

Factors Affecting In Vitro Growth and Zoospore Production by *Aphanomyces raphani*

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ABSTRACT

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A partially selective medium containing 150 µg/ml streptomycin sulfate and 10 µg/ml benomyl in radish agar facilitated the isolation of *Aphanomyces raphani* from infected radish roots. Oospores and zoospores formed readily on radish agar, but were completely suppressed in the presence of streptomycin sulfate or peptone. Maximum numbers of motile zoospores (5×10^4 /ml) were produced 24

hr after transferring 2-day-old mycelial mats grown at 24 C in radish peptone broth into sterile distilled water; water at 20 C and pH 4.0-5.3 provided optimum conditions. Washing the mycelial mats reduced the number of zoospores produced. Zoospore production and mycelial growth was reduced by boron and inhibited by tap water.

Additional key words: black root of radish.

Aphanomyces raphani Kendrick, the cause of the black root disease of radish, is a serious pathogen in several radish-producing areas in the U.S. and around the world (1, 4, 6, 11). Until recently, growers in New Zealand associated root blackening of radish with boron deficiency (15); however, in heavily infested muck soils in Minnesota, application of boron failed to control black root (B. Jacobsen, *personal communication*). A more promising approach to the control of black root has been the development of a number of radish breeding lines with high resistance to *A. raphani* (5).

To advance the breeding stocks as rapidly as possible and to determine the mode of inheritance of resistance to *A. raphani*, a reliable greenhouse screening technique was needed. The motile zoospores of *A. raphani* are the most conveniently quantifiable propagules. Although procedures for inducing zoospores by *A. raphani* have been reported (3, 6), the cultural and environmental factors involved in zoospore production have not been critically defined; consequently, production of an adequate number of zoospores has not always been possible.

The production of abundant zoospores from *A. raphani* requires: (i) maintenance and growth of the fungus on solid agar medium, (ii) then growth of mycelium in broth culture, and (iii) finally transfer of mycelial mats to a zoospore-induction solution. We report herein suitable cultural conditions for each of these three stages. First, growth and maintenance of the fungus was evaluated on a number of agar media. The agar

medium that was optimum for growth then was used to determine those media most suitable for mycelial mat formation and for zoospore-induction solutions. Since boron had been associated with the disease (15), the effects of boron on growth and zoospore production of *A. raphani* also were studied.

MATERIALS AND METHODS

A single-zoospore culture of *A. raphani*, designated AR-15, was used throughout the present study. This culture was isolated from the roots of 2-wk-old seedlings of White Icicle radish grown in infested muck soil from Anoka County, Minnesota (5). A radish agar medium containing 150 µg/ml streptomycin sulfate (9) and 10 µg/ml benomyl was used for isolation of this fungus. Radish agar was prepared as described by Ghafoor (2) except that for the radish decoction 250 g of Scarlet Globe radish roots were steeped in 1 liter of water for 45 min at 100 C. Benomyl was added before autoclaving the medium and streptomycin sulfate was added just before the medium was poured into petri plates.

The growth of *A. raphani* on 10 agar media was compared at 24 C. The agar media tested were: potato-dextrose (PDA), malt, Czapek, nutrient, V-8, cornmeal, radish, radish dextrose (1% dextrose in radish agar), radish peptone (1% Bacto peptone in radish agar), and radish peptone dextrose (1% peptone and 0.5% dextrose in radish agar). Media were prepared as described in Tuite's manual (14). The growth of *A. raphani* on radish agar was studied at several temperatures between 4 C and 36 C. The colony diameter (including the 5-mm diameter

agar plug) for each of three plates of each medium was recorded daily.

An actively growing colony of *A. raphani* was maintained on PDA plates at 24 C by transfers of the fungus to fresh medium every 6 days. Unless otherwise indicated, zoospore production was studied by transferring a 5-mm diameter disk cut from the margin of the actively growing colony, into 25 ml of radish peptone broth (5 g peptone and the decoction from 250 g radish roots per liter of water) in a 125-ml Erlenmeyer flask. Flasks were kept at 24 C for 48 hr, after which each mycelial mat was transferred aseptically into 25 ml of sterile fresh distilled water (pH 4.7) in a sterile petri plate. The plates were held at 20 C for 24 hr. The number of zoospores in each plate was computed as an average of the zoospores counted in four standard hemacytometer fields. To facilitate counting, zoospore motility was slowed down by adding 0.05 ml of 70% chloralhydrate to each plate. Each treatment was replicated three times and every test was repeated at least four times. The data were analyzed using Duncan's new multiple range test.

Broth media examined for their effect on zoospore production and mycelial growth were: peptone (0.5% peptone), potato-dextrose (200 g potato tubers and 15 g dextrose per liter of water), peptone-dextrose (2% peptone and 0.5% dextrose), radish (decoction from 250 g of radish roots in 1 liter of water), radish-dextrose (0.5% dextrose in radish), radish-peptone (0.5% peptone in radish broth), and radish-peptone-dextrose (1% peptone and 0.5% dextrose in radish broth). The effects of peptone concentration in radish broth also were examined. All media were autoclaved at 121 C for 15 min. Two-day-old mycelial mats, grown in these media at 24 C, were transferred either to sterile distilled water for zoospore induction or dried in an oven at 70 C for weight determinations.

The solutions that were compared for effects on zoospore production were: distilled water, deionized water, tap water, Mitchell and Yang salt solution (MY) for zoospore production in *Aphanomyces euteiches* (8), and 100 and 200 $\mu\text{g/ml}$ solutions of NaCl (12). All the solutions were autoclaved at 121 C for 15 min.

The influence of the pH on zoospore production was studied by adjusting distilled water (pH 4.6 - 4.8) to the desired pH with either 0.1 N NaOH or 0.1 N HCl.

The influence of boron on growth and zoospore production of *A. raphani* was examined by adding boric acid to 50 ml of radish peptone broth in 250-ml flasks or to the distilled water zoospore-induction solution.

RESULTS

A. raphani grew most rapidly on radish-dextrose, potato-dextrose (PDA), and radish agar (Table 1). Consequently, PDA was used to produce mycelial mats in further studies. Oospores and zoospores were formed abundantly on radish agar and on radish-dextrose agar. Oospores also were formed, though much less abundantly, on cornmeal agar. Numerous oogonia were formed on potato-dextrose agar, but very few mature oospores were observed. Malt agar and Czapek's agar failed to support growth of *A. raphani*.

Peptone in the medium resulted in abundant aerial mycelium which collapsed and assumed a dense, moist,

creamy appearance. However, both oospore and zoospore production was completely suppressed by 0.01% peptone in radish agar. Oospore and zoospore production on radish agar also was suppressed by 100 $\mu\text{g/ml}$ of streptomycin sulfate.

Linear growth of *A. raphani* on radish agar was maximal at 24-28 C although the fungus grew at temperatures of 8 and 32 C (Fig. 1). Oospores and zoospores were formed at 16-28 C but were most abundant at 20-24 C. The diameter of mature oogonia, produced on radish agar at 20 C, varied from 24.2 to 43.6 μm (average 32.1 μm) and that of the oospores varied from 18.1 to 30.3 μm (average 23.3 μm).

Radish agar was the best medium for maintaining *A. raphani*; cultures remained viable and pathogenic after 5 mo of storage at 4 C. As previously reported (15), PDA was an unsuitable medium for long-term storage of *A. raphani*.

Broths that contained radish decoction were significantly superior ($P = 0.01$) to others tested for the production of zoospores (Table 2). Though potato-dextrose broth and peptone-dextrose broth supported abundant vegetative growth, few zoospores were produced. However, when mycelial mats grown in peptone-dextrose broth were transferred to MY-salt solution instead of distilled water, 5×10^4 zoospores/ml were produced.

Zoospore production and mycelial growth were increased considerably by the addition of 0.5% peptone to radish broth. Adding only dextrose to radish broth did not significantly improve mycelium growth and reduced the number of zoospores produced (Table 2).

Adding peptone either alone or in combination with dextrose resulted in a significant increase in mycelial growth. Vegetative growth increased with increasing concentrations of peptone; however, zoospore production was maximal from mats produced in 0.5% peptone (Fig. 2). Thus, radish peptone broth (0.5% peptone) was used throughout this study.

When mycelial mats of various ages were placed in the zoospore induction solution, 2- and 3-day-old mats produced maximum numbers of zoospores, whereas, 1-day-old and 4-day-old mats produced very few. Five-day-old mats failed to produce any zoospores and microscopic examination revealed no protoplasmic differentiation in the young hyphae.

Distilled water, deionized water, or dilute NaCl (100 $\mu\text{g/ml}$) all were suitable for the production of high numbers of zoospores from mycelial mats grown in radish peptone broth, but MY-salt solution, NaCl solution (200 $\mu\text{g/ml}$), and tap water were inferior to distilled water. In tap water, zoosporogenesis progressed to the production of primary spores which then often burst. The effects of tap water were irreversible. Mycelial mats, which had been submerged in tap water for 24 hr failed to produce any zoospores when transferred to distilled water.

Washing of 2-day-old mycelial mats, grown in radish peptone broth, by transferring them to fresh changes of distilled water or MY-salt solution at 0, 2, and 4 hr significantly reduced the numbers of zoospores produced after 24 hr.

The temperature of the zoospore-induction solution had a profound effect on zoospore production (Fig. 1).

Zoospores were produced at 12-32 C, with the maximum numbers released at 20 C, 4 C below the optimum for vegetative growth. At 20 C, cytoplasmic differentiation occurred 4-5 hr after the mycelial mats were transferred into sterile distilled water. Two hr later, the primary spores began emerging from the sporangia and remained clustered for 2-3 hr before the motile secondary zoospores appeared. The total number of motile zoospores was highest 24-36 hr after the mycelial mats were transferred to water (Fig. 3).

Motility of zoospores was completely suppressed by placing them at 4 C overnight. However, large numbers of motile zoospores were present 12 hr after the temperature of the suspension was raised to 20 C. Zoospores in distilled water held at 4 C were pathogenic on radish seedlings for up to 45 days.

Although zoospores were produced at pH 3.5 - 9.0, maximum numbers were obtained between pH 4.0 - 5.3 (Fig. 4). Because phosphate, citrate, and borate

suppressed zoospore production, NaOH and HCl were utilized to adjust the pH of the induction solution.

Boron inhibited vegetative growth and, at 30 µg/ml of induction solution, virtually completely suppressed

TABLE 1. A comparison of linear growth and oospore production of *Aphanomyces raphani* on agar media after 7 days at 24 C

Medium	Colony diameter (mm) ^a	Oospore production ^b
Radish-dextrose agar	90	4
Potato-dextrose agar	88	1
Radish agar	83	4
Radish-peptone-dextrose agar	75	0
V-8 agar	57	0
Corn-meal agar	50	2
Radish-peptone agar	47	0
Nutrient agar	38	0
Malt agar	5	0
Czapek's agar	5	0

^aAverage of three colonies growing in 20 ml of agar in petri dishes in the dark. Diameter includes the 5 mm plug.

^bRelative abundance of oospores: 0 = no oospores formed; 1 = mostly oogonia; 2 = 1-20 oospores per microscope field; 3 = 20-40 oospores; and 4 = more than 40 oospores per microscope field (×10).

TABLE 2. Effect of types of broth media on growth and zoospore production of *Aphanomyces raphani*

Broth	Zoospores per ml (10 ³) ^a	Dry weight of mycelium (mg) ^b
Peptone	0 w ^c	0.54 w ^c
Potato-dextrose	1 w	4.25 y
Peptone-dextrose	5 w	5.50 y
Radish-dextrose	40 x	1.50 w
Radish-peptone-dextrose	43 xy	4.25 y
Radish	52 y	1.35 w
Radish-peptone	80 z	3.75 y

^aZoospores produced in distilled water at 20 C.

^bGrown for 48 hr at 24 C in 25 ml of broth in dark.

^cMeans with the same letter are not significantly different, P = 0.01 by Duncan's new multiple range test.

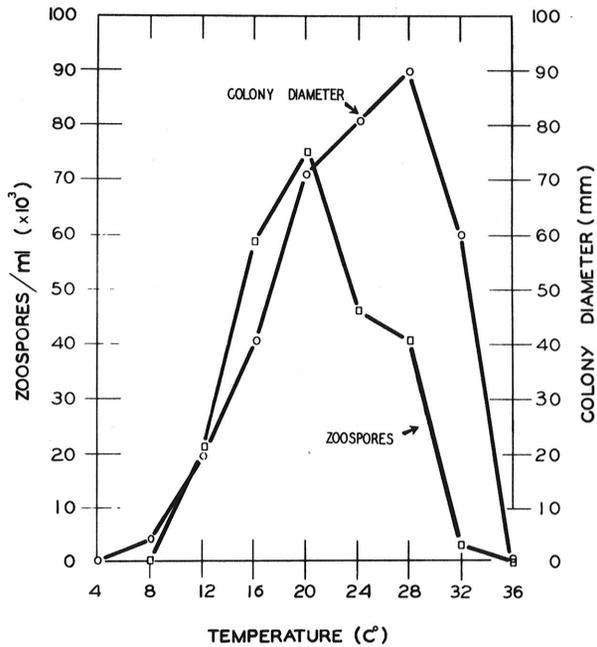


Fig. 1. Effect of peptone in radish broth on vegetative growth and zoospore production by *Aphanomyces raphani*.

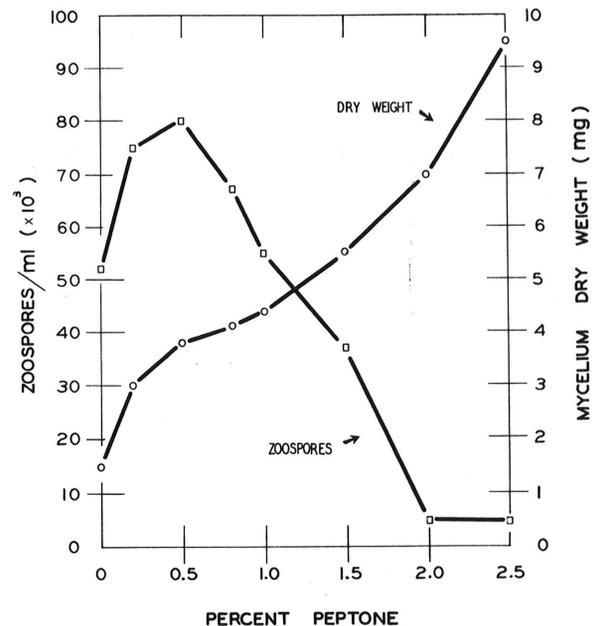


Fig. 2. The influence of temperature on growth and zoospore production by *Aphanomyces raphani*.

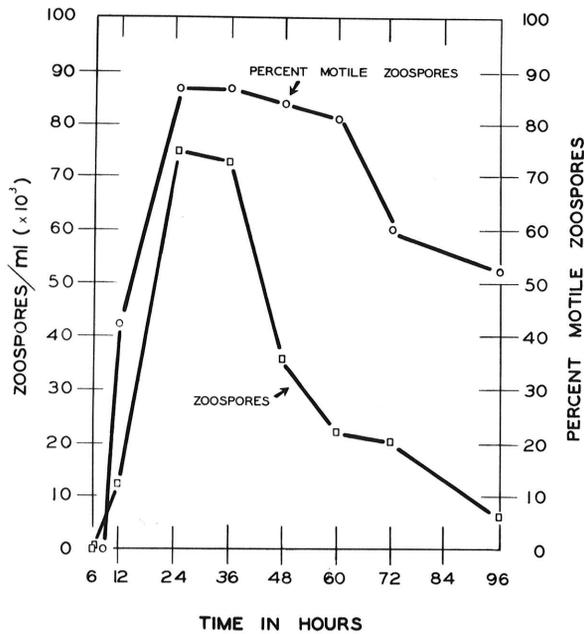


Fig. 3. Total and percentage of motile zoospores of *Aphanomyces raphani* at various times after mycelial mats were transferred to sterile distilled water at 20 C.

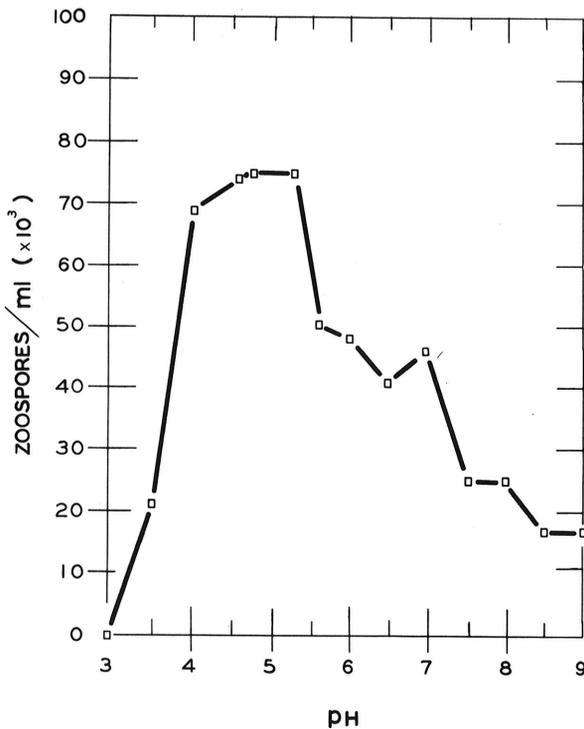


Fig. 4. The relationship of pH of the zoospore induction solution to zoospore production by *Aphanomyces raphani*.

zoospore release (Fig. 5). The few zoospores produced at boron concentrations of 20 and 30 $\mu\text{g/ml}$ remained in the primary cysts and never germinated or produced motile secondary zoospores.

DISCUSSION

By examining the effect of a number of factors on the production of zoospores in *A. raphani*, we have determined the conditions that reliably result in production of abundant supplies of zoospores necessary for the routine inoculation of radishes in our multi-disease resistance breeding program (5). We have produced sufficient zoospores (10^8) from one liter of broth medium to inoculate approximately 5,000 seedlings. That zoospore inoculum remains viable and virulent for at least 45 days at 4 C may be especially useful since seedlings produced over several weeks may be screened with a single large preparation of zoospore inoculum.

Many of the same nutritional or cultural requirements necessary for zoosporogenesis in other *Aphanomyces* sp. have proven to be necessary for *A. raphani*, and, in general, the relationship between vegetative growth and spore production adhere closely to Kleb's principles (7).

The enhancement of zoospore production by increasingly higher concentrations of peptone (up to 0.5%) suggests that an optimum balance is reached between factors governing vegetative growth and asexual reproduction (Fig. 2). The number of zoospores produced decline at peptone concentrations above 0.5% whereas vegetative growth continues to increase. We have not varied the concentration of radish decoction to determine

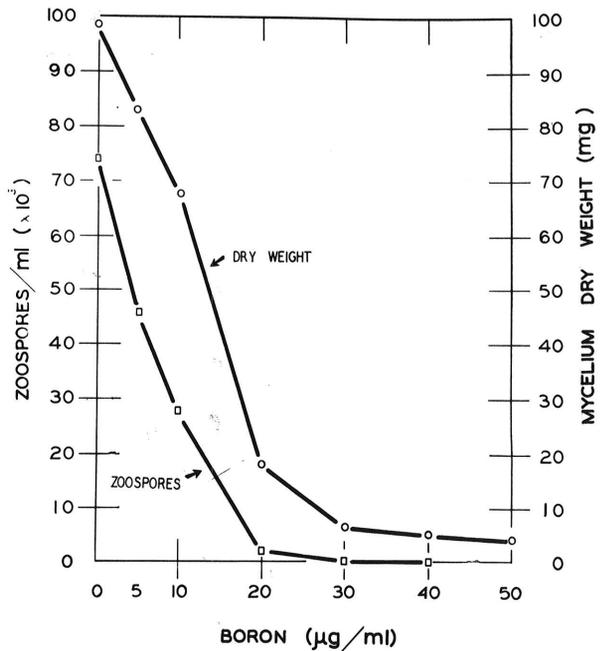


Fig. 5. Effect of boron on vegetative growth and zoospore production by *Aphanomyces raphani*.

whether an imbalance of factors or an inhibitory effect of increased amounts of peptone accounts for the decrease in zoospores.

Our data suggest that specific factors besides those essential to vegetative growth are required for secondary zoospore production. As might be expected, radish decoctions contain the appropriate balance of these unknown factors that are required to produce maximum numbers of both oospores and zoospores (Tables 1 and 2) (3, 6).

In addition to the effects of the growth medium on zoospore production, the constitution of the external bathing medium is critical to zoospore differentiation and release. It has been shown that various phycomycetes (10) are sensitive to low concentration of toxic ions commonly found in tap water. That such ions are absent from distilled water may explain why distilled water was more effective in stimulating zoospore production.

Although boron inhibited vegetative growth and zoospore production in *A. raphani* (Fig. 5), the concentrations needed for effective inhibition are unlikely to occur under field conditions. The fact that blackened, distorted, and cracked radish roots are common symptoms of boron deficiency (13) might explain the early confusion of these symptoms with those caused by *A. raphani*.

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