formation, and iii) sporangia production.

High percentages (50-75%) of germination were found in soil (natural or sterile) as compared to deionized water (30-35%). Sporangia produced in natural soil seldon germinated. Germination of oospores extended over several days and germination curves over time indicated that prolonged incubation could result in germination close to 100%.

Light stimulated germination of oospores incubated in sterile and in natural soil. However, germination of dark grown oospores under dark conditions was also relatively high. Light was inhibitory to sporangia production, but soybean seedlings reversed this effect. The optimum temperature for germination was found to be 20-24$^{o}$C.

Soybean seedlings stimulated rapid and high germination of oospores incubated in soil extract under light or darkness. This stimulation was not specific to soybean or restricted to the roots. All germination stages were similarly affected by the presence of seedlings. Finally, oospore germination was inhibited by glucose (0.5 mg/ml) or by a concentrated soil extract.

To Betty and Freddy
and in memoriam of
Marcelino, Ana Balbina, and Narcisa

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# INTRODUCTION

Phytophthora root and stem rot, incited by Phytophthora megasperma var. sojae is one of the most important diseases of soybeans (Glycine max (L) Merr.) in Michigan as well as other soybean growing areas of the United States and Canada.

The disease was first reported in 1955 (135) although it was observed in 1948 in Indiana and in 1951 in Ohio. By 1958, the disease was present in Illinois, North Carolina, Missouri, and Southwestern Ontario (56).

The causal organism was first identified as Phytophthora cactorum (Leb. & Cohn) Schroet. (55, 122). The fungus was later described as Phytophthora sojae by Kaufmann and Gerdemann (68). In 1959, Hildebrand proposed the presently accepted trinomial name, Phytophthora megasperma Drechs. var. sojae Hildeb. (56). Recently, Kuan and Erwin (78) proposed the name Phytophthora megasperma f. sp. glycinea. Although the disease is widespread and causes severe damage to soybeans at all stages of growth, comprehensive knowledge of economic losses has not been obtained.

Resistance to the disease was found in 1957 (11) and determined to be inherited through a single dominant gene, designated Rps (45). This resistance was

incorporated into several varieties which have desirable agronomic characteristics. Resistant isolines were released to growers shortly after 1963. These cultivars contained the $Rps_1$ gene, which conferred resistance to what has since been known as P. megasperma var. sojae race 1. This resistance was soon broken by the development of new physiological races of the pathogen which caused disease in race 1-resistant cultivars. Since the discovery of race 2 in Mississipi (96) races 3, 4, 5, 6, 7, 8, and 9 have been identified (48, 80, 115, 119). The occurrence of physiological races complicates the attempt to control Phytophthora root and stem rot by means of race specific resistance. Consequently, research efforts have been directed to the development of suitable commercial varieties resistant to one or several races of the pathogen.

The proliferation of P. megasperma var. sojae races singly or in combination in infested fields (18, 87) has also encouraged the development of methods for their direct detection and identification by means of differential soybean varieties (34, 87).

The hypocotyl inoculation technique (68) has been the conventional method for evaluating resistance or susceptibility of soybeans to P. megasperma var. sojae This method is reliable for differentiating resistance from susceptibility (69), but does not detect intermediate reactions or types of resistance designated as

"field tolerance" or "field resistance." This type of resistance seems to differ from the race-specific resistance used in breeding resistant cultivars (108, 142). Other criticisms of the hypocotyl inoculation technique have been summarized by Eye et al. (35).

The type of resistance designated as "field tolerance" or "field resistance" has been reported for soybean (108, 142), but neither its genetic nor its physiological bases are well understood. Our understanding of "field tolerance" has been hindered by lack of evidence obtained under controlled conditions and because reliable laboratory methods have not been available for obtaining data on specific parameters associated with this interaction. The development of a method for evaluating "field tolerance" of soybeans to P. megasperma var. sojae under controlled conditions would therefore be desirable.

Phytophthora megasperma var. sojae, the causal agent of the Phytophthora disease of soybeans, is primarily a root pathogen. It attacks the roots of susceptible varieties and plants may die at any stage of development. The pathogen causes pre-emergence and post-emergence damping-off of seedlings, a root rot and very often a stem canker that results in wilting and death of plants from early stages of growth to maturity (56, 68, 122). The disease is considered to be primarily a problem of lower-lying, slowly drained, heavy-textured

soils, though it also appears in well-drained areas. However, the most serious damage occurs under waterlogged soil conditions (37, 56, 68, 71, 74, 137). Soil porosity and soil temperature were important factors affecting disease severity (71); disease was more severe at 15°C and in the soil having greatest porosity.

Soybean tissue infected by P. megasperma var. sojae has been shown to be a source of primary and secondary inoculum, oospores and zoospores, respectively. Herr (54) and Skotland (122) reported observing oospores in infected soybean tissue. Sporangia were formed by flooding infected tissue in natural or steamed soil and the presence of the pathogen was confirmed by a diseased plant bioassay (74).

Oospores formed in soybean tissue may remain associated with it for variable lengths of time until they are set free in the soil by the activities of soil microorganisms. They are thought to be the primary survival propagules in soil (147), and are the major potential source of variation in nature.

Formation, development and germination of oospores of P. megasperma var. sojae, as well as other Phytophthora species, are known to be affected by several environmental factors. The fungus produced more oospores in culture under darkness than under continuous light (44, 72), but low light intensities were not unfavorable

for the formation of oospores (101).

Development of oospores is affected by temperature. Erwin and McCormick (33) reported that a large number of oospores produced by an isolate of P. megasperma var. sojae at 30°C were malformed ("vacuolated") and did not germinate. Highest percentage of germination (69%-78%) at 20-22°C in water, was obtained with oospores produced at 27°C (33).

Oospore production was stimulated by the presence of sterols in the culture medium (31, 101, 137), and higher numbers were also produced under dark than under continuous light (75, 101).

Light enhances germination of oospores of most Phytophthora species, including P. megasperma var. sojae, especially of those oospores produced and matured under continuous dark (10, 33, 99, 101).

Finally, soaking the oospores of P. megasperma var. sojae in water for 48 hours at 36°C has been reported to increase their subsequent germination at room temperature (88).

Though the germination of oospores of P. megasperma var. sojae and other Phytophthora species has been studied in culture media (147), little research has been directed to elucidate the behavior of these spores in their natural habitat, the soil (127). Understanding the biology of fungal propagules in soil, especially those of plant pathogenic fungi, is important for the

study of their ecology and because understanding derived therefrom may lead to better measures for controlling the disease.

The present investigation was undertaken to i) develop a laboratory method for studying "field tolerance" in soybeans to Phytophthora megasperma var. sojae, and ii) to study the germination of oospores of this pathogen in soil.

# PART I

## A LABORATORY METHOD FOR ASSESSING "FIELD TOLERANCE" TO PHYTOPHTHORA MEGASPERMA VAR. SOJAE IN SOYBEAN SEEDLINGS

# LITERATURE REVIEW

## The pathogen

Phytophthora root and stem rot, caused by *Phytophthora megasperma* Drechs. var. *sojae* Hildeb., is one of the most important and serious diseases of soybeans (*Glycine max* (L.) Merr.). It attacks plants in all stages of growth, causing pre-and post-emergence damping-off of seedlings and a root and stem rot that result in wilting and death of plants from early stages of growth to maturity (56, 68, 122).

The disease was first reported in 1955 in Ohio, and the causal agent was identified as a species of Phytophthora (135). Subsequently the fungus was incorrectly named *Phytophthora cactorum* (Leb. and Cohn.) Schroet. (55, 122), and *Phytophthora sojae* Kaufmann and Gerdemann (68). Hildebrand (56) proposed the presently accepted trinomial *Phytophthora megasperma* Drechs. var. *sojae* Hildeb. However, the name *P. megasperma* f. sp. *Glycinea* has recently been proposed (78).

Resistance in soybeans to *P. megasperma* var. *sojae* was found in 1957 (11) and determined to be controlled by a single dominant gene. This resistance was bred into soybeans with desirable agronomic characteristics, but soon it was broken by the development of new physiological

races of the pathogen. Races 1 to 9 (Table 1) have now been identified (48, 80, 96, 119, 145), and the disease is found practically in every major soybean growing area of the United States and Canada (18, 48, 70, 80, 119, 145).

P. megasperma var. sojae attacks primarily the roots of susceptible cultivars causing a severe rot that often advances into the stem. It forms resistant sexual structures (oospores) in diseased tissue (122, 123, 124), these persist in soil for long periods of time (See Part II) and are the major potential source of variation in nature (147).

Table 1. Chronological list of races of Phytophthora megasperma var. sojae

| Race[a] | Year | Location | Reference |
|---|---|---|---|
| 1[b], 2 | 1965 | Mississippi | 96 |
| 3 | 1972, 1974 | Ohio, Indiana | 4, 115 |
| 4 | 1974 | Kansas, Indiana | 4, 119 |
| 5, 6 | 1976 | Ontario (Canada) | 48 |
| 7, 8, 9 | 1977 | Indiana | 80 |

[a]Race number was given in the first reference listed.

[b]Since the discovery of race 2 in 1965, (96) previous isolates of the pathogen have been designated race 1 on the basis of varietal reaction.

Inoculation methods

Inoculation methods have been developed to test for pathogenicity and to identify the reaction of different soybean varieties to P. megasperma var. sojae. These techniques can be broadly grouped in two categories: i) direct methods in which the pathogen is directly brought into contact with seeds or seedlings of soybeans, and ii) indirect methods, in which the pathogen is inoculated to a substrate (solid or liquid) where soybeans are or will be planted.

Direct methods include: seed infestation (68); mycelial sprays onto foliage (95); dipping the root system in a mycelial suspension (68); hypocotyl inoculation with pieces of mycelium (68) or with infested toothpick splinters (57, 68) or by injecting small amounts of mycelial suspensions (48); and inoculation by placing infested soil or cotton balls impregnated with the pathogen on branch axils of old plants (95).

Indirect methods of inoculation have included planting seeds or seedlings in soil, soil-sand-vermiculite etc., previously infested with oospores, zoospores, or mycelial suspensions of Phytophthora megasperma var. sojae (56, 96, 145). Adding the pathogen to soil or other substrata sustaining soybeans (34, 68) is also considered an indirect method. Inoculation methods whereby plants were grown in either sand or soil (56, 67, 69, 96, 145) or liquid cultures (69, 70) infested with a mycelial

suspension were developed for pathogenicity tests or to identify sources of resistance. However, no clear advantage of one method over another was found (56, 70, 96).

Keeling (69) compared three methods for inoculating soybeans representing a wide range of germ plasm, using races 1 and 2 of P. megasperma var. sojae: i) hypocotyl inoculation, ii) seed planted in infested sand, and iii) plants grown in liquid medium. He concluded that hypocotyl inoculation was the most reliable method and was less subject to experimental variation in inoculum concentration, environment, and differences in seed or seedling vigor. This technique has been the most commomly used to evaluate resistance in soybeans to P. megasperma var. sojae (5, 45, 56, 68, 70, 79, 80, 81, 96, 119, 145).

The method was devised by Kaufmann and Gerdemann (68) and consists of inserting a small piece of mycelium into a longitudinal incision in the hypocotyl of young plants (usually 10 days old) midway between the soil line and the cotyledons. The wound is covered with petrolatum to prevent desiccation of the inoculum and plant tissues. Seedlings are grown for 4-6 days in a greenhouse at 24-27$^{o}$C. Plants are then classified as resistant (no external symptoms present) or susceptible (dead).

The hypocotyl inoculation method is reliable for differentiating resistance from susceptibility (69, 142)

but does not detect intermediate reactions or types of resistance designated as "field tolerance" (142).

The Rps gene

Using the hypocotyl inoculation method, Bernard et al. (11) studied the inheritance of resistance to Phytophthora root and stem rot. They found that resistance in several soybean cultivars including Mukden was controlled by a single dominant gene, and proposed the symbol Ps and ps for the alleles conditioning resistance and susceptibility, respectively.

Race 2 of P. megasperma var. sojae was reported in 1965 (96). It was isolated from field-infested soybeans (D60-9647) which were resistant to previous isolates of the pathogen. Other lines were resistant to races 1 and 2, or were susceptible to both races. Inheritance studies (45) revealed that the resistance in cultivar Semmes to races 1 and 2 was controlled by the single dominant gene, Rps (formerly Ps), which was dominant to $rps^2$ gene in D609647, and that $rps^2$ was dominant to rps (formerly ps) gene in susceptible lines. These genes formed an allelomorphic series: Rps, $rps^2$, rps. The genotypes given by Hartwig et al. (45) were as follow: Semmes, Rps Rps (resistant to races 1 and 2); D60-9647, $rps^2$ $rps^2$ (resistant to race 1, susceptible to race 2); and Hood, rps rps (susceptible to both races).

Schmitthenner (115) demonstrated the existence of race 3 in Ohio in 1972. He proposed that an

additional locus was involved in resistance to race 3, since the isolates studied caused disease on cultivars with the Mukden-type resistance. Soybean lines with the Arksoy and D60-9647-types of resistance were resistant to race 3.

Race 4 was reported by Schwenk and Sim (119) who showed that Arksoy, Mack, Picket 71, and D54-2437 were susceptible to this physiological race, but D60-9647 was resistant to races 1, 3, and 4. In addition, Athow et al. (4) noted that the cultivars Altona, Sanga, and certain other lines were resistant to races 1, 2, 3, and 4.

A second major gene (Rps2) was detected by crossing CNS with lines with the Arksoy-type resistance and with D60-9647 (70). Root inoculation with races 1 and 2 indicated that the resistance in CNS was controlled by a gene at a different locus than Rps and $rps^2$.

Mueller et al. (97) demonstrated that the proposed allelomorphic series $Rps_1$, $rps_1^2$, and rps could not explain the reactions of soybean cultivars with the Mukden-, Arksoy-, D60-9647- and Altona-type resistances to race 1, 2, 3, and 4. Their data indicated that the resistance of Mukden, P. I. 54615-1 (Arksoy-type) and P. I. 84637 (D60-9647-type) was controlled by a different single dominant allele located at the same locus in each variety. To differentiate these alleles they proposed that the allele in P. I. 54615-1 be designated $Rps^c$,

and the allele in P. I. 84637 be changed from $\underline{rps}^2$, to $\underline{Rps}^b$ to form the allemomorphic series $\underline{Rps}^a$, $\underline{Rps}^b$, $\underline{Rps}^c$, and $\underline{rps}$, represented by Mukden, P. I. 84637, P. I. 54615-1, and Harosoy, respectively. They also noted that the resistance in P. I. 86972-1 to races 1, 2, 3, 4, 5, 8, and 9 was controlled by a single dominant allele located at a different locus, and suggested the designation Rps3.

Laviolette et al. (8) extended these inheritance studies of resistance in soybeans to races 5 and 6 which had been reported by Haas and Buzzell (48), and to races 7, 8, and 9 reported by Laviolette and Athow (80). They found that the gene $\underline{Rps}^a$ in Mukden confers resistance to races 1 and 2 only. The gene $\underline{Rps}^b$, which conditions resistance to races 1, 3, 4, also gave resistance to races 5, 6, 7, 8, and 9. The gene $\underline{Rps}^c$ in P. I. 54615-1, which gives resistance to races 1, 2, and 3 also conferred resistance to races 6, 7, 8, and 9. The genes $\underline{Rps}^a$, $Rps^b$, $Rps^c$, are located at the same locus. The independent gene Rps3 in P. I. 86972-1, conditions resistance to races 1, 2, 3, 4, 5, 8, and 9.

Finally, Athow et al. (5) demonstrated that the variety Tracy which is resistant to all nine races of P. megasperma var. sojae, has two independent dominant genes for resistance. One gene (Rps3) controls resistance to all races except 6 and 7, and the second gene ($Rps^b$) provides resistance to all except race 2.

The following alleles have thus far been found in soybeans for resistance to P. megasperma var. sojae, three of which are located at the same locus:

$Rps_1^a$ (Mukden-type) for resistance to race 1 and 2.

$Rps_1^b$ (D60-9647-type) for resistance to all except race 2.

$Rps_1^c$ (Arksoy-type) for resistance to all except 4 and 5.

$Rps_1^c$ (Arksoy-type) for resistance to races 1 and 2 to root inoculation.

Rps3 (P. I. 86972-1-type) for resistance to all except 6 and 7.

"Field tolerance"

Field reistance also called "field tolerance" to P. megasperma var. sojae has been reported for soybeans (106, 107, 108, 142). This resistance seems to differ from race-specific, monogenic resistance commonly used in breeding resistant cultivars. Varieties such as Wayne, Woodworth and Williams typify this reaction (142). Such varieties lack major genes for resistance to Phytophthora root rot and give a susceptible (kill) reaction by hypocotyl inoculation, yet survive and yield better than susceptible varieties in the field.

The methodology for identifying and quantifying "field tolerance" has veen laborious and consists of advancing, by single seed descent, progenies of crosses that have Wayne in their pedigree. In the F4 to F6 generations, single plant progenies are evaluated for their reaction to various races of P. megasperma var.

*sojae* by hypocotyl inoculation. The susceptible lines are then evaluated in the field for vigor and yield at a location where the disease is severe. Performance is compared with the "field tolerant" variety Woodworth (142).

Apparently, the "field tolerant" reaction in soybeans has not been routinely demonstrated under controlled conditions because laboratory techniques have not been available. However, some factors affecting this interaction were studied in the greenhouse by placing lima bean cultures or soil infested with *P. megasperma* var. *sojae* in a layer below the seed prior to planting (36). For example, expression of "tolerance" was best in steamed soil, but poor drainage, ethazole and spore washings of *Glomus macrocarpus* var. *macrocarpus* suppressed this reaction.

## MATERIALS AND METHODS

### Soybean varieties

The soybean (*Glycine max* (L) Merr.) cultivars used routinely were Hark, Williams, and Agripro 26, which have known levels of "tolerance" to races 1, 3, and 6 of *Phytophthora megasperma* Drechs. var. *sojae* Hildeb. in the field (Table 2). According to field tests these varieties have respectively little, moderate, and high "field tolerance," and are fully susceptible by hypocotyl inoculation (Table 3).

Other varieties tested were: Wayne, Harosoy 63, Agripro 25, Woodworth, SRF307P, Hodgson, OX-20-8 and Corsoy. The variety Tracy, which is resistant to all known races (1 to 9) of *P. megasperma* var. *sojae*, was used in some experiments.

Seeds were surface-sterilized (5% sodium hypochlorite for 2 minutes), thoroughly rinsed with distilled water and germinated for 2-4 days, at 28°C, in plastic trays (17 X 25 X 6 cm) containing moistened vermiculite (3 parts of vermiculite: 2 parts of water, v/v). Usually, the seeds were soaked for at least 2 hr in water through which air was bubbled, to promote uniform germination. Seedlings with hypocotyls of the

same length, usually 5.0±0.5 cm to 7.0±0.5 cm, were chosen for assessing disease reaction.

Table 2. Evaluation of root and stem rot of soybean caused by Phytophthora megasperma var. sojae[a]

| Soybean variety | Tolerance ratings for the years[b] | | | |
|---|---|---|---|---|
| | 1973 | 1974 | 1975 | 1976 |
| Hark | 2 | | | |
| Wayne | 1 | 3 | 2 | 3 |
| Williams | 1 | | 1 | 3 |
| Agripro 26 | | 1 | | 2 |

[a]Tolerance ratings from the Ohio performance trials (106, 107, 108).

[b]Tolerance ratings for 1973:
1= No dead plants after flowering, some stunting may be evident.
2= Occasional dead plants after flowering; stunting may be considerable.
3= Plants die throughout the season.

Tolerance ratings for 1974, 1975, and 1976:
1= None-trace dead plants.
2= Up to 2% dead plants, no stunting or chlorosis.
3= Up to 10% dead plants, slight stunting or chlorosis.
4= Up to 50% dead plants, moderate stunting and chlorosis.
5= Over 50% dead plants, severe stunting and chlorosis.

## Pathogen isolates

Phytophthora megasperma var. sojae races 1, 2, 3, 4, 5, 7, 8, and 9 were used. Isolates of races 1 to 5 were obtained from Dr. A. F. Schmitthenner of Ohio State

University and isolates of races 7, 8, and 9 were obtained from Mr. F. A. Laviolette of Purdue University. Each isolate was maintained as a stock culture on lima bean agar (Difco Laboratories, Detroit, Michigan) plates or on a selective medium (117) at room temperature ($24\pm1^{o}C$), and was subcultured in lima bean agar bimonthly.

Table 3. Differential response of soybean cultivars to races 1 to 9 of Phytophthora megasperma var. sojae.

| | Race reaction[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Soybean variety | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Hark | S | S | S | S | S | S | S | S | S |
| Williams | S | S | S | S | S | S | S | S | S |
| Agripro | R | S | S | S | S | S | S | S | S |
| Wayne | S | S | S | S | S | S | S | S | S |
| Tracy | R | R | R | R | R | R | R | R | R |

[a]S= Susceptible; R= Resistant. Disease reaction was assessed by the hypocotyl inoculation technique (68).

## Production of inocula

The method of Eye et al. (34) was used for the production of zoospores of P. megasperma var. sojae. Lima bean agar plates, each containing 15 ml medium, were inoculated at three equidistant points with mycelial

discs (5 mm diameter) and incubated 3-4 days at 24°C. To induce production of sporangia and release of zoospores, the resulting colonies, when about 2 cm in diameter, were flooded with 30 ml sterile distilled water for 20-25 minutes; this was then decanted and replaced with fresh sterile water. This process was repeated 5 times. The cultures were then flooded with 10 ml of sterile water for 5-6 additional hours to induce sporangia formation and zoospore release. Zoospores were collected and their concentration was determined with a hemocytometer or by the microsyringe method (77). A small amount of 0.1% cotton blue in lactophenol (w/v) was used to stop motility and facilitate counting. Desired inoculum densities were made by serial dilutions of zoospore suspensions of known concentration.

Oospores were produced in cultures grown in clarified V8-$CaCO_3$ broth, 10-15 ml/plate, containing 30 µg cholesterol/ml (50). Plates were inoculated with 1 ml of a zoospores suspension ($10^3$-$10^4$/ml) or with 2 mm diam. discs from the edges of actively growing colonies. Plates were incubated at 24±1°C for 4-6 weeks under dark conditions. Mycelium was killed by freezing at -10°C for 12-15 hrs. Thawed mycelial mats were rinsed twice with sterile distilled water and were ground for 10-20 minutes in a Sorvall Omnimixer, with the container suspended in an ice-water bath (Figure 1).

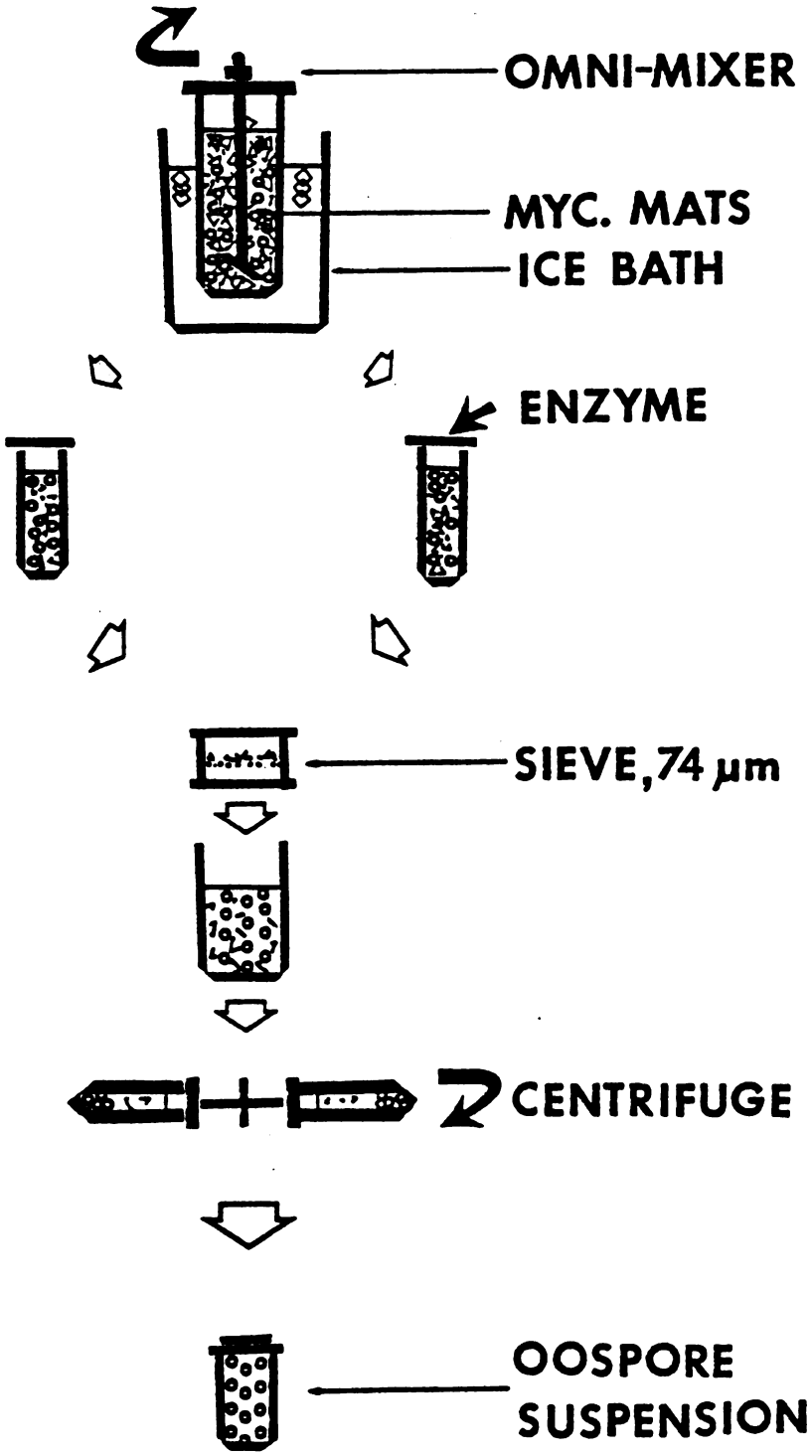



Figure 1. Isolation of oospores of Phytophthora megasperma var. sojae.

The homogenate was diluted and sieved (74 μm meshes). The filtrate containing the oospores was centrifuged at 320 X g for 15 seconds. The pellet was recentrifuged 2-4 times to obtain mycelium-free oospores. Oospores were aseptically stored in distilled water at 5°C. Desired spore concentrations were made by counting in a hemocytometer or by the mycrosyringe method (77).

Infestation of soil with oospores

Steamed greenhouse mix (peat, soil, sand: 1,1,1:v/v/v) was infested by thoroughly mixing 100 g with a fresh suspension containing $10^4$-$10^5$ oospores/ml. Final concentrations were $10^3$-$10^4$ oospores/g soil. The infested soil samples were kept at 4-5°C or used the same day of preparation after 2-4 hr of air-drying. Desired inoculum concentrations were made by diluting appropriate amounts of stock samples with steamed greenhouse mix.

The slitted cup method: a method for detecting "field tolerance" of soybeans under laboratory conditions

Soybean seedlings 2-4 days old were planted in slitted styrofoam cups (200 ml) filled with premoistened coarse vermiculite (Figure 2). The cups were placed 0.5 to 1.0 cm deep in plastic trays 17 X 25 X 6 cm containing 100 g steamed greenhouse mix and 200-250 ml $H_2O$. Inoculum consisted of 40 zoospores or 20-40

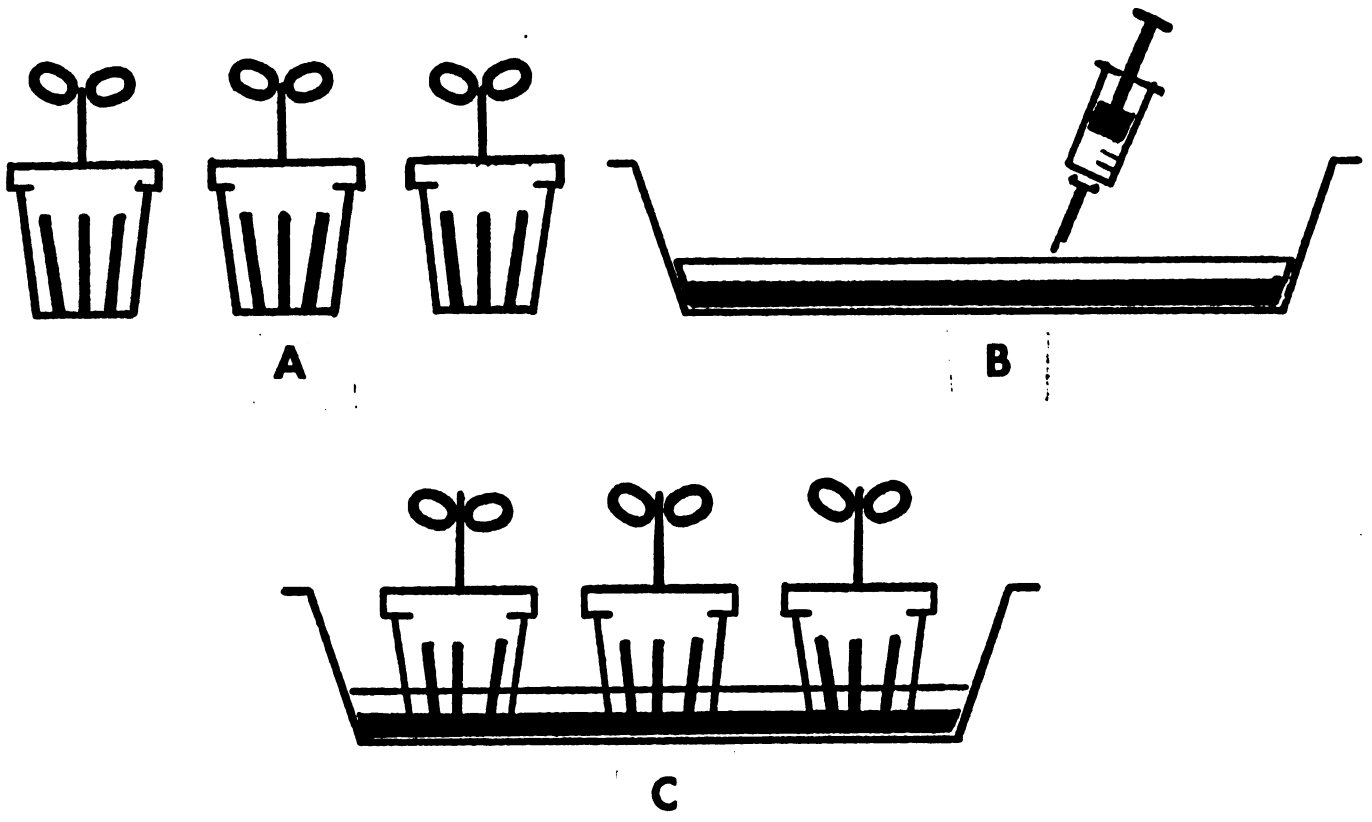



Figure 2. Diagramatic representation of the "slitted cup method":
A. slitted styrofoam cups containing soybean seedlings.
B. infestation of soil suspension.
C. Experimental arrangement for infection.

oospores/g soil. Soil was infested 2-3 days after direct seeding or during transplanting.

The plants were allowed to grow for 15-20 days in a growth chamber at 28°C and a 12 hr photoperiod. the light source consisted of cool white fluorescent lamps and incandescent light bulbs (17,000 lux).

During the first three days the volume of suspension in each tray was kept constant by adding water. From then until the completion of the experiment enough water was added daily to maintain flooded conditions.

Criteria used for assessing disease reaction

The criteria used for assessing reaction of soybean seedlings to P. megasperma var. sojae, were overall plant length and dry weight. The lengths of individuals plants were measured, from the apical bud of the stem to the tip of the main root. Plants from each cup were oven-dried f or 12 hr at 80°C, and weight determined.

The appearance of diseased roots was not used to assess infection to avoid subjectivity, and because root rot was similar among the varieties used.

Infection studies

Experiments were done to i) study the survival of zoospores added to soil, ii) determine the survival of natural inoculum under flooded conditions, and iii)

establish the flooding interval (prior to host presentation ) required to induce high percentages of infection in soybean seedlings placed in naturally infested soil or in soil artificially infested with oospores.

In these experiments the methodology used was similar to that described by Eye et al. (34). Soil was placed in 10 cm diameter petri dishes (10-25 g/petri dish) and 20-30 ml of distilled water were added. When soil was to be artificially infested with oospores (500-1250/petri dish) or zoospores ($5 \times 10^3$/ petri dish) the inocula were added with the water. The petri dishes were covered with glass tops and incubated at $24 \pm 1^{\circ}C$, on the laboratory bench, under ambient light conditions (130 lux).

Soybean seedlings (variety Hark) were used to detect the pathogen and evaluate the effect of various treatments. The seedlings were placed (5/petri dish) on the soil surface with hypocotyls submerged in the flooded soil sample. Seedling infection and severity of disease were evaluated after 3 to 4 days' incubation.

Seedlings severely diseased were stunted, failed to develop lateral roots and had necrotic flaccid hypocotyls. Severity of disease was evaluated using a disease index from 0 to 6, where: 1= healthy, 3= infected but lateral roots present, 5= seedling severely diseased with large brown necrotic lesion, and lateral roots

absent, 6= killed seedlings, hypocotyl completely rotten.

Infection was also determined by microscopic observation of sporangia production and/or presence of oospores (or oogonia) in diseased tissue. Seedlings were briefly rinsed, placed in tap water and observed under a stereomicroscope for the presence of sporangia. Seedlings apparently diseased but with no sporangia were flooded with water overnight for sporangial development. The presence of at least one sporangium was indicative of infection. The presence of oospores or oogonia was determined by firmly pressing diseased root tips between two microscope slides and observing with the microscope.

Statistical analysis

Experimental data of Part I were analyzed without transformation. Values are presented as means of 2 or 3 replications (each of these is the average length or dry weight of 5 plants). Significant differences among treatments were estimated using Tukey's w procedure or the Student-Newman-Keul's test (134). Experiments were done at least twice to verify the results.

## RESULTS

Prior to investigating "field tolerance" of soybeans to Phytophthora megasperma var. sojae under controlled conditions, several experiments were aimed to determine the survival of zoospores and oospores of this fungus in soil, and to define the most appropriate conditions for disease expression.

### Survival of zoospores in soil

To study the survival of zoospores of P. megasperma var. sojae, ten grams of natural or steamed soil (Capac loam) were flooded with 15 ml of distilled water in petri dishes, infested with 1 ml of a suspension containing $5 \times 10^3$ motile zoospores, and then incubated at 24°C. The pathogen was detected by placing 2-day-old soybean seedlings in the soil at intervals up to 16 days (Table 4).

P. megasperma var. sojae was detected in steamed soil after 16 days of incubation (the duration of this experiment), but in natural soil seedling infection was reduced to 10% at 8 days and to 0% at 16 days.

### Effect of flooding naturally infested soil on survival

To determine the survival capabilities of P. megasperma var. sojae under flooded conditions, 10 g

Table 4. Survival of zoospores of Phytophthora megasperma var. sojae in flooded soil using seedling of Hark soybeans as baits.

| Incubation time (days) | Seedlings infected, %[a] | |
|---|---|---|
| | Natural soil | Steamed soil |
| 0 | 100 | 100 |
| 2 | 100 | 100 |
| 4 | 80 | 100 |
| 8 | 10 | 100 |
| 16 | 0 | 100 |
| Control | 0 | 0 |

[a]There were 5 seedlings/plate, and 3 replications per treatment.

portions from each of two naturally infested soils, were flooded with 25 ml distilled water in petri dishes. Plates were incubated at 24°C for various time intervals up to 60 days (Table 5). The presence of the pathogen was determined using soybean seedlings as baits.

P. megasperma var. sojae was detected in soils A and B after being flooded for 49 and 60 days, respectively. Apparently, this condition did not have a detrimental effect on survival in soil B. However, in soil A infection and disease were less severe after 49 days of flooding than after 25, suggesting a reduction of

infective propagules.

Table 5. Survival of Phytophthora megasperma var. sojae in two naturally infested soils under flooded conditions[a]

| Criteria[b] | Flooding intervals, days | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Soil A[c] | | | | Soil B[d] | | | |
| | 2 | 25 | 33 | 49 | 0 | 20 | 40 | 60 |
| Infection[c] | 100 | 100 | 96 | 72 | 100 | 100 | 100 | 100 |
| Disease index[d] | 4.9 | 6 | 5.7 | 4 | 6 | 6 | 6 | 6 |

[a]Ten g of naturally infested soil were flooded with 25 ml distilled water and incubated at 24°C for the time intervals shown.

[b]Infection, %: values represent percentage of infected seedlings (Var. Hark). There were 3 replications with 5 seedlings/replicate. Disease index: 0 = healthy; 3 = infected, but lateral roots present; 5 = severe disease; 6 = plants killed (= rotten).

[c]Soil A, sandy loam collected in Ingham Co., Michigan, was infested with race 1.

[d]Soil B, Capac loam collected in Shiawasee Co., Michigan, was infested with race 4.

Effect of flooding interval, and sequential use of two sets of seedlings on infection of soybeans in soil artificially infested with oospores of Phytophthora megasperma var. sojae

Ten g portions of steamed soil artificially infested with oospores of P. megasperma var. sojae (50 oospores/g soil), were flooded with 20 ml distilled

water in petri dishes. Soybeans were added at intervals from 0 to 4 days. In one treatment a second set of seedlings replaced an initial set after two days. Disease was assessed by symptoms and by microscopic observation of sporangia on infected seedlings after four days of incubation.

Maximum infection (100%) occurred when soybean seedlings were placed in the soil after a minimum of two days of continuous flooding (Table 6). Low infection (Hark, 33%; Williams, 6.5%) was obtained when seedlings were applied immediately after flooding. However, there was no need to add a second set of seedlings to induce severe disease or to obtain high percentages of infection. These results were also indicative that oospores added to soil germinated under flooded conditions in the absence of the host (See Part II).

Effect of races 3 and 7 on the growth response of three soybean varieties.

To test the effect of races 3 and 7 of P. megasperma var. sojae on the growth response (measured as total length and dry weight) of soybeans, the varieties Hark, Williams, and Agripro 26 were selected and the "slitted cup method" (Figure 2) was followed.

Growth of Agripro 26 was less affected than the other two varieties by both races of the pathogen (Figure 3). Williams was intermediate in response and Hark was most strongly affected. For example, with race

Table 6. Effect of flooding interval prior to seedling placement, and sequential use of two sets of seedlings on infection of soybeans in soil artificially infested with oospores of Phytophthora megasperma var. sojae race 3.[a]

| Flooding interval prior to seedling placement (days) | Sets of seedlings | | Infection of indicated set of seedlings, %[b] | | | |
|---|---|---|---|---|---|---|
| | | | Hark | | Williams | |
| | 1 | 2 | 1 | 2 | 1 | 2 |
| 0 | + | - | 33 | | 6.5 | |
| 0 | +[c] | + | 0 | 100 | 0 | 100 |
| 2 | + | - | 100 | | 100 | |
| 4 | + | - | 100 | | 100 | |

[a]Steamed greenhouse mix (10 g/plate) infested with 500 oospores was flooded, and two-day-old soybean seedlings were placed in each plate at times indicated.

[b]Values represent the average of three replications with five plants per replication. Infection was determined four days after planting.

[c]Two days after planting, the first set of seedlings was replaced by the second set. Seedlings removed were incubated in water for two more days.

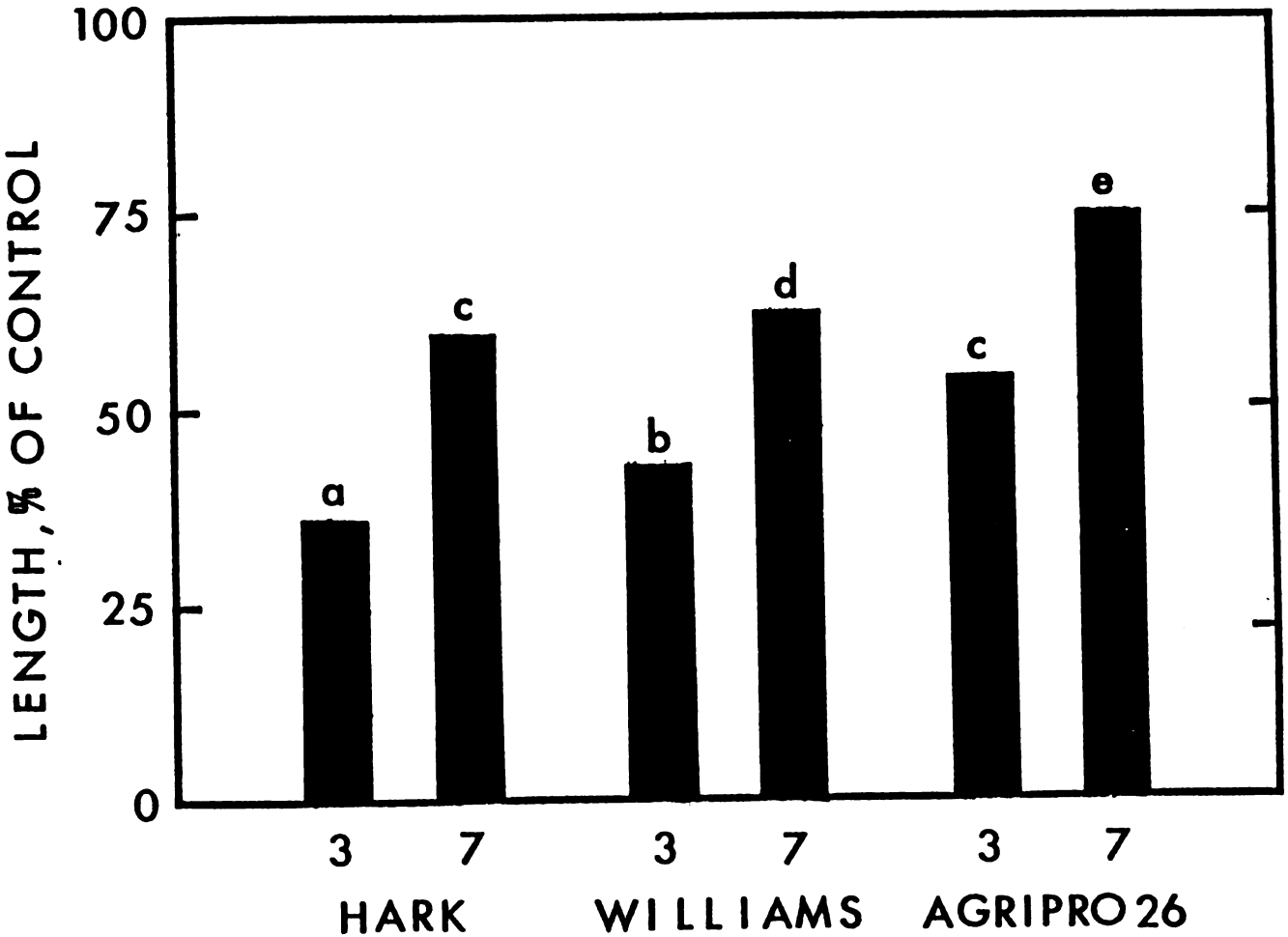



Figure 3. Effect of races 3 and 7 of Phytophthora megasperma var. sojae on the growth response (total plant length) of the soybean varieties Hark, Williams, and Agripro 26. Bars with the same letter do not differ significantly ($P = 0.05$) by the Student-Newman-Keul's test. There were 3 replications per treatment and the values are the means of 20 plants.

3 the reductions in growth as compared with uninoculated control plants, were 45% for Agripro 26, 57% for Williams, and 64% for Hark. With race 7, corresponding reductions were 26%, 34%, and 41%. A similar trend was observed when dry weights were measured (Figure 4).

These results indicate that the "field tolerant" variety Agripro 26 withstands the infection caused by two races of this pathogen much better than the more susceptible variety Hark. Williams, which also has demonstrated some "field tolerance" in the field, though less than that of Agripro 26, showed an intermediate response in these experiments.

Effect of natural inoculum of Phytophthora megasperma var. sojae on expression of "field tolerance" in soybeans.

To determine the effect of natural inoculum of P. megasperma var. sojae on growth of the "field tolerant" varieties Agripro 26 and Williams, soil from an infested (race 4) field was sieved and diluted with sterile sand. Each dilution was separately flooded with sterile water and incubated for three days at 24°C to promote oospore germination. Seedlings of the varieties Hark, Williams, Agripro 26, and Tracy were transplanted into cups containing vermiculite and placed in the flooded soil dilutions. Lengths of individual plants were measured after 15 days of growth at 28°C in a growth chamber with a 12 hr photoperiod.

Inoculation of soybean varieties (resistant and

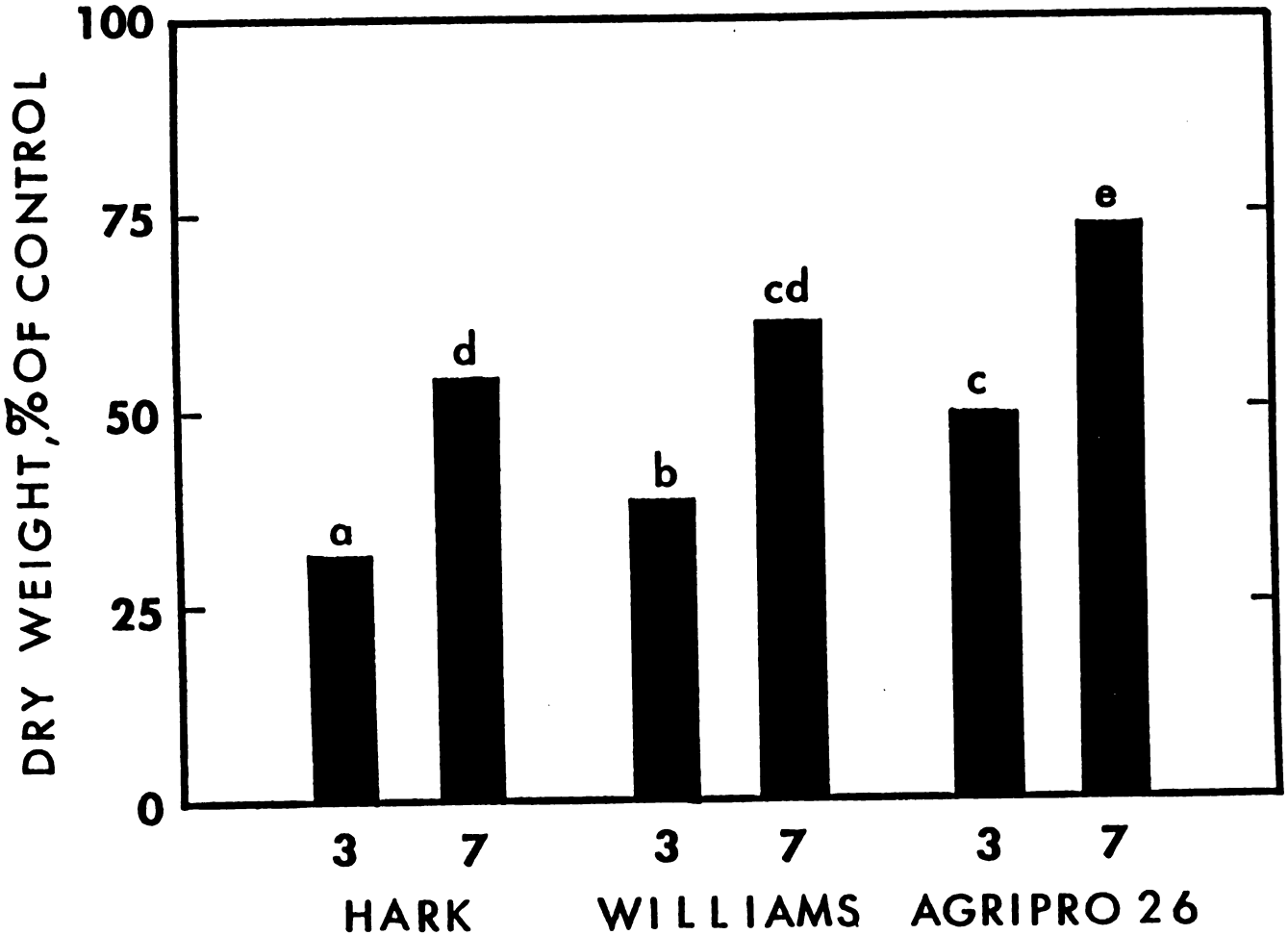



Figure 4. Effect of races 3 and 7 of Phytophthora megasperma var. sojae on the growth response (dry weight) of the soybean varieties Hark, Williams, and Agripro 26. Bars with the same letter do not differ significantly ($P = 0.05$) by the Student-Newman-Keul's test. There were 3 replications per treatment and the values are the means from 20 plants.

susceptible) with natural inoculum demonstrated that the varieties Agripro 26 and Williams were less affected than the more susceptible variety Hark (Figure 5). Agripro 26 showed less reduction in length than Williams. Varieties differed ($P = 0.05$) from each other at all inoculum densities. A concentration of 20 parts of infested soil per 80 parts of sand was enough to induce severe disease specially on Hark soybeans and to allow clear separation of the "field tolerant" varieties Agripro 26 and Williams from the susceptible Hark.

Effect of age on disease reaction of soybeans to Phytophthora megasperma var. sojae.

To determine the effect of age of the soybean plant upon infection by P. megasperma var. sojae, seedlings of the variety Hark at different stages of growth were inoculated with zoospores of race 3 (150 zoospores/g soil) in the greenhouse. Dry weight and length of plants were measured 8 days after inoculation.

Mean lengths and weights of inoculated plants were less ($P = 0.05$) than those of controls when inoculated at ages up to 8 days (Figure 6). Thereafter, differences were not significant, even though root infection of inoculated seedlings was still evident. Infection was determined by visual observation or by the presence of oospores in diseased root tissue.

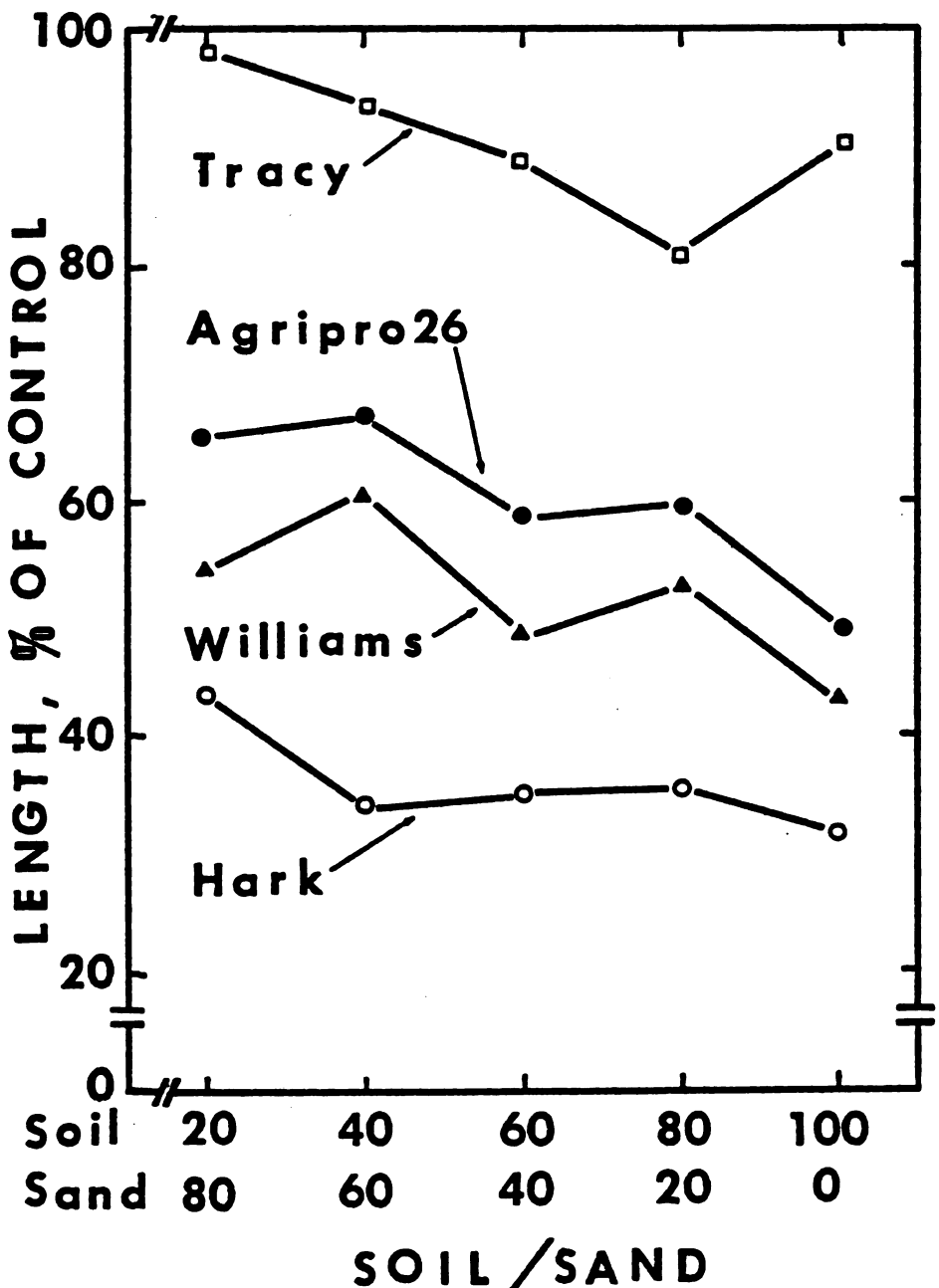



Figure 5. Effect of Phytophthora megasperma var. sojae race 4 present in a naturally infested soil on the total length of "field tolerant" soybean varieties. Each point is the average of 4 replications with 5 plants/replication. Any two varietal means differed significantly ($P = 0.05$) at any given inoculum concentration.

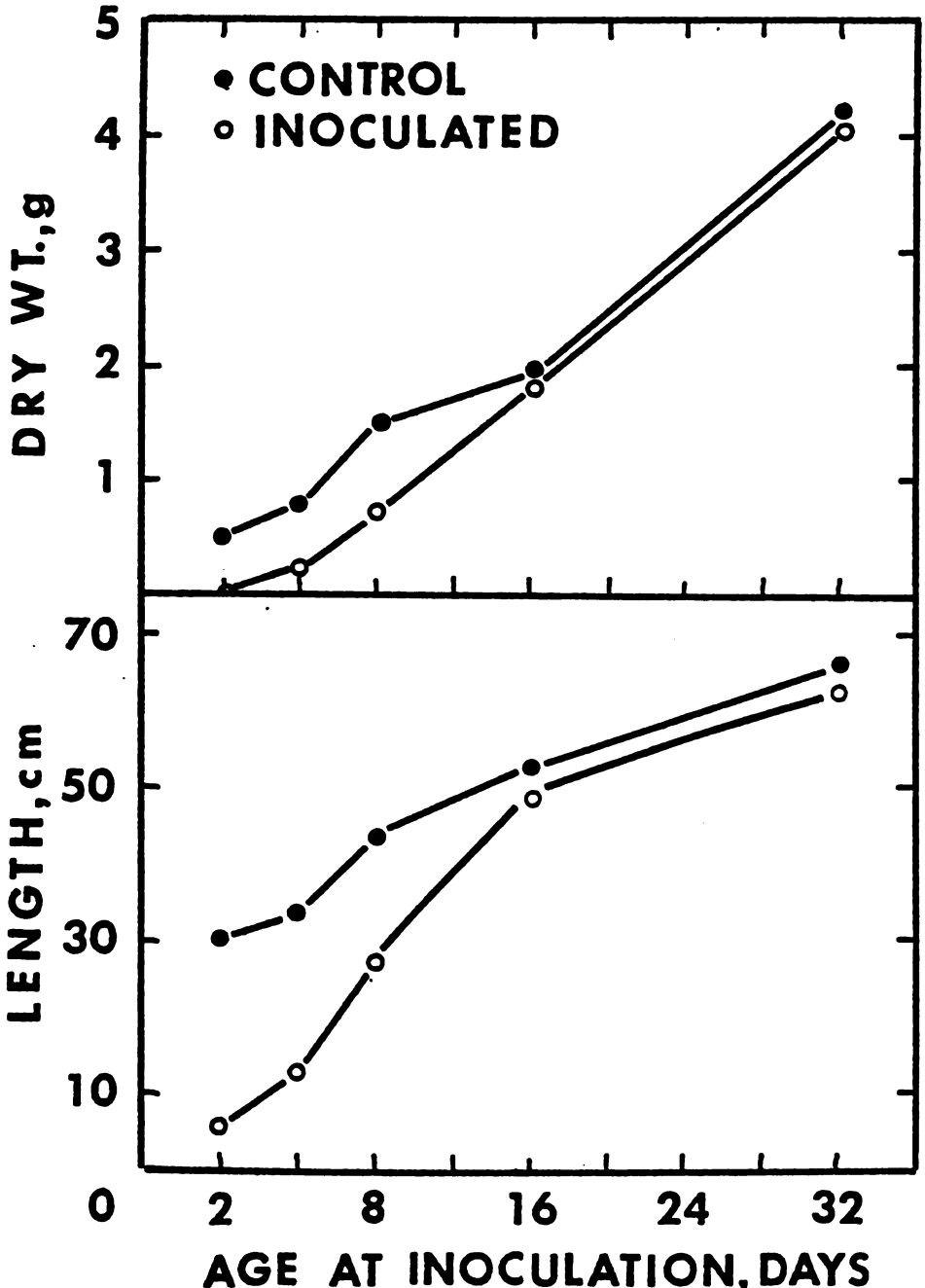



Figure 6. Effect of age at time of inoculation on disease reaction of Hark soybeans to race 3 of Phytophthora megasperma var. sojae 8 days after inoculation. Least significant ranges (P= 0.05) by Tukey's $\bar{w}$ procedure were 0.28 for dry weight and 4.5 for length.

Response of soybean varieties to races of Phytophthora megasperma var. sojae.

An experiment was done to determine the response of the varieties Hark, Williams, Agripro 26, and Tracy to races 1-5 and 7-9 of the pathogen using the "slitted cup method." In this experiment a higher concentration of inoculum than heretofore, ca. 200 zoospores/ml of soil suspension was used. Disease was assessed by the percent of plants killed after 15 days in a growth chamber (Table 7).

The number of dead plants of the variety Agripro 26 was negligible as compared to those of Williams or Hark. The average number of plants killed by all races was similar for Hark and Williams but significantly lower in Agripro 26 ($P = 0.05$). These results suggest that with high inoculum concentrations the varieties with intermediate levels of field tolerance, such as Williams, may behave as fully susceptible varieties.

Effect of races 3 and 7 of Phytophthora megasperma var. sojae on other soybean varieties susceptible to the pathogen.

Experiments were conducted to screen soybean varieties which might carry levels of resistance to P. megasperma var. sojae. The varieties tested using the "slitted cup method" were: Agripro 26, Woodworth, Wayne, SRF307P, Harosoy 63, all of which have shown some degree of "field tolerance" (106, 107, 108). The varieties Hodgson, Hark, and Ox-20-8, which are fully susceptible

Table 7. Varietal response to races of *Phytophthora megasperma* var. *sojae*

| Race[b] | Plants killed, %[a] | | | |
|---|---|---|---|---|
| | Hark | Williams | Agripro 26 | Tracy |
| Control | 0 | 0 | 0 | 0 |
| 1 | 100 | 80 | 0 | 0 |
| 2 | 40 | 80 | 0 | 0 |
| 3 | 30 | 0 | 0 | 0 |
| 4 | 70 | 60 | 10 | 0 |
| 5 | 60 | 70 | 0 | 0 |
| 7 | 50 | 30 | 0 | 0 |
| 8 | 80 | 60 | 10 | 0 |
| 9 | 20 | 10 | 0 | 0 |
| Mean | 56.2a | 48.7a | 2.5b | 0 |

[a]Values are the average of two replications with 5 plants each. Means followed by the same letter are not significantly different ($P = 0.05$) by the Student-Newman-Keul's test.

[b]Inoculum consisted of ca. 200 zoospores/ml of soil suspension.

were included for comparison. Inocula consisted of zoospores (100 zoospores/g soil) of races 3 and 7.

The results obtained further confirm previous observations that certain soybean varieties which give a fully susceptible reaction by hypocotyl inoculation, show some degree of resistance to P. megasperma var. sojae, when inoculated via roots with zoospores (Table 8). The varieties Agripro 25, Woodworth, Wayne, SRF 307 P, and Harosoy 63 were less susceptible than the variety Hark or the most susceptible Ox-20-8.

Table 8. Effect of races 3 and 7 of Phytophthora megasperma var. sojae on eight varieties of soybeans susceptible to the pathogen.

| | Length, % of control[a] | |
|---|---|---|
| Soybean variety | Race 3 | Race 7 |
| Woodworth | 73.4 | 67.0 |
| SRF 307 P | 72.7 | 65.3 |
| Agripro 25 | 67.2 | 66.8 |
| Wayne | 61.9 | 58.8 |
| Harosoy 63 | 61.3 | 58.3 |
| Hark | 53.7 | 52.1 |
| Hodgson | 51.2 | 52.2 |
| Ox-20-8 | 40.2 | 45.6 |

[a]Values represent the average of three replications with five plants per replication.

## DISCUSSION

Measurements of length and dry weight of soybeans inoculated via the roots with zoospores or oospores at controlled densities or with naturally infested soil, permitted identification of "field tolerant" varieties. Diseased plants produced results corresponding to reported "field tolerance" of the same varieties in field performance tests (106, 107, 108, 142).

Growth of the varieties Agripro 26 and Williams was less affected by races 3 and 7, than the variety Hark. In addition, disease was less severe in tha varieties Woodworth, Agripro 25, SRF 307 P, and Wayne (with known levels of tolerance in the field) than in Hark or Ox-20-8 (very susceptible).

The results reported in this study were obtained in a growth chamber under controlled conditions of light and temperature, using known concentrations of zoospores as inoculum (65). In addition to temperature (28$^{o}$C) and photoperiod (12 hr light cycle), the method exposes the natural infection court, the roots, to the inoculum, and it uses flooded soil conditions favorable for disease development. However, control of light and temperature may not be necessary since comparable data were obtained in the greenhouse, following the same procedure.

It is necessary to control the concentration of inoculum within reasonable limits since high levels overcame resistance in some soybean varieties to Phytophthora megasperma var. sojae (34). When the concentration of inoculum was increased to $10^3$ zoospores/g soil, the moderately "field tolerant" variety Williams showed a fully susceptible (kill) reaction. Finally, the distinction between the susceptible and "field tolerant" varieties was best observed 15-20 days after inoculation.

The response of the "field tolerant" variety Agripro 26 was maintained against 8 races of P. megasperma var. sojae. The variety Williams, however, demonstrated variability in its response against different races when a high concentration of inoculum was used, and it was not determined if this variation was due to race specific responses of this variety or to experimental error. It has also been reported that the levels of tolerance in a variety are the same against different races of the pathogen (36). However, my work shows that the varieties Agripro 26, and Williams differ quantitatively in their reactions to races 3 and 7.

During the study of "field tolerance" it was important to determine the effect of age of the soybean plant on infection, the effect of flooding interval, and the survival capabilities of zoospores and oospores of P. megasperma var. sojae in soil under flooded conditions. Zoospores of this fungus have been reported

to survive for a short time (92). In my work, these propagules remained viable and functional for aproximately a week in flooded natural soil. Mycelium-free oospores added to soil germinated readily as evidenced by soybean seedling infection. However, a minimum of two days of flooding were required to obtain high percentages of infection. The presence of seedlings during the first two days of flooding was not necessary in order to obtain high levels of infection.

Soil naturally infested with P. megasperma var. sojae, when flooded for 60 days, still caused severe disease in soybean seedlings. This is indirect evidence of oospore germination and indicates a tremendous capability of the fungus to survive apparently unfavorable environmental conditions (See Discussion Part II).

Herr (55) found that soybeans became more resistant to Phytophthora root rot as the plants aged. Results reported herein also indicate that susceptible cultivars become resistant to P. megasperma var. sojae as they age. Maximum susceptibility was found with seedlings from two to eight days of age. However, young or old (16-32 days) tissue harbored oospores of the pathogen. This may be important in the epidemiology of Phytophthora root and stem rot. Even though older infections may not result in severe disease, they may provide a means to produce inoculum by the pathogen.

# LITERATURE REVIEW

## Factors affecting germination of oospores

Dormancy of oospores is thought to be constitutive (22, 136). Low as well as non-synchronous germination of oospores, in many cases related to dormancy (144), has been a major problem for genetic and ecological studies of Phytophthora. Attempts to undertake population studies have been frustrated by the difficulties in germinating artificially as well as naturally produced inoculum. The nature and inherent opacity of soil have retarded these studies. An advance to overcome such problems was the application of fluorescent labeling techniques (139) to study saprophytic behavior in soil.

This review is restricted to the factors affecting germination of Phytophthora oospores, however some space also is devoted to Pythium oospores. The factors known to be involved in germination of Phytophthora oospores were reviewed by Zentmyer and Erwin (147) in 1970. They included light, sterols, temperature, maturity of the oospores, and the genetic proneness of the isolate to germinate (144, 147). However, other factors such as availability of free water, presence of specific ions, oxygen, pH, nature and concentration of substrate, host tissue, host exudates, and parasitism

# PART II

# GERMINATION OF OOSPORES OF PHYTOPHTHORA MEGASPERMA VAR. SOJAE IN SOIL

of oospores in natural soil are also important. Ribeiro (102) compiled information on the methodology for studying several phases of the life cycle of Phytophthora species. In addition, the informal publication "Phytophthora Newsletter" serves as a channel of information for those working on this genus.

Effect of light

Light as a factor affecting one or several stages of the life cycle of many fungi is well documented (20, 82 91). Studies on sporulation of Phytophthora have indicated that its response to light varies with the species (3, 10, 23, 60, 75, 86, 99). Sporangia production is stimulated by light in some (3, 15, 44, 51, 62, 86, 101), while other species are inhibited (23, 101) or fail to respond (3, 44, 141). Zentmyer and Ribeiro (145) demonstrated that sporangium production by Phytophthora cinnamomi was light variable, but not light-dependent.

Oospore production is also affected by light (59, 60, 61, 62, 75, 86, 94, 101, 103). For example, white light at high intensities reduced or inhibited oospore formation in Phytophthora cactorum and Phytophthora capsici; however, abundant numbers were produced under darkness or red light (44, 103). The production of oospores by intraspecific hybrids of some Phytophthora species was also inhibited by light, especially by blue and green light (113). P. drechsleri oospore production was inhibited by white light and

stimulated under continuous dark (75). In general, light of longer wavelengths stimulates oospore production while shorter wavelengths stimulates sporangium formation (101).

The role of light in the germination process has been extensively studied. Light, primarily in the blue and far red regions of the spectrum, stimulates germination of oospores (10, 60, 84, 99, 101). In addition, oospores produced and matured under darkness require light for germination (7, 10, 60, 75, 84, 86, 99, 101). However, germination of oospores of various Phytophthora species appeared to be independent of the quality and quantity of radiation received during gametogenesis (101).

Effect of light intensity

Although exposure to light increases germination of dark-grown oospores of most Phytophthora species, a low number (sometimes a relatively high number) of them have been reported to germinate under dark (99, 101) or at a very low light intensities (86). For example, dark-grown oospores of P. megasperma var. sojae race 1 germinated under darkness (after isolation) from 8-30%, race 2 from 5-15%, and P. cinnamomi from 2-16% (99). In another study, isolated dark-grown oospores of P. megasperma var. sojae, P. cinnamomi, and P. palmivora germinated in darkness ca. 50%, 10%, and 10%, respectively (101).

Few studies have been reported on the effect of light intensity either on oospore formation (i.e., during gametogenesis (60, 101), or on germination (7, 99). The range of light intensities used to date for studying germination of oospores of Phytophthora is from 0.1 $\mu W/cm^2$ (=0.68 lux) (99) to 10,830 lux (7). Banihashemi and Mitchell (7) reported very little effect of light intensities ranging from 104 lux to 2,165 lux on the germination of isolated P. cactorum oospores. However, there was a noticeable increase at 2,165 lux. Germination increased as light intensity increased up to 4,330 lux and then declined at higher intensities. After 92 hours of incubation, only 3.9% germination occurred at 104 lux while maximum germination (87.9%) was recorded at 3,240 lux. At 10,830 lux 66.4% of the oospores germinated (7). In spite of the low germination at 104 lux after 92 hr of incubation (7), P. cactorum oospores were reported to germinate 90% after 60 days under 0.5 ft-c (=5.4 lux) in a medium containing beta-sitosterol (86).

The effect of near UV, blue, red, far red, infrared, dark, and daylight at three different light intensities on germination of P. cinnamomi, P. palmivora, P. capsic, and P. megasperma var. sojae oospores was studied by Ribeiro et al. (101). They noted that germination of irradiated oospores under darkness was independent of the light quality and

quantity (intensity) received during gametogenesis. For example, the oospores of P. megasperma var. sojae germinated ca. 50% at almost all light intensities (including dark) of the different light wavelengths used.

The data indicate that germination of mature oospores of Phytophthora increases as the light intensity increases (7). Prolonged (60 days) exposure to low intensities gave 90% germination in situ of oospores of P. cactorum (86) , but further research with more species is required before any conclusion can be made that prolonged exposure to low light intensity can replace a short exposure to light of high intensity.

Effect of light quality

Fluorescent light has been shown to stimulate the germination of oospores of several Phytophthora species (7, 10, 31, 32, 33, 38, 39, 75, 103, 105, 111, 138, 147) either in situ or after isolation. For example, oospores of P. megasperma var. sojae failed to germinate in situ after 30 days under darkness, but germination occurred either in situ or after isolation when the cultures were exposed to fluorescent light for several days (10, 31, 33, 101). Dark grown oospores of P. cactorum germinated only 0.3% in the dark, while 99% germinated under fluorescent light (7).

The stimulatory effect of fluorescent light on oospore germination has also been reported for P. hevea (10, 84), P. drechsleri (32, 38, 39, 75, 147), P. capsici

(98, 101, 111, 138), P. citricola (53), P. cinnamomi (10), P. cactorum (7, 10, 84), P. infestans (10, 103, 104, 105), P. erythroseptica (10, 84), and P. palmivora (10, 101).

The spectral regions reponsible for the stimulatory effect of light on the germination of oospores are the blue (400-480 μm) and the far red (700-1000 μm), (7, 10, 19, 99, 101, 103). Regardless of the type of media used either for production and/or germination, the blue and far red regions of the spectrum were the most effective for inducing germination of P. cactorum oospores produced under darkness (7, 10, 19). In addition to P. cactorum, increased germination of oospores under blue, red, or far red light has been demonstrated for P. infestans (103), P. Cinnamomi (99, 101), P. capsici (99, 101), and P. palmivora (101).

Effect of duration of exposure to light

Information on the effect of duration of exposure to light on the formation (i.e., during gametogenesis) of oospores of Phytophthora, and during their germination is scanty. Apparently the effect of duration of exposure to light of dark-grown mature oospores was studied only for P. drechsleri (75) and for P. cactorum (7). Klisiewicz (75) reported that dark-grown oospores of P. drechsleri which received 0, 0.1, 4, 10, and 12 hr of fluorescent light (45 ft-c,

= 484.4 lux) germinated 4%, 15%, 15%, 25%, and 17% respectively.

Increased percentages of germination of P. cactorum oospores were obtained by increasing the duration of exposure to fluorescent light (7). Isolated oospores were incubated in water at 22°C under 2,594 lux for various intervals and then transferred to darkness. Oospores exposed to light for 0 hr did not germinate, those exposed for 12 hr germinated 0.3%, and those exposed for 48 hr and 108 hr germinated 69.7% and 99.2%, respectively. This information, although limited to one species in particular (7), suggest that there is a threshold of time of exposure to light that has to be reached before high and consistent germination of mature oospores can be obtained.

Effect of sterols

The role of sterols on growth and reproduction of Phytophthora and Pythium is well documented (6, 29, 31, 46, 47, 49, 50, 52, 64, 83, 86, 93) though their mode of action is incompletely understood (52).

Since Phytophthora species have an absolute requirement for sterols for sexual reproduction (52), their effects on the germination process are more difficult to interpret, especially in those studies (104, 105) in which media of unknown chemical composition were used. The addition of beta-sitosterol to chemically defined media seemed to increase germination of Phytophthora cactorum

oospores (10, 86). However, concentrations of beta-sitosterol at 0.5 and 10 mg/l in a glucose-asparagine agar medium each gave 90% germination in the culture after 60 days exposure to dim light (less than 5 f-c.) (86).

Effect of carbon and nitrogen source

The nutritional requeriments of Phytophthora have received considerable attention (21, 25, 30, 85, 100, 114, 146). Species and interspecific hybrids of this genus are normally grown on a variety of natural (100, 114) and chemically defined media (21, 25, 84, 100). In addition to carbon and nitrogen sources, small amounts of sterols are added to synthetic media to assure sexual reproduction. Oospores produced on both natural and defined media have been used for germination studies (7, 84), though variation in size and abundance have been reported (100, 146). The assumption is made that the nutritional status of the oospores varies accordingly (146).

Few systematic studies have been done to determine the influence of the carbon and nitrogen sources on the germinability of the oospores. Leal et al. (84) presented evidence that germination of oospores of P. hevea, and Phytophthora erythroseptica was dependent on the carbon and nitrogen sources present in a chemically defined medium containing beta-sitosterol. P. hevea germinated better when the medium contained glucose and valine as carbon and nitrogen sources, respectively. Germination was high (50%) when mannitol was used as the

carbon source as compared to 8% with xylose. Highest germination of P. erythroseptica was obtained whe fructose was used as carbon source.

Effect of V-8 juice

Among the natural media for growing Phytophthora (32, 38, 98, 100, 102, 114, 138), V-8 juice, with or without agar (per liter: 200 ml V-8 juice, 3 g $CaCO_3$, and ± 20 g agar) has been probably the most commonly used. Various reports indicate that the concentration of this medium influences the germinability of oospores produced therein (103, 104, 105, 111). In situ germination of oogonia (accepted now as true oospore germination) of P. infestans was higher (up to 39.2%) at 20°C under a 12 hr photoperiod, when the concentration of cleared V-8 juice in the agar was reduced to half-strength (103). Somewhat opposite to this, oospores of P. infestans and P. capsici produced in clarified V-8 juice agar germinated less than those produced in unclarified V-8 juice agar (105, 111); in both cases germination took place in water. However, when clarified V-8 juice agar was supplemented with beta-sitosterol, germination in water increased from 20% to 34% (104). Although germination in unclarified V-8 juice agar was similar to that in clarified V-8 juice plus beta-sitosterol (104), greatest germination (64%) of isolated Phytophthora infestans oospores in water was obtained when unclarified V-8 juice was amended with beta-sitosterol (104, 105). These

observations may reflect the higher natural sterol concentration of unclarified V-8 juice agar.

Effect of low nutrient substrates and other components of culture media

Prior to the discovery that light stimulates germination, attempts were made to induce oospores to germinate by means of physical as well as chemical treatments, but little success was accomplished (112, 125, 140). Oospores were germinated in water and in other substrates generally low in nutrients (7, 33, 38, 75, 100, 104, 105, 112, 125, 127, 139, 140). Dung infusion and potato soil leachates increased germination of oospores of P. infestans, but the percentages were low and inconsistent (112, 125). However, there was an indication that low concentrations of horse-dung infusion induced better germination of oospores than did higher concentrations (125).

Natural soil or extracts from it, both considered to be poor energy sources, supported abundant germination and production of sporangia by oospores of P. cactorum (6, 7, 127). In the former study, germination was influenced by the type of substrate and by several components of culture media as antibiotics, sugars, amino acids, and growth retardants.

Calcium, supplied either as $CaCl_2$ or as $Ca(NO_3)_2$, at concentrations ranging from 10-200 µg/ml was shown to be required for germination of Pythium aphanidermatum

oospores (130). However, concentrations of $CaCl_2$ above 25 µg/ml were inhibitory to Phytophthora cactorum oospore germination (7). Below this concentration calcium was stimulatory.

Antibiotics have been shown to affect germination of oospores of P. cactorum (7). Pimaricin at 100 µg/ml reduced germination by 80%, but had little effect at 50 µg/ml or less. Vancomycin did not affect germination at 200 µg/ml, but it was decreased 40% by polymyxin B at 200 units/ml (7). Neither pimaricin nor vancomycin inhibited germination of oospores of Pythium aphanidermatum, P. ultimum, and P. myriotylum (6).

Sugars inhibited germination of P. cactorum oospores (7), but were stimulatory to Pythium aphanidermatum oospore germination (130). Germination of P. cactorum was decreased by 50% by 0.5% glucose, and by 50% by 1% sucrose (7). However, glucose and sucrose at 1% (w/v) in water stimulated germination (74% and 92%, respectively) of snail-ingested oospores of Pythium aphanidermatum (130).

Glycerides and fatty acids were reported to enhance germination of Phytophthora oospores (10). In this brief report mention was made that germination in situ of P. cactorum oospores was stimulated by methyl linoleate and triolein.

Effect of host tissue

When oospores of Phytophthora species, formed and matured in host tissue, have been mechanically isolated, relatively few have been shown to germinate (27, 63, 75, 109). However, in some cases germination was equal or higher than that of oospores produced in laboratory media (63, 75). For example, oospores of Phytophthora drechsleri grown in corn meal agar germinated poorly, generally less than 5% in water, while oospores from safflower stems germinated 10% or higher (75). In addition, germination of oospores of P. megasperma var. sojae isolated from infected soybean seedlings was similar (ca. 30%) to that of culture (V-8 juice)-grown oospores (63).

Germination of oospores of P. megasperma isolated from alfalfa seedlings was also low (5% or less) as compared to that (90% or higher) of oospores produced in V-8 juice agar and passed through land snails (109). Finally, 5% to 60% germination in water agar was obtained using oospores of Phytophthora fragariae isolated from strawberry roots (28); however, no data have been reported on the germination of culture-grown oospores of this pathogen.

Under natural conditions, oospores formed in host tissue have survival value and constitute the main source of variation for these pathogens. Consequently,

the use of inoculum produced in diseased tissue for germination as well as for ecological studies could help to more realistically understand the behavior of Phytophthora in soil. Moreover, studies on the mechanisms by which oospores embedded in tissue remain dormant, are needed. This is important, not only from the scientific point of view, but because its intrinsic potential for controlling disease.

Effect of soil, soil extract, and host exudates.

The study of saprophytic behavior of Phytophthora and Pythium in the soil environment has been retarded by lack of adequate techniques. This obstacle has been partially overcome by the use of fluorescent brighteners (140) immunofluorescent techniques (90). Mycelium, zoospores, chlamydospores or oospores have been added to soil and their fate followed, usually by a soil smear technique (139). Few reports, however, have appeared on the germination of oospores added to or isolated from soil (66).

Oospores of Pythium aphanidermatum, obtained by passage through water snails, were capable of either direct or indirect germination in flooded field soil (131). Mode of germination however, was dependent on the presence or absence of an exogenous source of nutrients and presence or absence of free water in soil. In the presence of nutrients, oospores germinated by a

germ tube which continued to grow, and in the absence of exogenous nutrients, sporangia production and zoospore release from germinating oospores occurred at the surface of saturated soils (139).

Moisture condition and nitrogen amendment both influenced the germination of Pythium aphanidermatum oospores in soil (132). Oospores germinated (ca. 90%) directly in asparagine-amended soil, (100 µg asparagine/g soil) maintained at -0.1, -0.01, or 0 bars. Germination also occurred in asparagine-amended soil, maintained at -0.1 and -15 bars, but both the rate and percentage were considerably reduced, especially at -15 bars (132). Oospores of P. ultimum germinated after incubation in non-sterile saturated soil, and in soil agar if a source of nutrients was provided (89). Though high soil moisture (-0.3 or -0.5 bars) was needed, no addition of nutrients was required by oospores of Phytophthora cactorum to germinate and produce sporangia (127). Moreover, prolonged incubation in soil increased their subsequent germination in water agar. Contrary to these results, germination of oospores of Phytophthora megasperma f. sp. medicaginis was not stimulated by the presence of soil (9).

An extremely important factor that directly affects oospore germination in natural soil is the widespread occurrence of oospore parasites (58, 128, 129). Oospores of P. megasperma var. sojae, P. cactorum,

and Pythium sp. were parasitized by Chytridiomycetes, hyphomycetes, Oomycetes, Actinomycetes and bacteria. The amount of parasitism and the type of parasites involved were dependent on soil moisture (129). Since germination of oospores is known to occur in natural field soil (127, 132, 139), the presence of these mycoparasites will reduce the number of germinable propagules. Additional references dealing with oospore parasites were listed by Sneh et al. (129).

Soil extract, (prepared by mixing a soil-water suspension, usually 1:1, w:v, for a period of time and then separating the liquid) has been succesfully used for germinating oospores of Phytophthora and Pythium (7, 89). Autoclaved or non-autoclaved soil extract increased the germination of Phytophthora cactorum oospores to over 80% as compared to 1.3% in sterile distilled water (7). Besides, oospores germinated 16% in soil extract at a sub-optimal temperature (28$^{o}$C), while no germination occurred in distilled water. Non-sterile soil extract also transformed normal, thich-walled oospores of Phythium ultimum to thin-walled oospores that germinated when a supply of nutrients was provided (89). Prior incubation of oospores of P. aphanidermatum, P. ultimum, and P. myriotylum in non-sterile soil extract also increased their subsequent germination on a nutrient medium (6).

The germination of oospores of Phytophthora and Pythium as affected by the presence of, o by exudates from, roots or other host organs has not been studied extensively. After passage through water snails, oospores of Pythium aphanidermatum germinated over 80% in the immediate vecinity of bean seeds, sugarbeet seeds, and 2-week-old sugarbeet seedlings (131, 133). Pythium mamillatum oospores were also stimulated to germinate in response to root exudates (8). However, the oospores of Phytophthora megasperma f. sp. medicaginis were not stimulated to germinate by the presence of alfalfa roots (9). Moreover, germination of oospores of Pythium myriotylum was equally poor when applied directly to roots and hypocotyls of bean plants or when incubated in bean root exudates (6).

Effect of age

According to Blackwell (13) oospores of Phytophthora cactorum required an "after-ripening" period of ca. 9 months, and although this period could be shortened by a cool treatment, germination was very low. Other reports also indicated that germination of oospores occurred only after they had aged for long periods of time. However, there is evidence which indicates that oospores produced by young cultures germinate if placed in a proper environment. For example, relatively young oospores of Phytophthora capsici germinated by producing

germ tubes, germ sporangia, or sessile sporangia (110). Germination of 1.5%-2.6% was obtained with 14-day-old oospores as compared to 19%-20.1% with oospores from cultures 60 days of age (6, 111).

Shaw (121) indicated that germination of oospores of Phytophthora cactorum, that had been ingested by the water snail Planorbarius corneus, increased with the age of the propagules, particularly in those oospores incubated under light. Highest germination was obtained with 25-day-old oospores. No germination occurred with 4-day-old oospores incubated for 8 days. It was also reported (75) that 106-day-old oospores of P. drechsleri germinated better (ca. 40%) than oospores from cultures 35 days old (ca. 20%). However, only 15% germination was obtained with oospores from cultures 56 days old aged at 3°C. Apparently aging improved germination, but long periods were not necessary for germination to occur (75). Germination of P. megasperma oospores of various ages in the feces of the land snail Helix aspersa increased with age of the culture (109). Seven-day-old oospores germinated only 2.6% while 35 days old or older germinated over 90%.

Phytophthora cactorum oospores incubated in sterile distilled water for 7 days at 22°C under 2480 lux of cool-white fluorescent light increased from 14

to 38% as the age of the culture increased from 15 days to 5 months (7). The aging period was shortened, however, when oospores were treated with the enzyme complex Glusulase. Percentage germination increased to 76.1% when the oospores were treated with the enzyme.

Ayers and Lumsden (6) reported that among the conditions that favored increased percentages in Pythium aphanidermatum were the increased age of the cultures from which the oospores were harvested and a period of after-ripening in soil extract. For example, oospores from 3, 5, 10, and 18 day-old-cultures germinated 0, 30, 46, and 43% in a medium containing pimaricin and vancomycin. The oospores of P. ultimum required an after-ripening period (6, 26, 116) in order to germinate in soil extract (6).

Effect of temperature

The existing information on the germination of oospores of Phytophthora and Pythium as influenced by temperature is conflicting. Some reports indicate that germination is enhanced by exposing mature oospores to low (freezing) temperatures (13, 105), sometimes for prolonged intervals. Other studies report no beneficial effect with this treatment (6, 75, 147); however, freezing at -5°C to -20°C has been used to kill the mycelium associated with the oospores (7, 33, 111). Finally, other investigations showed that treating mature oospores at 33 or 36°C for several hours stimulated their

subsequent germination at moderate temperatures (75, 88).

Contrary to Blackwell (13) who indicated that chilling increased germination of mature oospores of Phytophthora cactorum, Zentmyer and Erwin (147) reported that preincubating oospores of P. drechsleri and P. megasperma var. sojae at 1°C did not increase their subsequent germination. Besides non-chilled oospores of P. megasperma var. sojae germinated up to 78% as compared to 25%-50% for oospores chilled at 9°C for at least one month and then incubated at 25°C (31).

Results (32, 105) which indicate that preincubating cultures containing mature oospores increased their germination at 20°C or 24°C, are difficult to interpret because other factors were not controlled. For example, a low temperature treatment (13 or 45 days at 4°C, before incubation) increased germination of oospores of Phytophthora infestans from 34% to 50% (105). However, the oospores had been produced in clarified V-8 juice supplemented with beta-sitosterol and had received a light treatment before refrigeration. Since these two factors, light and sterol, were not applied to oospores that were not chilled, no definite conclusion can be obtained from the data.

In another study (32) 22-day-old cultures of P. drechsleri were stored at 9°C for 90 days. After storage, oospores were extracted and incubated in water for 48 hr at 21-24°C. Germination varied from 20-80%

among experiments. Lowering the temperature may or may not have been responsible for stimulating germination since non-chilled oospores were not used. Besides, improvement in germination could also be attributed to aging.

It appears that temperature during the early developmental stages is an important factor for the subsequent germination of oospores (33, 103). Incubating cultures of P. megasperma var. sojae at 24°C and 27°C was more favorable for subsequent germination of oospores than 16, 18, or 30°C. Germination was 69-78% with cultures previously incubated al 27°C; and although an isolate grew well and produced oospores at 30°C, a large number of them were malformed and did not germinate.

High temperature treatment (33-36°C) after maturation may also increase germination of oospores (75, 88). Long et al. (88) reported that soaking the oospores of P. megasperma var. sojae for 48 hr in water at 36°C before incubation on 1.5% water agar increased their germination up to 50±10% as compared to ca. 20% with non-treated oospores or with oospores soaked at 25°C. Soaking the oospores at 36°C for 48 hr also reduced the variability in germination.

Variable temperature ranges for the germination of oospores of a few species of Phytophthora and Pythium

have been found (7, 75, 130). The effect of temperature on germination of Phytophthora drechsleri was determined with isolated oospores from 35 and 102-day-old cultures (75). Oospores incubated in water agar, pH 7.5, over a range of temperatures from 15 to 30°C germinated within 72 hr at all temperatures. Optimum for germination was at or near 24°C. Incubation of cultures at 33°C for 2-8 days prior to separating oospores, stimulated their germination, whereas chilling at 3°C for 7 days did not. Oospores of Pythium aphanidermatum germinated over a range from 15 to 45°C with an optimum at 35°C. This range also coincided with that for linear mycelial growth (130). However, the optimum temperature for germination of oospores of Phytophthora cactorum (22°C) was lower than the optimum for mycelial growth (28°C) (7). Under optimum conditions of substrate and light, germination at 20°C and 28°C in an autoclaved soil extract on water agar was 90% and 6%, respectively.

Effect of other factors on oospore germination

Enzymes (7, 88, 109, 143, 126), soil fauna (41, 109, 121, 130), pH (in soil and in culture) (60, 75, 102, 130), or centain compounds (139) used as tools for ecological studies have also been shown to affect germination of Phytophthora and Pythium. A brief account of these reports, as well as of those dealing with oogonium germination (53, 103) is presented.

Finally, air-drying, has been reported to enhance germination of Phytophthora drechsleri (75), Pythium aphanidermatum, and P. ultimum but not of P. myriotylum oospores (6).

Oogonium germination. Germination of oogonia has been observed of culture of P. infestans, P. citricola (53), Pythium aphanidermatum, Pythium ultimum, and P. myriotylum (6). The evidence presented suggests that the same environmental factors affect germination of oospores and oogonia similarly. However, oogonia has been regarded as artifacts caused by nutrient deficiency in artificial culture (6). Romero and Gallegly (103) interpreted their observations as oogonium germination, since oospore cell walls were not evident within the oogonia, and the cultures from single germinating oogonia did not demonstrate genetic recombination. This interpretation was challenged by Galindo and Zentmyer (38, 39) who obtained evidence of germination of young (14-day-old) oospores of Phytophthora drechsleri, and by Satour and Butler (111) who germinated what they considered to be relatively young oospores of Phytophthora capsici. The phenomenon described (103) for P. infestans was finally considered to be true oospore germination. Both authors have now accepted this interpretation (40, 104).

Oospore passage through snails. Land and water snails have been used as effective tools for separating

oospores from mycelium as well as for inducing germination of oospores of Phytophthora and Pythium (41, 109, 121, 130). Gregg (41) observed germination of young oospores of Phytophthora erythroseptica and P. cactorun after they have passed through the digestive tract of the land snail Helix aspersa. Besides, H. aspersa, Rumina decollata another land snail, and the water snail Helisona tenue, were used to induce high germination of oospores of P. megasperma (109). Mechanically separated oospores germinated 5% or less as compared to 93.4% of ingested oospores. Similarly, germination of Pythium aphanidermatum was increased to 94± 2% by passage of oospores through live water snails (Planorbis sp.), while non-ingested oospores germinated about 20% only (130). Although it was not clear whether passage through the water snail Planorbarius corneus affected the percentage germination, Shaw (121) reported up to 67% germination of ingested oospores of Phytophthora cactorum Finally passage through water snails (Helisoma sp.) did not increase germination of oospores of Aphanomyces eutiches (12)

Effect of enzyme treatment. It has been demonstrated that enzymes used to lyse mycelium in order to isolate oospores, affect their germination either positively or negatively (7, 88, 109, 126). For example, Glusulase (a mixture of 181,500 units of

glucuronidase/ml and 84,180 units of sulfatase/ml) treatment increased germination of young oospores of Phytophthora cactorum, but no differences in final germination were observed when cultures 5 weeks old or older were pretreated with the enzyme complex. Treated and non-treated oospores were incubated in sterile distilled water at 22°C under 2480 lux cool white fluorescent light for 7 days (7).

Although increased rates of germination of Glusulase-treated oospores were observed (7), lysing mycelium of Phytophthora cactorum with a mixture of cellulase and hemicellulase at a concentration of 0.5% each did not increase germination of oospores (126). Germination of treated and non-treated oospores was 40% after incubation on 0.8% water agar under fluorescent light.

Oospores of Phytophthora megasperma isolated with the enzyme complex beta-glucuronidase/aryl sulfatase or with the snail enzyme beta-glucuronidase germinated 52.3% to 60% in water as compared to non-enzyme treated oospores that germinated less than 5% (109).

Contrary to the results obtained with P. megasperma (109), the use of an enzyme treatment for promoting germination of Phytophthora megasperma var. sojae had a null or inhibitory effect (88). In these

experiments pretreatment of oospores with Helicase or beta-glucuronidase at concentrations ranging from 0.025% to 5% for 1-72 hr at 25, 30, and 36°C did not stimulate germination; on the contrary, germination relative to control oospores (incubated in water or buffer) was decreased. Purified beta-glucuronidase also did not improve germination. However, oospores of Pythium aphanidermatum treated with the enzyme mixture obtained from the land snail, Helix pomatia, germinated up to 75%; non-treated oospores germinated 15% (130).

Effect of brighteners. Tsao (139) reported significant differences in germination of oospores that were labelled or non-labelled with 280 ppm of the brighteners Calcofluor White M2R New. Germination of labelled oospores of Phytophthora megasperma var. sojae in water was 37.1% as compared to 24.1% for non-labelled oospores. The mode of action of this brightener on the germination process is unknown.

Effect of pH. In general, oospores of Phytophthora and Pythium species germinate over a wide range of pHs, but the response varies with the species. For example, Phytophthora dreshsleri germinated over a pH range from 4.5 to 9.5 with an optimum near 7.5 (75), while P. palmivora germinated over a narrowe range (pH 5 to 6) (60). P. cactorum germinated over a range of pH from 7.6 to 10, P. Capsici from 4.5 to 7.3, and P. infestans

from pH 2 to pH 10 (102). Pythium aphanidermatum germinated over a pH range of 5.2 to 8.6, with an optimum between 6 and 8 on corn meal agar (130). Pythium ultimum oospores germinated over a range of pHs from 4.5 to 7.5 with a maximum at pH 7, in soil adjusted with $CaCO_3$ or $Al_2(SO_4)_3$ (89).

## MATERIALS AND METHODS

### Isolates

Oospores of Phytophthora megasperma var. sojae races 1, 3, and 9 were used. The isolates of faces 1 and 3 were obtained from Dr. A. F. Schmitthenner of Ohio State University, and the isolate of race 9 was obtained from Mr. F. A. Laviolette of Purdue University. Stock cultures of these isolates were maintained and sub-cultured as described in Part I.

### Production and isolation of oospores

Oospores used for germination studies were produced as described in Part I except for the light and temperature regimes.

Zoospore-inoculated plastic petri dishes containing 20 ml V-8 juice broth amended with cholesterol (per liter: 100 ml V-8 juice, 1.0 g $CaCO_3$, and 30 mg cholesterol dissoved in 1.5 ml ethanol over heat) (6) were sealed with Parafilm and placed under a light source or enclosed in several layers or aluminum foil (dark treatments). Two different light sources, each with four fluorescent lamps, were suspended over a laboratory bench that was covered with aluminum foil. A summary of the features of these light sources is presented in Table 9.

The distances between the light sources and the cultures were adjusted so that light intensities were the same. The average intensity for each source was 4125 lux as determined with a light meter (Weston Electrical Instrument Corp., Newark, N.J., U.S.A.). The temperatures inside and outside of the culture plates

Table 9. Characteristics of two light sources used for studying the germination of Phytophthora megasperma var. sojae oospores.

| Characteristic | Light source[a] | |
|---|---|---|
| | 1 | 2 |
| Type of lamps and make | F40/CW ITT | Gro-lux WS 40W Sylvania |
| Distance (cm) to the surface of the cultures | 50 | 47 |
| Average light intensity[b] (lux) at culture level | 4125 | 4125 |
| Spectral peaks (μm)[c] | 370<br>400<br>435<br>550<br>575 | 400<br>430<br>530<br>650 |
| Temperature of cultures, C.[d] | 26.4 ± 0.9 | 26.8 ± 0.8 |

[a]Four fluorescent lamps each.

[b]Determined with a light meter.

[c]From Iscotables (2).

[d]Determined with thermister probes placed within the petri dishes.

were measured with a 12 channel tele-thermomether (Yellow Springs Instruments Co., Inc.). Temperatures inside culture plates were monitored by thermister probes inserted into separate cultures through small holes in tops of petri plates. Dark treatment petri plates were brought closer to the light source until they reached a temperature equal to that of plates directly exposed to light.

Oospores for germination experiments were isolatted as described in Part I. Dark-grown oospores were isolated under a "safe light" which was obtained by passing light from an incandescent light bulb (15 W) through a yellow filter (Kodak Safelight Filter Wratten Series OA). For some experiments, instead of freezing the mycelium mats to kill the mycelium, the enzyme complex beta-glucuronidase/aryl sulfatase, obtained from Helix pomatia (Sigma Chemical Company, St. Louis, Mo.) was used to digest mycelium and antheridial remains. The mycelial mats from 3 culture plates were rinsed in sterile distilled water and ground for 10 min. in 0.1 M sodium acetate buffer, pH 5.0 (43) (Figure 1). Approximately 20 ml of the homogenate was treated with 0.25 ml of enzyme solution and incubated at 37°C for 8 hr. The digested material was passed through a sieve (74 µm meshes) to remove non-lysed mycelium. Three low speed centrifugations (320 X g, 15 seconds each) were used to

concentrate the oospores and to remove the enzyme solution.

Oospores used for germination studies (treated or non-treated with the enzyme) were germinated immediately after isolation or stored in sterile water at 5°C for no longer than 5 days.

Techniques for the germination of oospores

Oospores were germinated on Millipore membrane filters (0.22 µm pore size, 25 mm diam.), which were placed on the surface of 10 to 15 g natural or steamed flooded soil (28) or flooded sterile sand in 10 cm diam. petri dishes. In some experiments, 2-day-old soybean seedlings were also present in the petri plates. The membranes were removed after 5-7 days, cleansed of soil by flotation en petri dishes containing distilled water, then stained with rose bengal, and examined microscopically.

The effect of several substrates on oospore germination was studied using oospores embedded on flooded water agar. The oospores were first transferred to the agar by applying them to Nuclepore membranes (0.4 µm pore size, 1.5 X 1.5 cm), (Figure 7). The membranes were inverted in 60 mm diameter plastic petri dishes (3 membranes/plate) containing 5 ml 1.5% water agar at approximately 40°C. When the agar had solidified (5-10 min.), the outline of each membrane was traced on the

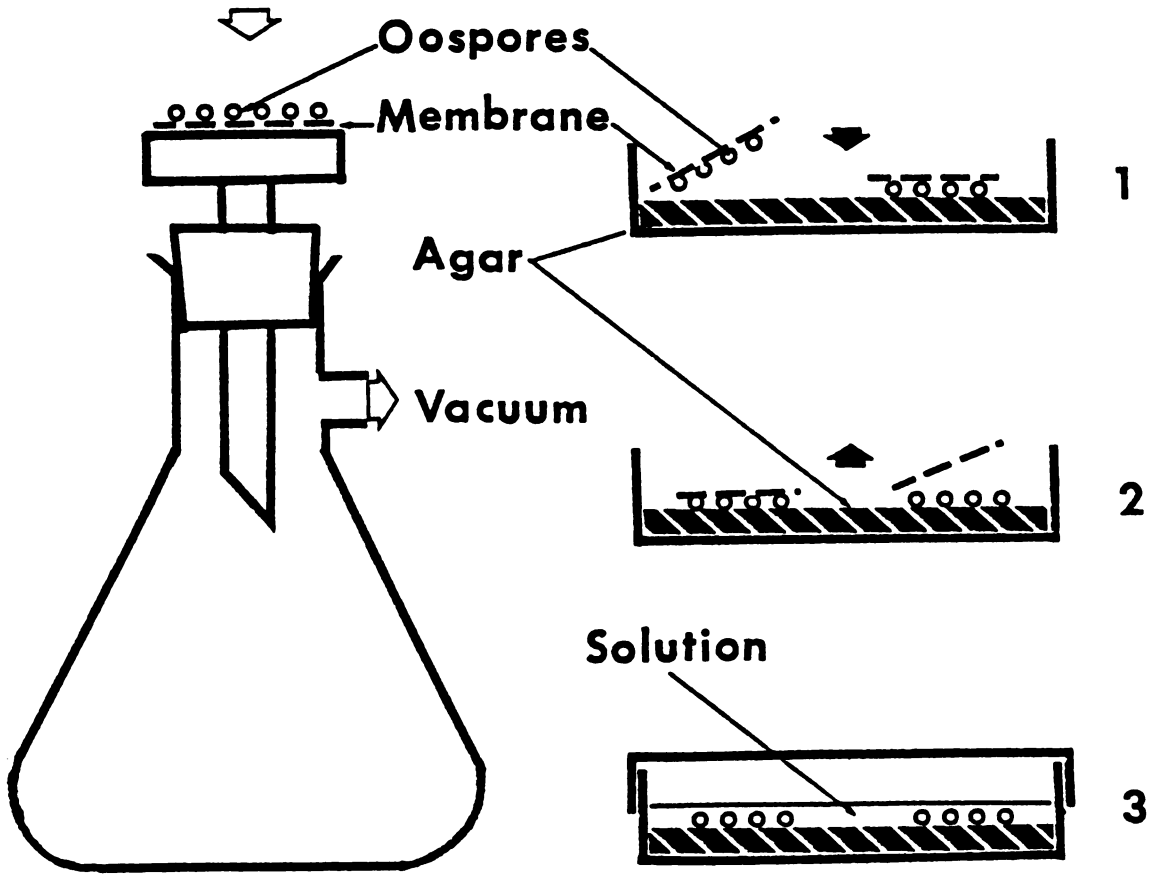



Figure 7. Method of transferring oospores of Phytophthora megasperma var. sojae, for germination studies, using Millipore membranes.

bottom of the plate to facilitate localizing the propagules. The membranes were then aseptically removed leaving the oospores embedded on the agar surface.

Among the substrates (2 ml/dish) used were: a) distilled deionized water, b) tap water, c) soil extracts, and d) sugar solutions. Soil, natural, steamed, or sterile was also used at 1.0-1.5 g/plate. Care was taken to place the soil sample at some distance from the embedded propagules. Germination was determined microscopically after 4-6 days.

Natural soil (0.1-0.2 g/petri dish) was smeared in the bottom of 60 mm diameter 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 by rolling a clean moistened swab over the soil surface until it no longer was difficult to observe the oospores trough the microscope. The plates thus prepared were air-dried on a laboratory bench before adding the oospores (Figure 8, A).

Soil films were prepared by pipetting 1 ml aliquots of an aqueous suspension of field soil into 60 mm diameter glass petri dishes. The plates were air-dried for 2-4 hr, and the peripheral portion of the film was removed with a swab leaving a circular area covered with soil. Swirling the plates with 1 ml of water was sometimes necessary to remove large sand particules. The soil suspension (1%-2%) were prepared with natural soils

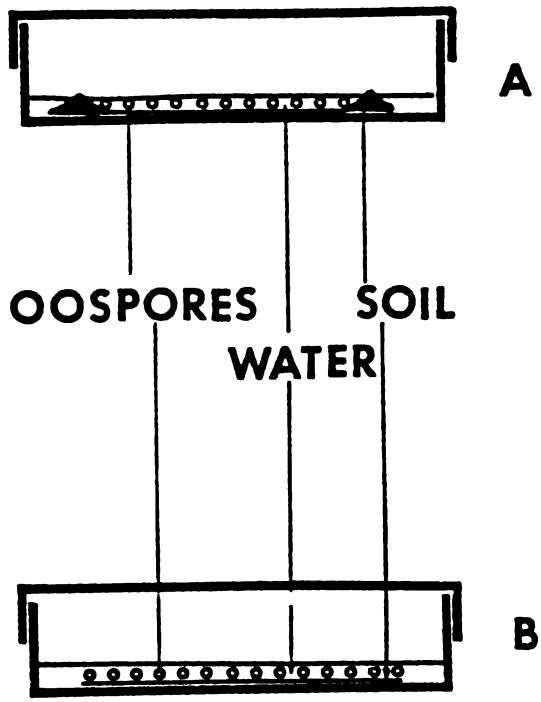



Figure 8. Techniques for the germination of oospores of Phytophthora megasperma var. sojae.
A. Soil smear technique.
B. Soil film technique.

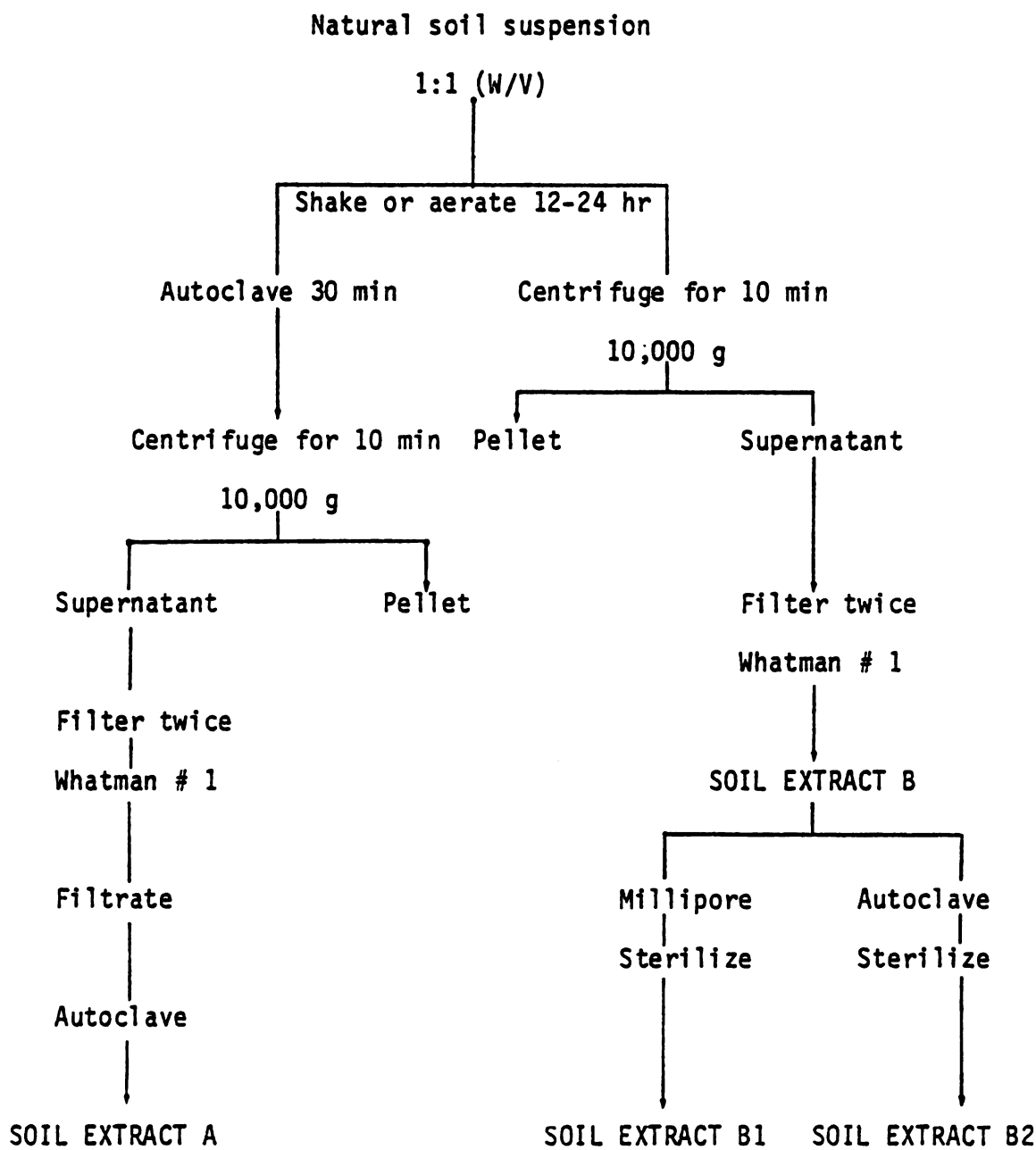
previously mixed for 30 min. in a rotating drum, passed through an 850 μm (20-mesh) sieve, then ground with a mortar and pestle.

Soil films were also prepared from the supernatant liquid of sedimented soil suspensions. Natural soil was suspended in water (1:1, w/v), aerated overnight and sedimented for 30 min. The supernatant was decanted and filtered through two layers of chessecloth. One ml aliquots of this filtrate were dispensed into glass petri dishes which then were air-dried. Plates used as controls in every case, were autoclave-sterilized for 1 hour (Figure 8, B).

Preparation of soil extracts

Soil extracts were prepared according to the procedure described in Figure 9. The soils used to prepare soil extracts were collected from Michigan fields naturally infested with P. megasperma var. sojae. They were stored in polyethylene plastic bags at 5°C. Before use, the soils were thoroughly mixed for 30 min. in a rotating drum, passed through a 2 mm (9-mesh) sieve and air-dried. To prepare a natural soil suspension, 1000 g soil were thoroughly mixed with 1000 ml distilled water. This suspension was then shaken for 12-24 hr. Half of this suspension was used to prepare soil extract A and the other half to prepare soil extract B.

Soil extract A was prepared by autoclaving the soil suspension for 30 min. followed by centrifugation




Soil extract A = Extract of autoclave-sterilized soil suspension, (1 g soil/1 ml Water)

Soil extract B = Extract of natural soil, (Non-sterile).

1 = Extract of natural soil, (Millipore-sterilized).

2 = Extract of natural soil, (Autoclave-sterilized).

Figure 9. Preparation of soil extracts for oospore germination.

at 10,000 X g, for 10 min., then passing the supernatant through Whatman #1 filter paper to remove light-weight particles of organic matter. The filtrate was then autoclaved for 15 minutes.

Soil extract B was prepared by first passing the natural soil suspension through three layers cheesecloth to remove coarse particles, centrifuging the filtrate at 10,000 X g for 10 min., and passing the supernatant twice through Whatman #1 filter paper. Soil extract B was sterilized either by passage through a Millipore filter (0.22 μm pore size) or by autoclaving to obtain soil extract B1 or soil extract B2, respectively. Soil extracts, sterile or not, were kept at 4°C until use.

Preparation of soybean seedlings and root exudates

Soybean seedlings were sterilized by a modified version of the method of Schlub et al. (118), (Figure 10). Dry seeds were placed in capped flasks (500 ml) containing 1-2 ml propylene oxide dispensed in a test tube. Seeds never came in contact with liquid. After 12-14 hr, the caps were replaced with sterile cotton plugs or loose caps of aluminum foil to allow dispersal of propylene oxide. Propylene oxide-treated seeds were further sterilized for two minutes with 5% sodium hypochlorite (NaOCl) and then rinsed copiously with sterile distilled water. Sterilized seeds were germinated in 1% nutrient agar. After 2-4 days, 90%-100% of seedlings were free of contaminant bacteria or fungi. Seedlings were discarded

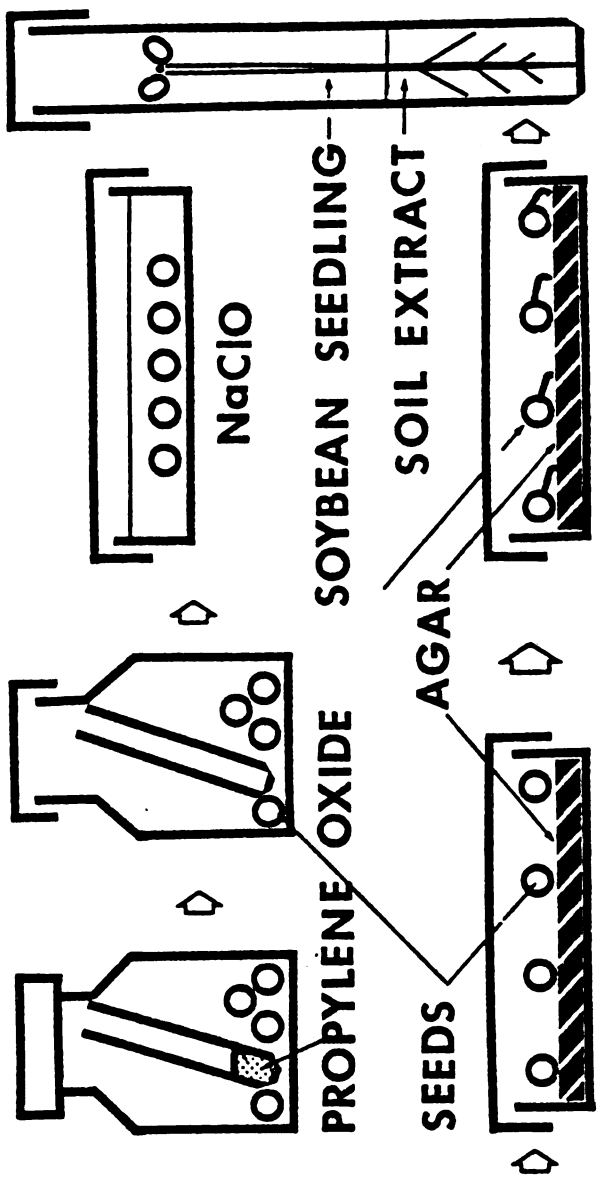



Figure 10. Preparation of aseptic soybean seedlings and root exudates for the germination of Phytophthora megasperma var. sojae oospores.

when contaminated.

To collect root exudates, aseptic 2-4-day-old soybean seedlings were placed in petri plates (5 seedlings/plate) containing 10 ml of an autoclaved extract of natural soil, or in sterile capped 20 ml test tubes (1 or 3 seedlings/tube) containing 4 ml of the same soil extract (soil extract B2, Figure 9). In this case the radicle or each seedling was partially submerged (1/2 to 3/4 of its length). Plants were grown for 5-10 days under continuous light at 4125 lux provided by four Sylvania Gro-Lux WS 40 watt lamps.

The exudates were assayed for sterility by streaking a small amount from a single test tube or plate, on 1% nutrient agar. Contaminated exudates were discarded and sterile exudates were pooled, filtered to remove sloughed plant material, and stored at 5-10$^{o}$C.

Criteria for evaluating germination of oospores

The structural changes occurring during the germination process (13, 33, 147) were observed with a microscope at X100 magnification. After specified incubation intervals, two phases (activation and germination) were recognized and the germinating oospores were classified (7) as follows:

a) Thick walled, dormant oospores with fine granular cytoplasm (no changes were evident after isolation).

b) Activated, thin-walled oospores with coarse granular appearance of the cytoplasm.

c) Germinated oospores with germ tube.

d) Germinated oospores with germ tube bearing sporangia.

These stages represent a sequence, with each being depending upon prior development of the preceding stage. Oospores (300/replicate) were counted in each of three or four replicate petri dishes and the percentages of the different categories were determined. All experiments were repeated at least twice.

Statistical analysis

Data from experiments dealing with oospore germination were transformed to arcsin $\sqrt{X}$, (X= percentage germination) for analysis of variance (134). Treatments in all experiments were replicated at least three times. Significant differences among treatments were estimated by Tukey's w procedure. All experiments were done at least twice to verify the results.

## RESULTS

### Effect of enzyme treatment

Dark-grown cultures of race 1 of P. megasperma var. sojae were processed under dark with or without the enzyme complex beta-glucuronidase/aryl sulfatase (2000 units of enzyme activity/ml) in 0.1 M sodium acetate buffer at pH 5.2. The oospores obtained therefrom were incubated in a sterile extract of natural soil for 5 days in light or in darkness.

Lysing mycelium with the enzyme reduced germination of oospores of P. megasperma var. sojae slightly but did not suppress their ability to respond to fluorescent light (Table 10). In addition, the number of activated oospores was lower with treated oospores than with oospores mechanically isolated. These results seem to indicate that the enzyme treatment affected the pregermination stages.

### Germination of oospores in soil

To study germination in natural soil, the soil-film technique was used. Dark-grown oospores from 26-day old cultures of P. megasperma var. sojae race 1, isolated with the enzyme mixture beta-glucuronidase/aryl

Table 10. Effect of enzyme treatment and light regime on the germination of oospores of Phytophthora megasperma var. sojae.

| Light[a] | Enzyme[c] | Germination, %[b] Activated | Total |
|---|---|---|---|
| + | + | 3.3 A | 61.9 A |
| + | - | 18.4 B | 70.3 B |
| - | + | 4.2 A | 18.1 D |
| - | - | 7.0 A | 24.3 C |

[a]The light source consisted of four ITT 40/CW fluorescent lamps suspended 50 cm above the cultures (4125 lux).

[b]Values represent the average of 3 replicates with 300 oospores/replicate, including activated oospores ($P = 0.05$).

[c]Beta-glucuronidase/aryl sulfatase in sodium acetate buffer, pH 5.2.

sulfatase, were germinated in sterile or non-sterile soil (20 mg soil/plate). Oospores placed to germinate in deionized sterilized water were used as a control (Table 11).

In spite of the fact that some oospores were parasitized, natural soil supported almost as much germination (70.4%) as sterile soil (77.4%). High numbers of oospores with sporangia were found in plates containing either sterile (31.6%) or non-sterile soil (38.4%). The number of activated oospores was also high

Table 11. Germination of oospores of Phytophthora megasperma var. sojae in soil

| Substrate | mg/ml | Germination, %[a] Activated | With germ tube | With sporangia | Total |
|---|---|---|---|---|---|
| Water | | 24.9 A | 4.1 A | 5.0 A | 34.0 A |
| Sterile soil | 20 | 18.6 A | 27.2 B | 31.6 B | 77.4 B |
| Natural soil[b] | 20 | 23.2 A | 8.8 A | 38.4 B | 70.4 C |

[a]Means in the same column followed by the same letter are not significantly different ($P = 0.05$). Values represent the average of 3 replications with 300 oospores/replication.

[b]Parasitized oospores were included among those non-germinated.

in both natural or sterile soil, and did not differ significantly. However, more oospores with germ tubes were found in sterile soil (27,2%) than in water (4.1%) or natural soil (8.8%).

Another experiment was done to compare germination of oospores of isolates of races 1, 3, and 9 of P. megasperma var. sojae in soil smears. In sterile soil, races 1, 3, and 9 germinated 70.3%, 40.2%, and 59.5%, respectively. In natural soil these isolates germinated (in the same order) 73.2%, 38.3%, and 57.2%. There were no differences between soil conditions in total germination (all stages), but differences ($P = 0.05$) among isolates were significant.

Since only one experiment was performed and only one type of soil was used (Capac loam), no definite conclusion can be drawn as to whether or not there is a specific germination response of the isolates in a particular soil, or if the relative germinability of the different races of the pathogen is consistent in different soils.

## Germination of oospores in extract of natural soil

Oospores of P. megasperma var. sojae isolated with the enzyme mixture beta-glucuronidase/aryl sulfatase from 40-day-old cultures were germinated in glass petri plates containing 1.5 ml of natural soil extract (extracts B and B2, Figure 9). Sterile deionized water was used as a control.

Non-sterile extract of natural soil was found to be more stimulatory than sterile extract for inducing germ tube formation and sporangia production by germinating oospores (Table 12). Total germination (all stages) was 57.6% in the non-sterile extract and 39% in the sterile extract. The total germination percentage (33.9%) in the control was given almost exclusively by oospores that underwent activation (30.7%). The low number of activated oospores in sterile (6.6%) or non-sterile (3.7%) extract of natural soil reflects a high rate of change to the germ tube stage, 32.3% and 44.3%, respectively. By contracts only 3.2% of the oospores reached this stage in water.

Time course of oospore germination

To determine the time course of germination, an aqueous suspension of dark-grown oospores of P. megasperma var. sojae was incubated under light in petri dishes containing sterile soils films. Germination stages were followed for twelve days at two-day intervals (Figure 11).

The number of oospores in the different germination stages increased as the time of incubation increased up to the 12th day (the duration of this experiment). After two days, the number of oospores with germ tubes lagged behind the total number of activated oospores and the number producing sporangia lagged behind those that

Table 12. Germination of oospores of Phytophthora megasperma var. sojae in soil extracts

| Substrate | Germination, %[a] | | | |
|---|---|---|---|---|
| | Activated | With germ tube | With sporangia | Total |
| Water | 30.7 A | 3.2 A | 0.0 A | 33.9 A |
| Extract of Natural soil:[b] | | | | |
| Sterile (B2) | 6.6 B | 32.3 B | 0.1 A | 39.0 B |
| Non-sterile (B) | 3.7 B | 44.3 C | 9.6 B | 57.6 C |

[a]Means in the same column followed by the same letter are not significantly different ($P = 0.05$)

[b]See Figure 9 for the preparation of soil extracts.

had produced a germ tube. By the 12th day, total activation (sum of all stages) had increased to ca. 70%. Since germination had not leveled off after 12 days, it could probably be expected that after a longer period of time almost all, if not all, viable oospores would have germinated.

Effect of substrate concentration on oospore germination

To study the effect of substrate concentration on the germination of oospores of Phytophthora megasperma var. sojae race 1, one ml of the following solutions containing ca. $10^4$ axenic oospores/ml were separately added to sand films: i) deionized water, ii) extract of

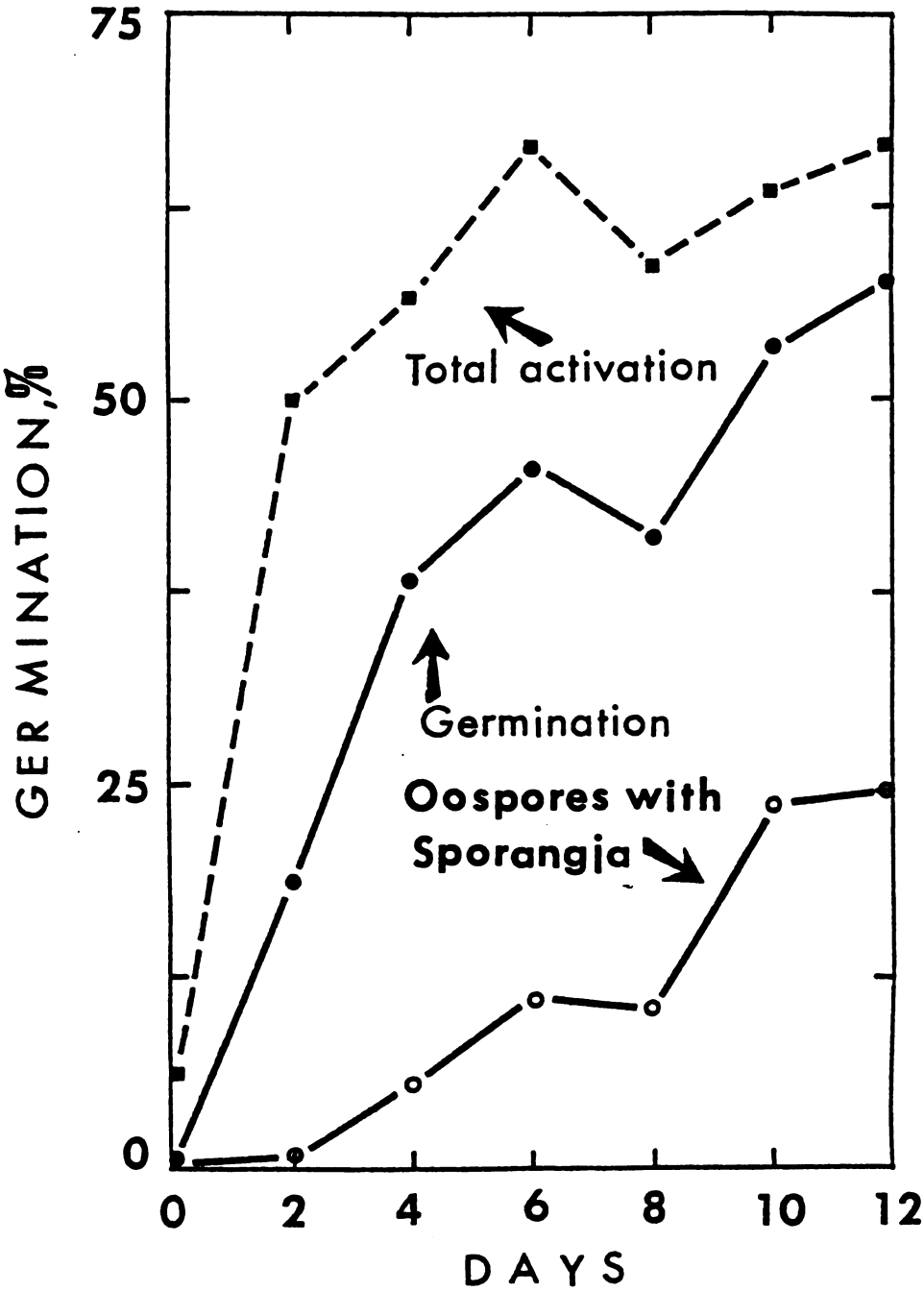



Figure 11. Time course of germination stages of Phytophthora megasperma var. sojae incubated in sterile soil films. Total activation refers to the sum of all oospores producing a germ tube including those which had formed sporangia; germination refers to those that had produced a germ tube but no sporangia; and oospores with sporangia refers only to those which had formed sporangia.

autoclaved soil (extract A, Figure 9) at concentrations of 1:1 and 1:10, iii) extract of natural soil (extract B2, Figure 9), and iv) glucuse at 0.5 mg/ml and 0.005 mg/ml.

Concentrated substrated (extract of autoclaved soil, 1:1 and glucose at 0.5 mg/ml) were inhibitory to oospores germination (Table 13). Oospore germination was reduced to 7.8% by a concentrated extract of autoclaved soil as compared to 52.6% with 1:10 dilution of the same extract. Glucose at 0.5 mg/ml was less inhibitory than the concentrated extract of autoclaved soil, but reduced germination to 30.1% as compared to 56.1% with 0.005 mg/ml. Similar germination (48.2-53.5%) was obtained in water, 1:10 dilution of extract of autoclaved soil, and in (1:1, w/v) extract of natural soil.

Effect of light on oospore germination

To test the effect of light on the germination of *P. megasperma* var. *sojae*, five-week-old oospores were produced under darkness and isolated with the enzyme complex beta-glucuronidase/aryl sulfatase. One ml aliquots of an oospore suspension ($10^4$ oospores/ml) were dispensed into 60 mm diam. glass petri plates each containing autoclave-sterilized films of soil (0.01 g soil/plate). Plates thus prepared were covered with plastic petri dish tops, sealed with Parafilm and exposed to the following light regimes: i) 6 days under darkness, ii) 6 days under

Table 13. Effect of substrate concentration on the germination of oospores of Phytophthora megasperma var. sojae.

| Substrate[a] | Concentration | Germination, %[b] |
|---|---|---|
| Water | | 48.2 B |
| Extract of autoclaved soil (A) | 1:1 | 7.8 D |
| | 1:10 | 52.6 AB |
| Extract of natural soil (B2) | 1:1 | 53.5 AB |
| Glucose | 0.5 mg/ml | 30.1 C |
| | 0.005 mg/ml | 56.1 A |

[a]Substrates were added to autoclave-sterilized films of sand. See Figure 9 for the preparation of soil extracts A and B2.

[b]Values represent the average of three replicates with 300 oospores/replicate. Means followed by the same letter are not significantly different ($P = 0.05$).

continuous light, iii) 4 days under darkness followed by two days under light, and iv) 4 days under light followed by two days under darkness (Table 14).

More oospores in the activated stage were found under light regime number three than in any other treatment. Continuous fluorescent light was stimulatory to germ tube formation, and more germ tubes were produced with longer exposure to light. The number of oospores with germ tubes produced under 0, 2, 4, and 6 days exposure to light was 7.5%, 15.9%, 17.2%, and 32.0%

Table 14. Effect of fluorescent light on the germination stages of oospores of Phytophthora megasperma var. sojae.

| Light regime[b] | Germination, %[a] | | | |
|---|---|---|---|---|
| | Activated | With germ tube | With sporangia | |
| 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 column followed by the same letter are not significantly different ($P = 0.05$). Values represent the average of three replications with 300 oospores/replication.

[b]The light source consisted of four cool-white fluorescent lamps which provided 4125 lux.

respectively.

The production of sporangia, however, was inhibited by continuous light. Under light only 5.8% of the germinated oospores produced sporangia as compared to 22.5% under continuous darkness, or 27.4% under four days of light followed by two days under darkness. Finally, there was a stimulatory effect of light on the total percentage of germinating oospores. About 50% of the oospores were germinated (activated oospores plus oospores with germ tubes plus oospores with germ tubes bearing sporangia) in those treatments which included an exposure to light, as compared to only 34.7% under continuous darkness.

Effect of light and darkness on germination of oospores in soil

Oospores from cultures 15 days old (grown for 10 days under dark plus 5 days under fluorescent light) were used to test the effect of light and soil condition (sterile or non-sterile) on the germination stages of P. megasperma var. sojae, race 9 (Table 15).

The effect of light on the germination stages of oospores incubated in natural or sterile soil was similar. For example, there were as many oospores with germ tubes under light in sterile soil (18%) as in natural soil (19%), and similar number of oospores with sporangia were counted in natural soil (19.4%) and in sterile soil (24.1%) under darkness. However, oospores placed under

Table 15. Effect of light and darkness on germination of oospores of Phytophthora megasperma var. sojae in the presence of natural or sterile soil.

| | Germination, %[a] | | | |
|---|---|---|---|---|
| | Sterile soil | | Natural soil | |
| | Light | Dark | Light | Dark |
| Activated | 34.0 A | 16.0 B | 35.6 A | 19.5 B |
| W/germ tube | 18.0 A | 4.7 B | 19.0 A | 12.1 C |
| W/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 row followed by the same letter are not significantly different ($P = 0.05$). Values represent the average of 3 replication with 300 oospores each.

darkness in natural soil had more germ tubes (12.1%) than those under darkness in sterile soil (4.7%); a large number had moved on to sporangia formation.

The production of sporangia, as previously shown under "Effect of light on germination," was also inhibited by light in these experiments. For example, there were more oospores with sporangia in sterile soil under dark (24.1%) than under light (2.2%), and in natural soil under dark (19.4%) than under light (1.1%).

Effect of soybean seedlings on oospore germination

Experiments were done to test the effect of soybean seedlings on germination of oospores in light and in darkness using extract of natural soil Figure 9, B2) as the substrate. The oospores were obtained from cultures 40 days of age, grown and extracted under darkness.

Presence of seedlings greatly enhanced germination of oospores at all stages. Whereas the results previously reported were obtained 5-7 days after exposure of the propagules to a particular treatment, the data on germination of oospores of race 9 of P. megasperma var. sojae in these experiments were taken after only 36 hr incubation (Table 16).

Soybean seedlings increased total oospore germination under fluorescent light as well as under darkness. However, in the presence of seedlings germination in light exceeded that in darkness. The change fron the activated stage to the production of sporangia was greater and faster with seedlings than without seedlings. When seedlings were present, activation and germ tube production were similar under light or darkness. Although the number of sporangia was higher under light than under darkness, 18% had produced sporangia under darkness. This behavior seems to indicate that soybean exudates reverse the inhibitory effect of light on sporangia production.

Table 16. Germination of oospores of *Phytophthora megasperma* var. *sojae* in the presence of soybean seedlings.

| | Germination, %[a] | | | |
|---|---|---|---|---|
| | With seedlings[b] | | Withour seedlings | |
| Stage | Light[c] | Dark | Light | Dark |
| Activated | 15.0 A | 15.0 A | 30.4 B | 33.3 B |
| W/germ tube | 16.7 A | 12.3 A | 8.5 B | 1.0 C |
| W/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 row followed by the same letter are not significantly different ($P = 0.05$). Values represent the average of three replications, with 300 oospores/replication.

[b]Three-day-old aseptic soybean seedlings (2/ replicate).

[c]4125 lux, provided by four cool white fluorescent lamps (ITT-F40/CW).

Additional experiments were done to determine the effects of wounded soybean and navy bean (*Phaseolus vulgaris*) tissue on germination of oospores. Soybean stem and leaf pieces, and navy bean stem tissue separately incubated with oospores in an extract of natural soil increased their germination. Stimulation of germ tube growth was sufficient to induce the development of small mycelial colonies.

An experiment was also done to determine the

effect of soybean seedlings on sporangia produced by germinated oospores in natural soil. Larger numbers of zoospores were released by sporangia in the presence of soybean seedlings than in their absence.

Effect of temperature on oospore germination

To study the effect of temperature on germination, oospores from 19-day-old cultures were incubated under darkness in soybean root exudates at 16, 20, 24, and 28°C. The number of oospores at different stages of germination was recorded after four days incubation.

Oospores of P. megasperma var. sojae germinated over a temperature range of 16 to 28°C, with an optimum at 20-24°C (Figure 12). All stages of germination were similarly affected by temperature. The number of oospores in each stage increased as the temperature increased from 16°C to 20-24°C, and then declined at a higher temperature (28°C).

The fact that some oospores germinated at relatively low temperatures (16°C) suggests that secondary inoculum could be produced early during the growing season when low temperature prevail and young susceptible host tissue is available.

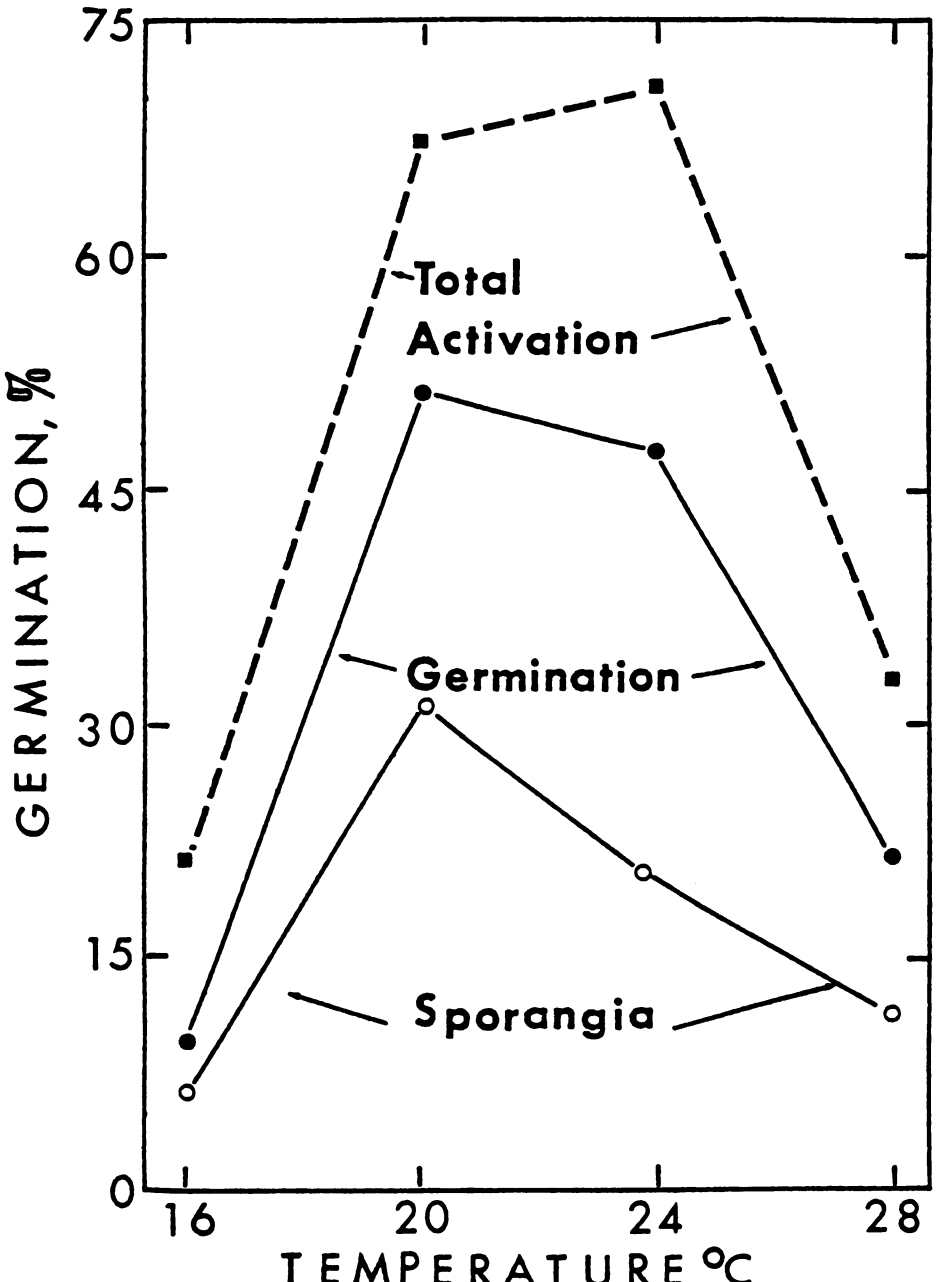



Figure 12. Effect of temperature on germination of oospores of Phytophthora megasperma var. sojae. Least significant ranges (Tukey's w procedure, P= 0.05) were 16.0, 26.4, 20.2 respectively for sporangia, germination, and total activation. Total activation refers to the sum of all germination stages, germination refers to all oospores producing a germ tube (including those which had formed sporangia), and sporangia refers to those which had produced sporangia.

## DISCUSSION

The study of oospore germination in soil has proven to be difficult nor only because the opacity of this substrate, which forced the use of brighteners (139) and immunofluorescent techniques (90), but also because of the inconsistent and often very low germination of natural as well as artificially produced inoculum. The use of thin films of soil or extracts of natural soil in this study, overcame the difficulties encountered in microscopic observation, and avoided addition of extraneous materials (139) which may complicate the interpretation of results.

Through this research it has been shown that: i) soil constitutes a more suitable substrate than water for the germination of the oospore of Phytophthora megasperma var. sojae, ii) Oospore germination was not subject to soil fungistasis, but was inhibited by certain concentrations of glucose and soil extract, iii) Germination of a population of oospores extends over several days, iv) Light stimulated germination of oospores in natural or sterile soil. However, relatively high numbers of oospores germinated and produced sporangia under darkness. Production of sporangia was inhibited by light, but this effect was

reversed by soybean seedlings. v) Soybean seedlings induce high and rapid germination of oospores of Phytophthora megasperma var. sojae; however, this stimulatory effect was not specific to soybeans or restristed to the root system. vi) The optimum temperature for oospore germination was found to be 20-24°C.

High percentages of germination were found when soil (natural or sterile) was used as compared with deionized water. In particular, the transformation of oospores from the activated stage to the formation of sporangia was low in water but high in soil. Consequently, soil supplied some kind of stimulus (which could be a mineral nutrient) required for germination. Low germination (37.1% of P. megasperma var. sojae oospores in water has been previously reported (139) and in a case (33) in which high germination occurred (77%) the oospores were probably contaminated with culture media. The equally high germination in natural and in sterile soil suggests not only a mineral requirement but perhaps a response to an environment with a low nutrient status resulting from competition. Germination might have been even higher in natural soil if parasites had not been present.

The germination of oospores incubated in soil films was not arrested by the fungistatic properties of natural soil (76), and the flooded conditions probably

served as a vehicle for a faster and more efficient diffusion (42) of appropriate concentrations of specific nutrients, either minerals, carbon sources, or perhaps metabolites produced by soil microflora.

Sporangia produced in natural soil seldom germinated but remained intact and did nor release zoospores. Though observations were made for only ten days, infrequent direct or indirect germination occurred. This persistence of the sporangia may indicate that they are more sensitive to fungistasis than oospores. The significance of this response fo the pathogen deserves further study. It could serve for example as a mechanism for building up the production of secondary inoculum or perhaps to extend the survival of the pathogen under conditions favorable for germination, such as high moisture levels or flooded conditions in soil.

The germination of oospores in soil extends over several days and it could be expected that prolonged incubation may result in germination percentages close to 100% (assuming 100% viability). This extended germination in time has been attributed to a normal delay of gametic nuclear fusion and to environmental conditions (17). It would be of interest to determine the variation in age of a spore population, which could add information on the lack of synchrony of germination.

Light stimulated germination of P. megasperma var. sojae oospores incubated in sterile and in natural

soil. However, relatively high numbers of oospores also germinated and produced sporangia under darkness. Comparable results in water agar also incubated under darkness have been previously obtained (99, 101). The fact that oospores produced, isolated, and incubated in natural soil under darkness, were able to germinate, sheds some doubts on the importance of light for germination of oospores under field conditions.

Banihashemi and Mitchell (7) reported that production of germ tubes and sporangia by oospores of P. cactorum was not light dependent. Germ tube formation by germinating oospores of P. megasperma var. sojae was stimulated and sporangia production was inhibited under continuous light in my work. Light inhibition of sporangia formation by oospores confirms previous results showing the production of larger numbers of zoospores of this fungus on lima bean agar plates in darkness than in light (35).

The inhibitory effect of beta-glucuronidase/aryl sulfatase on germination of oospores of P. megasperma var. sojae, though statistically significant, was slight. However, Long et al. (88) reported deletereous effects on germination, but presented no data. The apparently lesser effect of these enzymes in my work may have been because the oospores were incubated in soil extract. This substrate also has been successfully used to germinate P. cactorum oospores (7). The enzyme apparently inhibited

activation of oospores, but it may also have resulted in an increased rate of change to other stages (germ tube formation and sporangia development). Increased rates of germination were observed with young oospores of P. cactorum treated with the enzyme complex Glusulase (7).

The optimum temperature for germination of oospores of P. megasperma var. sojae was found to be at 20-24°C, which almost coincided with the optimum (25°C) for mycelial growth (56). This behavior departs from that of P. cactorum whose optimum was 28°C for mycelial growth and 22°C for oospore germination (7) but coincided with P. drechsleri which has an optimum at or near 24°C for germination of oospores (75).

The fact that some oospores of P. megasperma var. sojae were capable of germinating at relatively low (16°C) and high (28°C) temperatures suggests an ecological adaptation of the fungus to produce infective inoculum throughout the growing season. In addition, successful host infection presumably assures high numbers of overwintering propagules in soil.

Direct evidence of the effect of host plants on germination of oospores of Phytophthora species has not been published. However, studies have indicated a positive host effect on the germination of Pythium oospores (8, 131, 133).

Soybean seedlings stimulated germination of oospores of P. megasperma var. sojae incubated in an

extract of natural soil either under light or under darkness. Oospores previously exposed to light or dark-grown oospores incubated under light or under darkness in the presence of seedlings were greatly stimulated to form germ tubes and sporangia in a short period of time (36 hr). All germination stages were positively affected by seedlings whether under darkness or under light. Moreover, light inhibition or sporangia formation was completely reversed by the presence of soybeans, and the stimulatory effect of light on oospore germination was partially replaced by seedlings when these were incubated with oospores under darkness.

Experiments remain to be done to more precisely define the role of soybean plants and light on the germination of oospores. For example, germination of dark-grown oospores should be tested with seedlings in natural soil incubated under darkness. Also, the conditions for zoospore release from sporangia in natural soil are not known. Possibly seedling exudates, or a carbon source added to sporangia produced by oospores in natural soil may stimulate zoospore release. In one experiment, zoospores were released in the presence of soybean seedlings.

The inhibitory effect of light on the formation of sporangia by germinating oospores of P. megasperma var. sojae and its reversibility by soybean root exudates apparently has never been reported before. In

addition, this is probably the first reported direct evidence on the germination of oospores of this pathogen in natural soil in the absence of host plants.

Stimulation of oospores of P. megasperma var. sojae to germinate in response to soybean exudates was not specific to soybean or restricted to the roots. It is not surprising that soybean stem and leaf pieces stimulated oospore germination since these tissues are normally infested by the pathogen under field (95) or greenhouse (68) conditions. The response of the oospores to germinate when intact seedlings, or wounded host or non-host tissue was present is similar to what occurs in other host-pathogen systems (120).

Inhibition of P. megasperma var. sojae oospore germination by glucose at 0.5 mg/ml was similar to that caused by the same sugar and by sucrose on germination of oospores of P. cactorum (7). P. megasperma var. sojae oospores were also inhibited by a concentrated extract of autoclaved soil suspension. The fact that a concentrated extract was also inhibitory while a dilute solution of the extract was not, and that a natural soil extract supported as good germination as 0.005 mg glucose/ml, suggests that germination of oospores is suppressed by high nutrient concentrations. Inhibition of germination by excess nutrient has also been shown for Glomus mosseae spores (24). Germination of oospores of

this mycorrhizal fungus was inhibited on agar media rich in nutrients and in water agar amended with dialysates from air-dry, autoclaved or chloropicrin-treated soils. By contrast, agar containing dialysates from natural soil or low concentrations of nutrients stimulated germination. The extract of autoclaved soil is considered a rich energy source as compared to extract of natural soil (76).

Substrate inhibition of oospore germination may be a mechanism which prevents oospores formed in host tissue from germinating readily in situ. When these propagules are released to soil by microbial degradation of plant tissue they apparently become prone to germinate especially under high moisture conditions and at very low nutrient concentrations. However, more research is needed to support this hypothesis.

Mechanisms such as constitutive dormancy, thick oospore walls, fungistasis through host tissue protection or inhibition, etc., offer plausible explanations for long term survival of ungerminated oospores. However, since oospores of Phytophthora megasperma var. sojae will germinate in soil in the absence of the host, this behavior would seem to be detrimental to survival. Consequently, special and perhaps subtle compensatory strategies must have evolved in order for this pathogen to avoid extinction.

P. megasperma var. sojae could possibly use

several means to cope with the threat of dying out in the absence of soybeans under circumstances conducive to oospore germination. Perhaps sporangia are strategic units for survival under flooded conditions or in soil high in moisture. Persistence of zoospore cysts, and mycelium are also reasonable possibilities for short term survival.

Production of secondary sporangia and zoospores in soil may be additional means of persistence. A secondary sporangium is defined as a sporangium produced by a germinated zoospore cyst; secondary zoospores are those produced by secondary sporangia. Under temperatures non-limiting for germination, zoospores released into the soil encyst and then germinate by a germ tube which may terminate in a small sporangium. At least some of these sporangia later release zoospores. Direct or indirect germination of sporangia and zoospore cysts is possible when metabolizable carbon sources, for example root exudates, become available to them.

Structures other than oospores are presumably vulnerable to desiccation as the soil dries. However, the presence of soybeans would assure survival of the pathogen and support the production of additional primary and secondary inoculum. Assuming oospores to be the only overwintering propagules, any of the means for short term survival discussed earlier could explain the fact that natural soil infested with P. megasperma var. sojae

caused 80%-100% infection of susceptible soybean seedlings after two months incubation of the soil under flooded conditions at 24°C (See Part I).

# APPENDIX

# APPENDIX

## GERMINATION OF OOSPORES OF PHYTOPHTHORA MEGASPERMA VAR. SOJAE IN SOIL

The observation of events occurring during germination of oospores was done under the light microscope (at X100) by placing them to germinate in soil films, soil smears, or in soybean root exudates in 60 mm diam. petri plates (See Parts I and II).

The germination process for this fungus has been partially described (33) and similar observations were reported for Phytophthora drechsleri (147) and for P. cactorum (13) in culture media. The developmental stages prior to that of dormancy (Figure 13. A, B, and C) have been described (13, 147) and need not be repeated.

In the dormant stage, the oospores are thick-walled and have a fine-textured cytoplasn in which one or two nuclei (=pellucid bodies) appear at the outside of a central to subcentral reserve body (=ooplast) (Figure 13. D). The oospore usually fills the oogonial cavity and its color varies from yellow to brown to dark-brown, apparently depending upon age and nature of substrate. Dark to dark-brown oospores are associated with naturally or artificially infested soybean root tissue.

Figure 13. Germination of Phytophthora megasperma var. sojae in soil. A, B, and C developmental stages prior to that of dormancy (D). E, activation stage; F and G, germ tube formation (= germination); H, sporangia development; I, abortive oospore; J, abortive sporangium; K, indirect germination of sporangia; L, encysted zoospore; M, zoospore cyst germination; N, secondary sporangium; O, indirect germination of secondary sporangium; P, extended proliferation of the sporangiophore; Q, direct germination of sporangium; R, formation of new sporangium by direct germination; S, zoospore germination and repetitive germination of the sporangium.

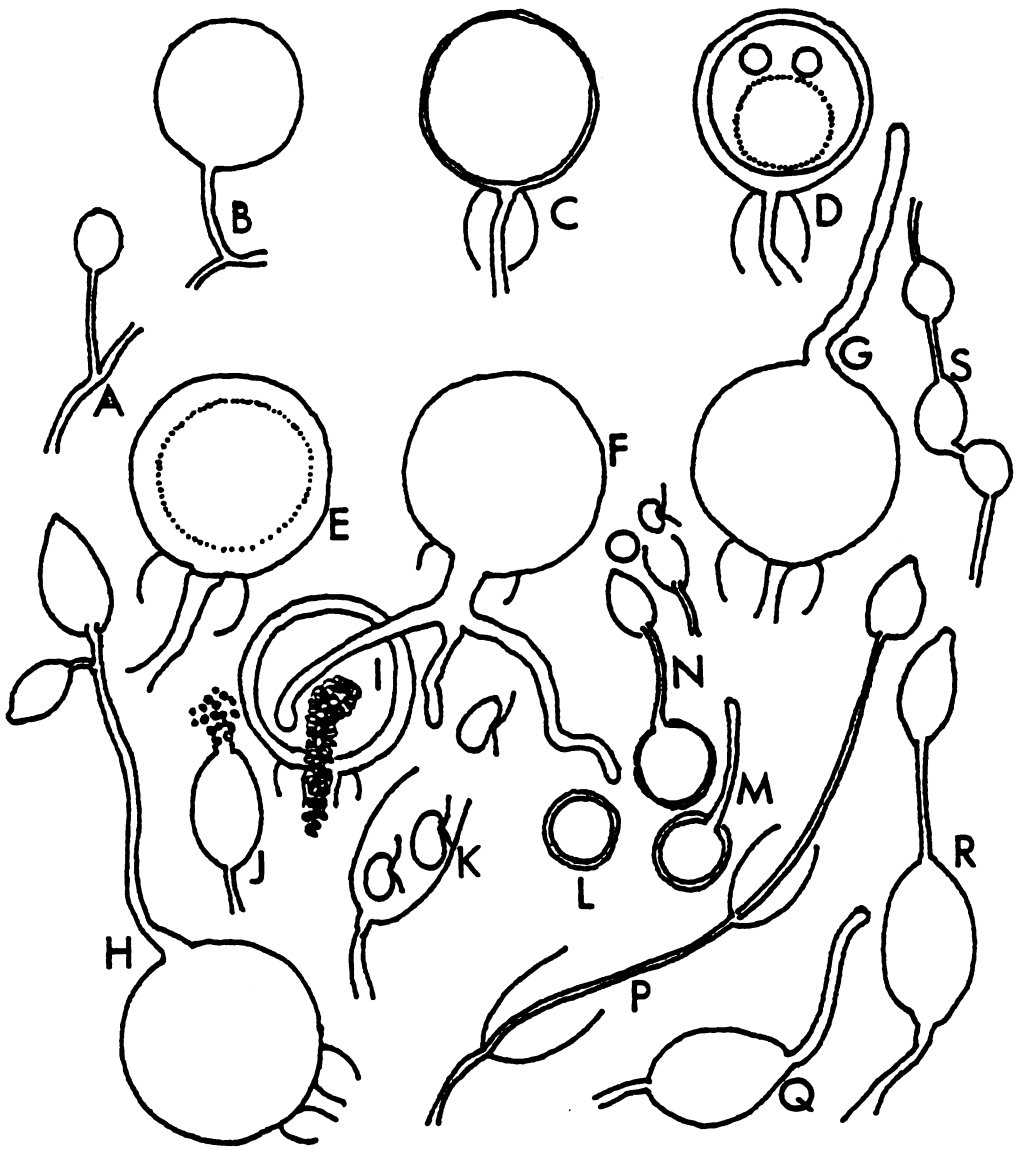



Figure 13

The germination process may conveniently be studied by assigning to it the following stages in order of development: i) activation stage, ii) germ tube formation, and iii) sporangia development. The protrusion of the germ tube has been used as criterion for germination studies (1, 136).

During the activation stage or "active pregermination stage" (33, 147) the central reserve body enlarges and desintegrates, the cytoplasm turns coarser in texture and the pellucid bodies fuse and "disappear" (are no longer discernible under the light microscope). Absorption of the inner layer, and consequent thinning of the oospore wall, also occuring at this stage, is a clear indication that germ tube formation (germination) is about to occur (Figure 13. E). At this stage swelling of the oospore takes place, probably by imbibition of water, nearly filling the oogonium.

The formation and protrusion of the germ tube occurs more frequently through the base (proximal end) of the oospore (antheridium-oogonium connection), but emergence at other sites is not uncommon (Figure 13. F and G).

After germination the contours of the disintegrating reserve body may still be visible. The cytoplasm flows into the germ tube as it enlarges and the development of the sporangium ensues. Oospores usually germinate by producing a single germ tube, but

this often branches giving rise to two or three (Figure 13. F). The germ tube presumably depending on the availability of nutrients, continues to grow or forms a sporangium. Close to the terminal sporangium a smaller sporangium usually develops (Figure 13. H).

As germination progresses the cytoplasm originally present in the oospore flows into the maturing sporangium where zoospores start to differentiate. The empty oospore remains attached to the sporangium, and if in natural soil, the "oospore shell" serves as a food base for soil microosganism. In these conditions the germ tube may also lyse after a period of time.

Mature zoosporangia germinate either directly by producing a germ tube or indirectly by releasing a variable number of zoospores. Direct germination (Figure 13. Q), gave rise after formation of a germ tube, to a second smaller sporangium (Figure 13. R), which may or may not germinate directly. These structures can easily be confused with hyphal swellings.

Indirect germination of sporangia, i.e., by differentiation and release of zoospores was the most frequently observed germination mode (Figure 13. K). In addition, extended proliferation of the sporangiophore (14) was also noticed (Figure 13. P). In this condition, after release of zoospores, the sporangiophore resumes growth inside the empty terminal sporangium and shortly after, a new but smaller sporangium develops. This in

turn releases zoospores.

Released zoospores (Figure 13. K), if not disturbed, swim for several hours. Encystment (Figure 13. L) is followed by germination (Figure 13. M), most often by a single germ tube. A germinated zoospore may form a small secondary sporangium (Figure 13. N) which eventually germinates indirectly releasing from one to three zoospores (Figure 13. O). Secondary sporangia may also germinate directly (Figure 13. S), somewhat replicating the pattern described for direct germination of sporangia originating from the oospores (Figure 13. Q and R).

Finally, abortive oospores and sporangia were observed (Figure 13. I and J), respectively. Protrusion of the oospore contents without germ tube formation was evident before and after thinning of the wall; similarly some mature sporangia released the entire protoplasm before zoospore development.

## PRODUCTION OF OOSPORES ON MILLET SEEDS

Millet (Setaria italica (L.) Beauv.) seeds (20-25 g) were water-soaked in 250 ml erlenmeyer flasks for 10-12 hr. The excess water was then decanted and the seeds were autoclave-sterilized for 30 minutes. Sterile seeds were inoculated with 5-10 ml of an axenic zoospore suspension (ca. $10^3$ zoospores/ml) and incubated at 24°C for 2-4 weeks.

To isolate oospores grown on millet seeds, 50 ml of sterile distilled water were added and the flasks shaken on a reciprocal shaker for 20-30 minutes. The liquid containing oospores, mycelium and sloughed seed material was decanted and passed through a sieve (74 µm meshes). The filtrate was centrifuged 3-5 times at 320 X g for 15 seconds each. Particles (mostly starch) that sedimented with the oospores were removed by passing the suspension through a finer sieve (28 µm meshes) which retained the oospores. These were transferred to vials containing distilled water and stored at 5°C.

Large numbers of oospores of Phytophthora megasperma var. sojae were produced on millet seeds. This method appears suitable for producing large number of propagules for germination studies, or for pathogenicity tests, as well as for inoculum for other

purposes. If care is taken to avoid contamination, after oospore extraction a second or a third crop of oospores was produced on the same seeds, without reinoculation.

Oospores produced on millet seeds germinated in flooded water agar as well as those produced in V-8 juice. The form and size of oospores produced on millet seeds varied more than those produced in liquid medium. This may have been a consequence of their production primarily on the surface of the seeds.

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