

## Factors Affecting Zoospore Production by *Phytophthora megasperma* var. *sojae*

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### ABSTRACT

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Cultures of *Phytophthora megasperma* var. *sojae* grown on lima bean agar in petri dishes were flooded with distilled water to induce formation of zoospores. Maximum numbers of  $10^4$ - $10^5$  zoospores/ml (15 ml of suspension per plate) were

*Additional key words:* soybean.

produced when 6-day-old cultures of isolates of races 1 to 6 received five changes of distilled water each at 30-min intervals, followed by incubation in darkness at 20 C for at least 8 hr.

*Phytophthora* root and stem rot of soybean, which is caused by *Phytophthora megasperma* (Drechs.) var. *sojae* Hildeb., is a widespread pathogen in the soybean-producing areas of the USA and Canada, where it may cause severe losses. Testing of the pathogenicity of isolates of the pathogen and evaluation of host resistance have been accomplished by placing mycelia in wounds made in hypocotyls of young plants (7). However, this method involves the use of atypical inoculum and an unnatural infection court. Moreover, it cannot be used to study environmental influences on the infection process, nor does it detect 'field tolerance' which differs from the race-specific resistance so far used in breeding resistant cultivars (10, 14). In nature, the inoculum consists of zoospores derived from germinated oospores (4) which infect the roots or hypocotyls below ground.

The use of zoospores in inoculation procedures requires that they be produced reliably and in adequate numbers. Zoospores of *P. megasperma* var. *sojae* have been produced after placing mycelial fragments in water or salt solutions (4, 5, 6, 11), but apparently no systematic studies of factors that may affect zoospore production have been made. Our purpose was to evaluate several factors that may affect zoospore production in *P. megasperma* var. *sojae* in order to consistently produce large numbers of zoospores of this pathogen.

### MATERIALS AND METHODS

**Maintenance of the fungus.**—*Phytophthora megasperma* var. *sojae* races 1, 2, 3, 4, 5, and 6 were used. Isolates of races 1 to 5 were obtained from A. F. Schmitthenner, and isolates of races 1, 2, and 6 were obtained from J. H. Haas. The isolates were maintained on cleared V-8 juice agar slants (per liter: 200 ml V-8 juice, 3.0 g CaCO<sub>3</sub>, and 20 g agar). In order to maintain

pathogenicity, new cultures were produced every 2-3 mo by placing soybean seedlings (cultivar 'Hark') on flooded cultures of the fungus on Difco lima bean agar, and 3 days later reisolating the fungus on a selective medium (12).

**Zoospore production.**—Mycelial disks (5 mm diameter) cut from the periphery of 4- to 6-day-old cultures on lima bean agar were transferred individually to the centers of new plates. After different periods of incubation the plates were flooded with distilled water to induce the formation of sporangia and zoospores. The following variable factors were tested: (i) the amount of agar (7-16 ml), (ii) culture age (2-10 days), (iii) various washing solutions, (iv) the number of water changes (0-8), (v) washing interval (2.5-60 min), (vi) incubation temperature (10-35 C), and (vii) light vs. darkness. When one condition was varied, others were kept more or less constant as follows: (i) amount of agar, 10 ml; (ii) culture age, 5 or 6 days; (iii) washing solution, distilled water; (iv) numbers of changes of washing solution, 4 or 5; (v) washing interval, 30 min; (vi) incubation temperature,  $23 \pm 2$  C; (vii) light conditions, ambient laboratory light (130 lux). An isolate of race 1 was used in all experiments. Other races were used to verify the method.

Zoospore concentrations were determined with a hemacytometer or by counting the numbers of spores in five different 2- $\mu$ liter aliquots, usually after 18-20 hr (8). Before zoospores were counted, motility was stopped by adding one drop of 0.1% aniline blue in lactophenol to 1 ml of zoospore suspension.

In all experiments, treatments were replicated at least three times, and experiments were repeated three or four times with similar results. Differences between means were detected using Tukey's *w* procedure following analysis of variance.

### RESULTS

Cultures containing 7, 10, 13, or 16 ml of lima bean agar were grown for five days when colony diameter were 7.8, 7.5, 7.3, and 7.3 cm, respectively. After flooding, zoo-

spore numbers per milliliter of suspension (15 ml/plate) were  $9.2 \times 10^5$ ,  $5.7 \times 10^5$ ,  $1.5 \times 10^5$ , and  $4.9 \times 10^4$  for 7, 10, 13, and 16 ml agar, respectively. Ten ml of agar was chosen for future work because cultures on that volume produced large numbers of zoospores, were convenient to dispense, and easily covered the petri dish.

Cultures 2 to 10 days old were evaluated for zoospore production. Maximum numbers were produced from 6-day-old cultures (Fig. 1-A). At that age, mycelia had completely covered the surface of the agar.

To test the effect of mycelium age in the same culture on zoospore production, three cultures were started by placing a disk of agar inoculum in the center of each plate. Each day's growth was marked with ink on the underside of the plates. After 6 days of incubation, 7-mm diameter disks ranging from 1 to 5 days old were cut from the rings of mycelium. Five mycelial disks of each age were transferred to sterile petri dishes and flooded with 15 ml of distilled water for 30 min, then flooded once more with 10 ml. The younger the mycelium, the greater was the zoospore production. One-day-old mycelium produced  $7.0 \times 10^3$  zoospores/ml ( $1.4 \times 10^4$ /disk), which was four times that produced by 5-day-old mycelium ( $1.8 \times 10^3$

zoospores/ml;  $3.7 \times 10^3$ /disk). Two-, 3-, and 4-day-old disks produced  $3.8 \times 10^3$ ,  $3.0 \times 10^3$ , and  $2.1 \times 10^3$  zoospores/ml, respectively.

The following washing solutions were evaluated for effects on zoospore production: (i) tap water, (ii) single distilled water, (iii) double distilled water, (iv) a salt solution [per liter: 0.294 g  $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$ , 0.247 g  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ , and 0.075 g KCl (9)]. Fifteen ml of distilled water was used in each of the first five washings for each treatment, but 10 ml of each test solution was used in the final flooding. Double-distilled water produced  $1.1 \times 10^5$ , single-distilled water  $7.3 \times 10^4$ , salt solution  $2.0 \times 10^4$ , and tap water  $5.3 \times 10^3$  zoospores/ml.

The effect of number of water changes on zoospore production was studied using 5-day-old cultures washed 0 to eight times at 30-min intervals. Maximum numbers of zoospores ( $3.5 \times 10^5$ /ml) occurred with five water changes (Fig. 1-B).

The optimum time interval between changes of water on zoospore production was determined by flooding (with 15 ml) 6-day-old cultures for intervals of 2.5 to 60 min. The times of flooding were so arranged that washing in all treatments was terminated at the same time. Numbers of zoospores increased as the time interval between washing lengthened up to 30 min (Fig. 1-C). However, a 60-min interval did not produce significantly greater numbers of zoospores than did the 30-min interval.

To test the effect of incubation temperature on production of zoospores, cultures were washed four times at 30-min intervals. The water used for the fifth and final flooding (15 ml) was adjusted to 10, 15, 20, 25, 30, or 35 C, and the plates were incubated at those temperatures in darkness maintained by covering the plates with aluminum foil. Plates incubated at 20 C yielded the greatest numbers of zoospores ( $1.7 \times 10^5$  zoospores/ml) (Fig. 1-D). This treatment also appeared to have the highest proportion of actively swimming zoospores although precise determinations were not made. Plates incubated at 30 C contained many dehiscent sporangia in which the zoospores remained trapped (6). Many zoospores germinated within the sporangia, and produced germ tubes which penetrated the sporangium walls. At 35 C no sporangium production was observed.

The effect of the same temperatures on mycelium growth rate on lima bean agar was also examined. Maximum colony diameter was obtained at 25 C (Fig. 1-D), confirming the results of others (5, 6, 11). Thus, the optimum temperature for colony growth was higher than that for sporangium development and zoospore production.

The effect of light on zoospore production was studied. Cultures, after flooding (15 ml), were incubated (i) under fluorescent light provided by Sylvania Gro-Lux WS 40-watt lamps (4,800 lux) for 20 hr, (ii) under fluorescent light for 10 hr followed by 10 hr in darkness, or (iii) in darkness for 20 hr. The temperature inside foil-covered and noncovered plates did not differ. Plates incubated in the dark produced 10 times more zoospores ( $1.3 \times 10^5$ ) than those incubated in light ( $1.2 \times 10^4$ ). The sequential light/dark treatment produced  $6.3 \times 10^4$  zoospores/ml.

To determine the length of time required for zoospore production, cultures exposed to the optimum conditions described above were examined 2, 4, 6, 8, and 18 hr after

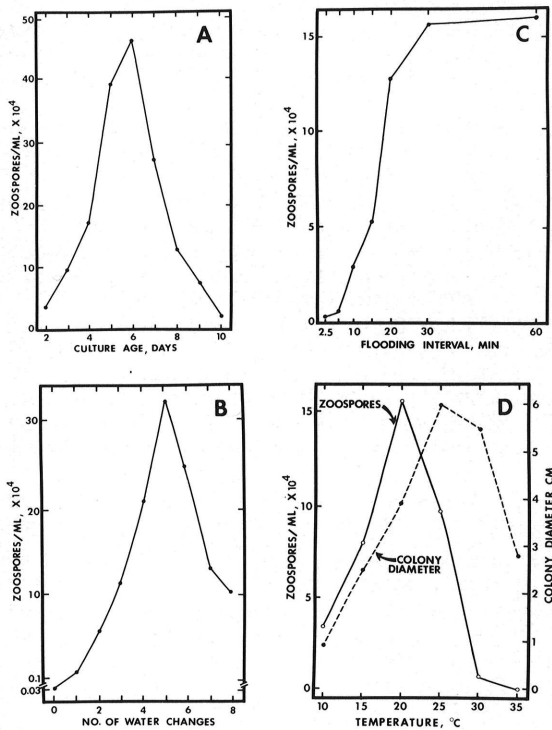


Fig. 1-(A to D). Factors affecting zoospore production by *Phytophthora megasperma* var. *sojae* race 1 on flooded (15 ml) lima bean agar cultures in petri dishes. **A**) Effect of age of culture. Least significant range (LSR) by Tukey's  $w$  procedure is  $6.6 \times 10^4$  ( $P = 0.05$ ). **B**) Effect of number of times the culture was flooded with distilled water (30 min each); the LSR is  $4.9 \times 10^4$  ( $P = 0.05$ ). **C**) Effect of flooding interval between water changes using 6-day-old cultures; the LSR is  $1.7 \times 10^4$  ( $P = 0.05$ ). **D**) Effect of incubation temperature (after the final flooding) on zoospore production and on mycelial growth; LSR for zoospores is  $2.0 \times 10^4$  and the LSR for growth is  $0.4$  ( $P = 0.05$ ).

the washing procedure was completed. After 2 hr of incubation in a flooded (15 ml) condition, sporangia had begun to appear, but no zoospores had been released. However, at 4 hr  $5.7 \times 10^4$  zoospores/ml were collected. Numbers increased progressively to a maximum of  $3.5 \times 10^5$ /ml after 18 hr. After the supernatant fluid was collected for each treatment (except the 18-hr treatment), the plates were flooded once more and allowed to stand overnight. All plates, regardless of treatment, produced a total of  $5.1 \times 10^6$  to  $6.2 \times 10^6$  zoospores in the two harvests.

Isolates of races 1 through 6 were treated under the optimum conditions as described above. All races produced large numbers of zoospores. Race 2 produced  $1.0 \times 10^5$ ; race 3,  $1.4 \times 10^5$ ; race 4,  $1.2 \times 10^5$ ; race 5,  $9.3 \times 10^4$ ; and race 6,  $2.1 \times 10^4$  zoospores/ml.

#### DISCUSSION

Large numbers of zoospores of *P. megasperma* var. *sojae* were readily produced in flooded cultures on lima bean agar, provided that culture age, number and frequency of water changes, temperature, and light conditions were appropriately standardized. Maximum numbers ( $10^4$  -  $10^5$ /ml) were produced when 6-day-old cultures received five changes of distilled water each at 30-min intervals, followed by incubation in darkness at 20 C for at least 8 hr. The number of zoospores produced on plates incubated in darkness was ten times greater than on plates incubated under continuous light. Light has been reported (i) to have no influence on the production of sporangia (3, 13), (ii) to be stimulatory (1, 2), and (iii) to be inhibitory (15) to several species of *Phytophthora*. The method yielded high numbers of zoospores ( $10^4$ /ml) in as few as 4 hr postwashing. Previous methods have required 24 to 72 hr of post-treatment before harvest (5, 6, 11). Furthermore, cultures may yield a second and third harvest of zoospores from the same petri dish after reflooding.

Although the determination of optimum conditions for zoospore production was done with an isolate of race 1, the applicability of the method was verified using isolates of races 2, 3, 4, 5, and 6, each of which produced  $10^4$ - $10^5$  zoospores/ml. Moreover, additional isolates of races 1, 3, 4, 5, and 6 have all produced similar high numbers of zoospores in other work (J. L. Lockwood and S. D. Cohen, unpublished).

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