In Vitro Zoospore Production, Motility, and Germination of Aphanomyces cochlioides[1]

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Introduction

Investigations of the black root disease of sugarbeets require a reliable technique for producing adequate numbers and volumes of *Aphanomyces cochlioides* Drechs. zoospores. Several techniques, for *in vitro* production of *A. cochlioides* zoospores have been described (4,8,9,10)[3], but these failed to yield adequate numbers in our tests. The primary reason for this failure probably was due to differences in water from various sources, especially tap water (2). Various additions to distilled or tap water have been made to favor zoospore production in the Saprolegniaceae, such as use of charcoal water (7), addition of NaCl to distilled, demineralized, or tap water (10), or use of a complete mineral solution plus methionine (7). Mitchell and Yang (5), studying *A. euteiches,* devolped a mineral solution which produced zoospore numbers equal to that obtained with lake water.

Our objective was not only to produce adequate numbers of *A. cochlioides* zoospores, but to do so using water or solutions of reproducible composition. Another objective was to ascertain the effects of various temperatures and time intervals (after the zoospore production period) on zoospore motility and germination. The concentrations of motile and nonmotile zoospores of *A. euteiches* were determined by Mitchell and Yang (5) immediately after production, but little information has been reported on zoospore behavior of *A. euteiches* or *A. cochlioides* with time following the production period. Germination data were taken at the same time as motility ratings.

Methods and Results

Zoospore Production

Basically, modified procedures of Schneider (10) were used. A single-zoospore culture of *A. cochlioides* was used throughout. Fresh transfers were made weekly to slants of Difco corn meal

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[3] Numbers in parentheses refer to literature cited.

agar and to inoculum-increase plates. Mycelial mats for zoospore production were cultured in 250-ml Erlenmeyer flasks containing 50 ml of various nutrient media. Liquid cultures were inoculated with agar discs cut from inoculum-increase plates with 6 mm diam cork borer and incubated 4 days at 24°C. The mats were washed with sterile double-deionized water and placed in sterile zoospore-production solutions (ZPS) for 16-18 hr at 24°C. Usually one mycelial mat/200 ml ZPS was used. Preliminary tests indicated that double-deionized water (DD) used for washing mycelial mats and for ZPS induced higher zoospore production than did glass distilled water, single deionized water, or tap water. At the end of the incubation period, zoospores were counted using Schneider's procedures (10).

The effect of composition of nutrient media used for mat production on zoospore numbers was tested. Five media were used: 1) 0.5% starch, 0.5% glucose, 0.1% yeast extract (7); 2) 12.6 g glucose, 10 g/liter yeast extract (6); 3) 0.3% Difco peptone (10); 4) and 5) 0.3% Difco soytone (10). The difference between media 4 and 5 was the source of soytone; 4 was recently purchased, whereas 5 had been stored in the laboratory for several years. Media 1, 3, and 5 were best for zoospore production (Table 1). The soytone medium was eliminated because the new and old sources differed. Peptone was selected for use because it was simpler to prepare than the starch-glucose-yeast extract medium.

Table 1.—Effect of mycelial-mat culture medium on zoospore production in *Aphanomyces cochlioides*.

Medium	Zoospore/ml
	x10³
1. Starch-glucose-yeast extract	23.3
2. Glucose-yeast extract	16.0
3. Peptone	23.3
4. Soytone (new source)	6.5
5. Soytone (old source)	19.8

$F=11.50^{**}$ LSD (0.05)=5.5

A comparison was made of DD with the salt solution (MY) developed by Mitchell and Yang (5) for *A. euteiches* zoospore production. My composition was: 0.194 g $CaCl_2$ 0.075 g KCl, 0.247 g/liter $MgSO_4 \cdot 7H_2O$. The pH was adjusted to 6.0. All combinations of wash and ZPS of the two solutions were used. The best combination for zoospore production was a DD wash for the mats and MY for the production solution (Table 2).

The influence of pH of the MY solution and of the peptone mycelial-mat culture medium on zoospore production was investigated. Large differences in zoospore numbers resulted from

Table 2.—Effects of double-deionized water (DD) Mitchell and Yang's salt solution (MY) in combinations of wash and production solution on zoospore production in *Aphanomyces cochlioides*.

Wash	Production solution	Zoospores/ml[a]
		X10[3]
DD	DD	23
DD	MY	72
MY	DD	34
MY	MY	33

[a] Average of two counts/duplicated flask.

varying the pH of these media (Table 3). A culture medium of pH 7 and a MY of pH 8 was adopted for regular use.

Table 3.—Effects of pH of mycelial-mat culture medium and pH of Mitchell and Yang's zoospore production solution (MY) on zoospore production in *Aphanomyces cochlioides*.

Culture medium pH	MY salt sol. pH	Zoospores/ml[a]
		x10[3]
6	6	16
6	7	36
6	8	42
7	6	30
7	7	50
7	8	71
8	6	46
8	7	19
8	8	40

[a] Average of two counts/duplicated flask.

Schneider (10) reported that aeration of ZPS during the production period increased zoospore concentrations two to three fold. We aerated the ZPS on a reciprocating platform shaker, but zoospore numebrs were lowered. Apparently this treatment was too damaging to the mats since they fragmented during shaking. To reduce damage to the mats, reagent bottles (2.5 liter) were rotated on a roller (18 rpm). Each reagent bottle contained 400 ml MY and two mycelial mats. Results of comparisons of zoospore counts from rotated (aerated) vs. non-rotated controls were as follows (average of two counts/duplicated bottle): a) rotated reagent bottles=282,000 zoospores/ml; b) non-rotated reagent bottles=55,000 zoospores/ml; c) Fernbach flasks =46,000 zoospores/ml. Rotation of reagent bottles was adopted as standard procedure.

The possibility that the aerated (rotated) ZPS might yield results different from the ZPS in non-aerated flasks was checked. In these tests, estimates of the percentage motility of zoospores and counts were made. Zoospore motility was estimated by placing a drop of the zoospore suspension on a glass slide and observing five 40X fields/drop.

Five mycelial-mat culture media were tested for their effects on percentage motility and numbers of zoospores produced in rotated bottles. The media included: 1) starch-glucose-yeast extract (same as in still-flask test); 2) standard peptone (control); 3) 2% peptone, 0.5% glucose (5); 4) 3 g maltose 1 g/liter peptone (3); and 5) 1 g peptone, 1 g yeast extract, 0.5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H20$, 2 g/liter dextrose (4). Considerable differences in percentage zoospore motility and concentration were evident (Table 4). Zoospore concentrations were higher than with non-rotated flasks, but media effects were relatively similar. Peptone was retained as the best medium for growth of mycelial mats.

Table 4.—Effect of mycelial-mat culture medium on zoospore motility and concentrations of *Aphanomyces cochlioides* in rotated reagent bottles.

Culture medium	Motility	Zoospores/ml[a]
	%	X10³
1. Starch-glucose-yeast extract	65	139
2. Peptone (control)	75	120
3. Peptone-glucose	60	113
4. Maltose-peptone	95	33
5. Peptone-yeast extract-glucose-salts	63	72

[a] Average of two counts/duplicated bottle.

The effect of composition of ZPS on zoospore motility and concentration was investigated using seven different ZPS aerated by rotation. Treatments were: 1) MY salt solution; 2) DD; 3) DD plus 120 mg NaCl/liter; 4) basal mineral salts (BMS) (1); 5) charcoal water (1 g activated charcoal/4 liters DD water, brought to boil, cooled and filtered through Whatman No. 1 filter paper); 6) tap water; and 7) tap water plus 120 mg NaCl/liter. MY was superior in percentage motility and zoospore numbers (Table 5). Tap water induced high zoospore concentations in contrast to previous still-culture tests, but zoospore motility was only moderate.

Table 5.—Effect of zoospore production solution composition on zoospore motility and concentrations in *Aphanomyces cochlioides*.

Production sol.	Motility	Zoospores/ml[a]
	%	X10³
1. MY	80	136
2. DD	1	39
3. DD + NaCl	45	99
4. BMS	1	51
5. CW	20	63
6. Tap water	55	130
7. Tap water + NaCl	18	55

[a] Average of two counts/duplicated bottle.

The rotated reagent bottle method of zoospore production generally yielded uniformly high numbers of zoospores, but

occasionally there was considerable variation among replications. Failure to remove all the culture medium by adequate washing has been reported to depress zoospore production (5). The effect of number of washes on zoospore production was ascertained using: 1) one wash of 30 min (mycelial mat placed on 500-600 ml sterile DD for 30 min, then transferred to ZPS); 2) two washes of 15 min each; and 3) three washes of 10 min each. Results were as follows: 1=249,000; 2=306,000; and 3=292,000 zoospores/ml (average of two counts/triplicated bottle). Washing procedures did not result in much change in zoospore production. Two 15 min washes were chosen as the standard treatment.

Because only two mycelial mats were used per bottle, the possibility that the occasional low zoospore concentrations might be due to injury to one or both mats was investigated by comparing 1, 2, 3, 4, and 6 mats per bottle (400 ml MY/bottle). Numbers of zoospores obtained on a per mat basis were: 1 mat=73,000; two mats=72,000; 3 mats=86,000; 4 mats=77,000; and 6 mats=62,000 zoospore/ml (averages of 4-6 replications). Three mats/bottle were used for routine zoospore production.

Use of chemically clean glassware and *A. cochlioides* cultures free of bacteria have given consistent results in routine zoospore production. All zoospores are now produced axenically, low concentrations most commonly have been associated with bacterial contamination.

Zoospore Motility and Germination

Motility was estimated by the technique described previously, with emphasis on changes in percentage motility with time after harvest. Zoospores were produced by standard techniques, thoroughly mixed, and aliquots were placed at each of seven temperatures. Estimates of percentage motility were made at seven time intervals (Table 6). The highest motility over the longest time was at 16°C. Motility dropped off rapidly with time at and above 24°C. Rather unexpected was the rapid loss of motility at 5°C.

Table 6.—Percentage of zoospores of *Aphanomyces cochlioides* motile at different temperatures after various times following zoospore harvest.[a]

Time	Temperatures						
	5C	12C	16C	20C	24C	28C	32C
Hr	%	%	%	%	%	%	%
2.5	55	60	75	75	70	65	10
5.	20	30	70	60	25	30	0
7.	5	20	55	45	0	10	0
8.5	1	20	40	30	0	5	0
24	0	10	25	15	0	0	0
32	0	10	15	10	0	0	0
48	0	5	5	0	0	0	0

[a] Average of three trials, rounded off to nearest 5%.

Estimates of the percentage of germinated zoospores were made when motility was estimated. Germination percentages, as affected by time and temperature, are presented in Table 7. Highest germination occurred between 5 and 55 hr. Increased germination at the higher temperatures may account for some of the loss of motility at these temperatures.

Table 7.—Percentage of germinated zoospores of *Aphanomyces cochlioides* at different temperatures after various times following zoospore harvest.[a]

Time	Temperatures						
	5C	12C	16C	20C	24C	28C	32C
Hr.	%	%	%	%	%	%	%
2.5	1	1	1	1	1	1	1
5.	1	1	5	3	3	5	9
7.	2	3	7	5	8	12	13
8.5	4	12	11	13	13	18	26
24	2	10	10	8	17	20	33
32	4	16	22	23	39	32	43
48	5	19	25	28	40	35	45
55	5	28	28	35	43	40	48

[a] Average of three trials, rounded off to nearest 1%.

Discussion

The peptone medium of Schneider (10) for growth of mycelial mats was the simplest medium that gave consistently good results. Unexpectedly, results with Difco soytone depended upon whether a new or old source of soytone was used. These sources differed in appearance (color and texture) as well as in suitability for zoospore production. The most recently acquired source of soytone was not satisfactory.

Double-deionized water was used to prepare the peptone medium, to wash mycelial mats, and to prepare the MY salt solution for zoospore production, because its composition varied only within narrow limits and, thus, constituted a reproducible water supply.

Increases in zoospore numbers of two to four fold have been reported for aerated vs. non-aerated ZPS for both *A. eutheiches* (3) and *A. cochlioides* (10). In these previous studies air bubblers were used for aeration, and no attempt was made to aerate the solutions by shaking or rotating the containers. The latter method is less cumbersome and can be used routinely. Rotated reagent bottles increased zoospore numbers 4-5 times over that of non-rotated control bottles. Initial attempts to aerate the solutions on a reciprocating shaker caused shredding of mycelial mats and depressed zoospore numbers. Apparently, damage to mycelial mats must be avoided.

The superiority of Mitchell and Yang's salt solution for zoospore production was most clearly evident when percentage motility was considered along with concentration. High motility is desirable for many black root investigations, i.e., zoospore migration studies.

Often, zoospores are not used immediately following their production. Therefore, the storage temperature or temperatures most suitable for maintaining motility are important considerations. One might reason that a low temperature would reduce zoospore activity and preserve their motility; however, 5°C reduced motility rather rapidly compared with the optimum 16°C. Temperatures of 24°C and above promoted germination which may account for some of the reduction in motility at these temperatures.

Summary

Adequate numbers ($2\text{-}3 \times 10^5$) and volumes of *Aphanomyces cochlioides* zoospores were obtained using 4-day-old mycelial mats grown on 0.3% peptone at 24°C. Mats were washed for two 15-min periods in double-deionized water, transferred (3 mats/bottle) to 400 ml of salt solution (0.194 g $CaCl_2$, 0.075 g KCl, 0.247 g/liter $MgSO_4 \cdot 7H_2O$, pH adjusted to 8), and rotated on a roller for 16-18 hr at 24°C. In addition to concentrations, the percentage of motile zoospores was estimated at harvest and after storage intervals at various temperatures. Motility at harvest was affected by mat-culture medium and zoospore production solution. Retention of motility after harvest was highest and longest at 12, 16, or 20°C, with 16°C being superior. Motility was lost rapidly at 24-32°C, and at 5°C. Most germination occurred at 24-32°C, with the highest at 32°C.

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Literature Cited

(1) AYERS, W. A., and G. C. PAPAVIZAS. 1965. An exocellular pectolytic enzyme of *Aphanomyces euteiches*. Phytopathology 55: 249-253.

(2) LANGE, N. A. 1952. Handbook of chemistry. 8th Edition. Handbook Publishers, Inc. Sandusky, Ohio. 1998 p.

(3) LLANOS, CARMEN M., and J. L. LOCKWOOD. 1960. Factors affecting zoospore production by *Aphanomyces euteiches*. Phytopathology 50: 826-830.

(4) MACWITHEY, H. S. 1961. *In vitro* inoculation of sugar beet seedlings with *Aphanomyces cochhioides* Drechs. J. Am. Soc Sugar Beet Technol. 11: 309-312.

(5) MITCHELL, J. E., and C. Y. YANG. 1966. Factors affecting growth and development of *Aphanomyces euteiches*. Phytopathology 56: 917-922.

(6) PAPAVIZAS, G. C., and C. B. DAVY. 1960. Some factors affecting growth of *Aphanomyces euteiches* in synthetic media. Am. J. Bot. 47: 758-764.

(7) REISCHER, HELEN S. 1951. Growth of Saprolegniaceae in synthetic media. I. Inorganic nutrition. Mycologia 43: 142-155.

(8) SCHNEIDER, C. L. 1954. Methods of inoculating sugarbeets with *Aphanomyces cochlioides* Drechs . Proc. Am. Soc. Sugar Beet Technol. 8: 247-251.

(9) SCHNEIDER, C. L. 1962. Some factors affecting zoospore production by *Aphanomyces cochlioides*. (Abstr.) Phytopathology 52: 166.

(10) SCHNEIDER, C. L. 1963. Cultural and environmental requirements for production of zoospores by *Aphanomyces cochlioides in vitro*. J. Am. Soc. Sugar Beet Technol. 12: 597-602.