Cultural and Environmental Requirements For Production of Zoospores by Aphanomyces cochlioides in Vitro

C. L. SCHNEIDER[1]

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Sugar beet strains developed at the Plant Industry Station, Beltsville, Maryland, have been screened in the greenhouse for resistance to the fungus, *Aphanomyces cochlioides*. In each test of 24 entries, large quantities of inoculum—approximately 100 million zoospores—are required (5)[2] . At the outset of the testing program, zoospore inoculum was obtained in accordance with a previously described method (4) whereby mycelial mats of the fungus are submerged in water at 20 to 25 C for about 16 hours. Wide variation in number of zoospores produced at different times indicated a need to determine the variables, besides temperature, that influence zoospore production.

It has been shown that zoospore production by a related fungus, *Aphanomyces euteiches*, is influenced by the type of medium which the mycelial mats are produced, temperature, age of culture, type of water, and aeration of water (2).

The experiments described herein were conducted to determine the degree to which the following variables influence zoospore production by *A. cochliodes*: age of culture, type, pH, and aeration of water; relative amounts of mycelium and water. An abstract of some of the results has been published (6).

Methods

Monosporous cultures, isolated from damped-off sugar beet seedlings and maintained on maize meal agar, were used. Mycelial mats were produced in flasks containing 0.3% peptone or 0.3% Soytone[3, 4]. Previous studies showed that addition of dextrose, maltose, or sucrose to broth did not enhance zoospore production (6). The size of the flask and amount of broth in which mycelial mats were produced varied from one experiment to another.

Zoospore production was induced by rinsing the mycelial mats and transferring them to flasks containing water at 20 to 25 C. Approximately 16 hours later, spore counts of 10 ml samples from each flask were made with a brightline counting chamber.

[1] Plant Pathologist, Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture, Utah State University, Logan, Utah.

[2] Numbers in parentheses refer to literature cited.

Zoospores were immobilized by the addition of 0.1 ml Roccal[4, 5] solution (800 ppm) to each sample in order to facilitate counting.

Results

Age of culture

Zoospore production was compared between cultures of different age. Flasks containing 125 ml nutrient broth were inoculated 5, 7, 11, 14, and 20 days before each subsequent mycelial mat, incubated at 20 C, was transferred to 250 ml tap water. Zoospore production was greatly influenced by age of culture. Average number of zoospores produced by 3 myclelial mats in each age group was as follows:

Age of culture (days)	Zoospores/ml (thousands)
5	41.4
7	65.6
11	10.4
14	4.8
20	0
LSD (P = .05) =	7.1

The decline in zoospore production, noticeable by the 11th day, can be delayed by refrigeration. On numerous occasions, broth cultures placed in the refrigerator at 5 C, on the fourth day after inoculation, produced over 80,000 zoospores/ml when transferred to water 14 days later.

Type of water

Zoospore production was compared in 3 types of water (tap, distilled and demineralized) alone and with NaCl (120 mg per liter) added. The water in which each mat was rinsed was of the same type as that in which it was subsequently submerged. The greatest number of zoospores was produced in tap water (Table 1). Zoospore production was increased by the addition of NaCl, especially in distilled and in demineralized water.

Table 1.—Zoospore production by mycelial mats of *Aphanomyces cochlioides* in 3 types of water, with and without NaCl.

Type of water	Zoospores/ml (thousands)[1]	
	+ NaCl (120 mg/liter)	Control
Tap	113.8	94.7
Distilled	94.0	33.5
Demineralized	70.9	30.6
LSD (P = .05)	18.9	

[1] Results expressed as average of 2 experiments, each with 4 replicates per treatment. Each replicate comprised one mycelial mat, produced in 30 ml broth, in 90 ml water.

[3] Trade name of an enzymatic hydrolysate of soybean meal prepared by Difco Laboratories, Detroit, Michigan.

[4] Mention of material and company name is for identification only and does not imply endorsement by U. S. Department of Agriculture.

[5] Trade name of a germicide containing benzalkonium chloride, prepared by Winthrop Laboratories, N.Y.

pH of water

Zoospore production was compared in demineralized water and in tap water adjusted to several pH values from 5.6 to 8.1 with M/3 KH_2PO_4 and M/3 Na_2HPO_4 buffer solutions. In both types of water, abundant zoospores were produced at pH 5.6 - 7.5 (Table 2). Zoospore production decreased noticeably at pH 7.8 and beyond.

Table 2.—Zoospore production by mycelial mats of *Aphanomyces cochlioides* in water of different pH.

Experiment No.	Type of water	Zoospores/ml (thousands) in water of indicated pH[1]								LSD
		5.6-5.7	5.8-5.9	6.0-6.1	6.4-6.5	7.0-7.1	7.4-7.5	7.8-7.9	8.0-8.1	(P=.05)
1	Demineralized	56.3	62.7	.....	64.3	.....	68.5	42.5	32.3	24.2
2	Tap	97.7	.....	118.0	142.2	127.5	123.5	70.7	.....	21.1

[1] Results expressed as mean of 3 replicates, each comprising one mycelial mat, produced in 30 ml broth, in 100 ml water.

Aeration of water

Tap water was aerated by bubbling air through it during the 16 hours that the mycelial mats were submerged. Air was introduced through glass tubing (4 mm diameter) at the approximate rate of 250 ml/min. In 4 experiments, zoospore production in aerated flasks was increased approximately 2 to 3 times over that in control flasks.

Table 3.—Zoospore production by mycelial mats of *Aphanomyces cochlioides* in aerated and nonaerated water.

Experiment number	Amount of water (ml)	Zoospores/ml (thousands)[1] produced in water treated as indicated.	
		Aerated	Control
1	150	105.5	32.5
2	300	48.9	18.8
3	1000	31.5	10.5
4	1000	102.2	48.8

[1] Results based on one replicate per treatment in each experiment.

Relative amounts of mycelium and water

Zoospore production was compared between flasks containing equal amounts of mycelium in different amounts of water and between flasks containing different amounts of mycelium in equal amounts of water. The mycelial mats when fully grown appear to occupy all of the volume of the broth, hence the approximate amount of mycelium constituting a mat can be designated by the volume of broth in which it was produced. The relative amounts of mycelium and water can thereby be expressed as the ratio of broth (ml) in which mats are produced and of water (ml) in which they are submerged.

With quantity of mycelium constant, zoospore production increased with added amounts of water, then leveled off after a mycelium-water ratio of 1:3 was attained (Table 4). With quantity of water constant, zoospore production increased with added amounts of mycelium when the mycelium-water ratio was 1:8 but not when the ratio was 1:4 and less (Table 5). The average number of zoospores produced per mycelial mat was greatest when the mycelium-water ratio was 1:3 to 4.

Table 4.—Zoospore production by mycelial mats of *Aphanomyces cochlioides* in different amounts of tap water.

Amount of water (ml)	Ratio[1] mycelium:water	Zoospores/ml[2] (thousands)	Total zoospores/ flask[2] (millions)
50	1 : 1	33.4	1.67
100	1 : 2	40.3	4.03
150	1 : 3	35.3	5.29
200	1 : 4	25.3	5.07
LSD (P = .05)			1.85

[1] Ratio between ml broth in which each mycelial mat was produced and ml water in which it was submerged.

[2] Results expressed as mean of 3 replicates; each comprising one mycelial mat, produced in 50 ml broth, in indicated amount of water.

Table 5.—Zoospore production by different numbers of mycelial mats of *Aphanomyces cochlioides* in equal amounts of tap water.

Number of mycelial mats	Experiment 1			Experiment 2		
	Ratio[1] mycelium:water	Zoospores/ml.[2] (thousands)	Zoospores[2] produced per mycelial mat (millions)	Ratio[1] mycelium:water	Zoospores/ml[3] (thousands)	Zoospores[3] produced per mycelial mat (millions)
1	1 : 3.3	82.8	8.28	1 : 8	59.0	14.75
2	1 : 1.7	92.6	4.63	1 : 4	119.9	14.99
3	1 : 1.1	83.1	2.77	1 : 2.7	96.9	8.16
4	1 : 0.8	92.2	2.31	.....	.....	.....
LSD (P = .05)		NS			35.8	

[1] Ratio between amount of water in which mycelial mats were produced and amount of water in which they were subsequently submerged.

[2] Results expressed as mean of 4 replicates; each comprising designated number of mycelial mats, produced in 30 ml broth, in 100 ml water.

[3] Results expressed as mean of 3 replicates; each comprising designated number of mycelial mats, produced in 30 ml broth, in 240 ml water.

Discussion

On the basis of the studies described, an improved methodology for production of zoospores in quantity by *A. cochlioides* has been established. Adequate quantities of zoospore inoculum have been consistently obtained from mycelial mats, not over

7 days old, submerged in a quantity of aerated tap water, with NaCl (120 mg per liter) added, equal to approximately 3 times the quantity of broth in which the mycelial mats were produced.

It is not fully known why more zoospores are produced in tap water than in distilled or in demineralized water. The presence of injurious elements or lack of essential ones has been cited as a possible cause of suppressed zoospore production by certain *Saprolegniaceae* in distilled water (3). The reason for increased zoospore production in water to which NaCl has been added, noted also by Sherwood (7) with *A. euteiches,* is, as yet, unknown.

In the laboratory where the preceding experiments were conducted, pH of tap water has been near optimum for zoospore production. Where pH of water is near 7.8 or above, reduced sporulation would be expected.

Increasing the amount of water in which mycelial mats are submerged would reduce the concentration of nutrients that may be carried from the broth by the mats. Increased zoospore production with increased amounts of water might therefore be associated with reduced concentration of nutrients in the water. Klebs (1) noted suppression of zoospore production by the water mold, *Saprolegnia mixta* with low concentrations of organic nutrients in the water.

Summary

Mycelial mats of *Aphanomyces cochlioides*, 5 to 7 days old, produced more zoospores than those 11 days old and older. More zoospores were produced in tap water than in distilled or demineralized water. NaCl (120 mg per liter) added to water in which mycelial mats were submerged enhanced zoospore production. Zoospore production at pH 7.8 and above was considerably less than at pH 5.6 to 7.5. The greatest number of zoospores per mycelial mat was produced when the quantity of water in which mycelium was submerged equaled 3 to 4 times the quantity of broth in which it was grown.

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